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Infectious Multiple Drug Resistance

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S Falkow

Pion advanced biochemistry series

4 INFECTIOUS MULTIPLE DRUG RESISTANCE

S.Falkow, Department of Microbiology, University of Washington, Seattle

This volume is a documented guide to the study of infectious multiple-drug resistance (R) factors and other bacterial plasmids. The first three chapters review the properties of the classical F-factor and the bacteriophage λ so that they may serve as the prototypes for the comparison of the broader spectrum of extrachromosomal elements that are found in Nature. From this base of information there follows an analysis in depth of the genetics, molecular nature, and replication of R-factors and other plasmids. Particular emphasis is placed on the more recent research developments, including the classification of R-factors by their incompatibility properties, electron microscope heteroduplex analysis, the use of restriction endonucleases for the dissection of the plasmid genome, and the use of R-factors and other plasmids for 'cloning' genetic material. The ecology of R-factors and their implication to human and veterinary medicine is covered in considerable detail with emphasis on the critical areas of nosocomial infection, the use of antibiotics in animal feeds, the dissemination of R-factors *in vivo*, as well as the biochemical basis and origin of R-factor-mediated drug resistance. Although the R-factors are employed as the general model system, specific chapters also deal with the plasmids of the staphylococci and plasmids, such as Ent and K88, which are known to contribute directly to bacterial pathogenicity.

The book will be of prime interest to microbiologists, clinicians, research workers, and students in the fields of medicine, public health, veterinary medicine, genetics, and molecular biology. It will also serve as a text for an introductory course on plasmids.

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Preface

A number of students, clinicians, and research scientists have been virtually forced to become familiar with infectious multiple drug-resistance (R) factors and other bacterial plasmids. This 'forced' interest has largely been the result of a growing awareness of the importance of these genetic elements to medicine, public health, veterinary medicine, genetics, and molecular biology. Because R-factors are, at present, principally studied by microbial geneticists and molecular biologists, it has often been a burden for the newcomer to relate the genetic, molecular, and functional properties of R-factors with their ecology and public health significance. Many students and microbiologists are already familiar with the classical sex factor, F, and the temperate bacteriophage λ found in the laboratory strain, *E. coli* K-12. In the first three chapters of this book the classical properties of F and phage λ are discussed and serve as the prototypes for comparison of the broader spectrum of extrachromosomal elements that have been found in Nature. I hope that, from this base of information, chapters 5, 6, 7, and 8 may help to put the specific genetic and molecular properties of R-factors and their genetic relatives into a clearer biological perspective. Finally, it has seemed to me that many microbial geneticists and molecular biologists have remained unaware of the full scope of the clinical implications of R-factors and other plasmids found in clinical isolates. Chapters 4, 9, 10, 11, and 12 were particularly included for this reason, as well as to serve as an overview for those who are concerned practically with the prevention and treatment of infectious diseases. But no matter for what reason or purpose that this book may be read, I hope that it will serve in some measure as a useful introduction to R-factors in particular and bacterial plasmids in general.

This volume was primarily written over the period of 1972–1974. Obviously it was not always possible to incorporate the very latest developments as the book drew close to completion. I have made every attempt to have the book reasonably current through June, 1974. One of the major difficulties in writing this book was the question of references. A number of my friends felt that there simply could not be enough. However, I did not in any way intend this book as a review of the literature but rather as an attempt to present the basic information that is available and to underscore the more abundant questions that remain. At the end of each chapter there is a list of recent general review articles dealing with the broad themes that were developed. Specific references are also cited for each chapter subsection. In the main, I have attempted to limit the references to the most current review articles, books, and research papers, or those references which, in my judgement, are most illustrative or contain valuable historical, conceptual, or methodological facts. I apologize now to those individuals who may be upset to find that their work has not been cited. I hope that they will understand. The reader is specifically warned that this book contains

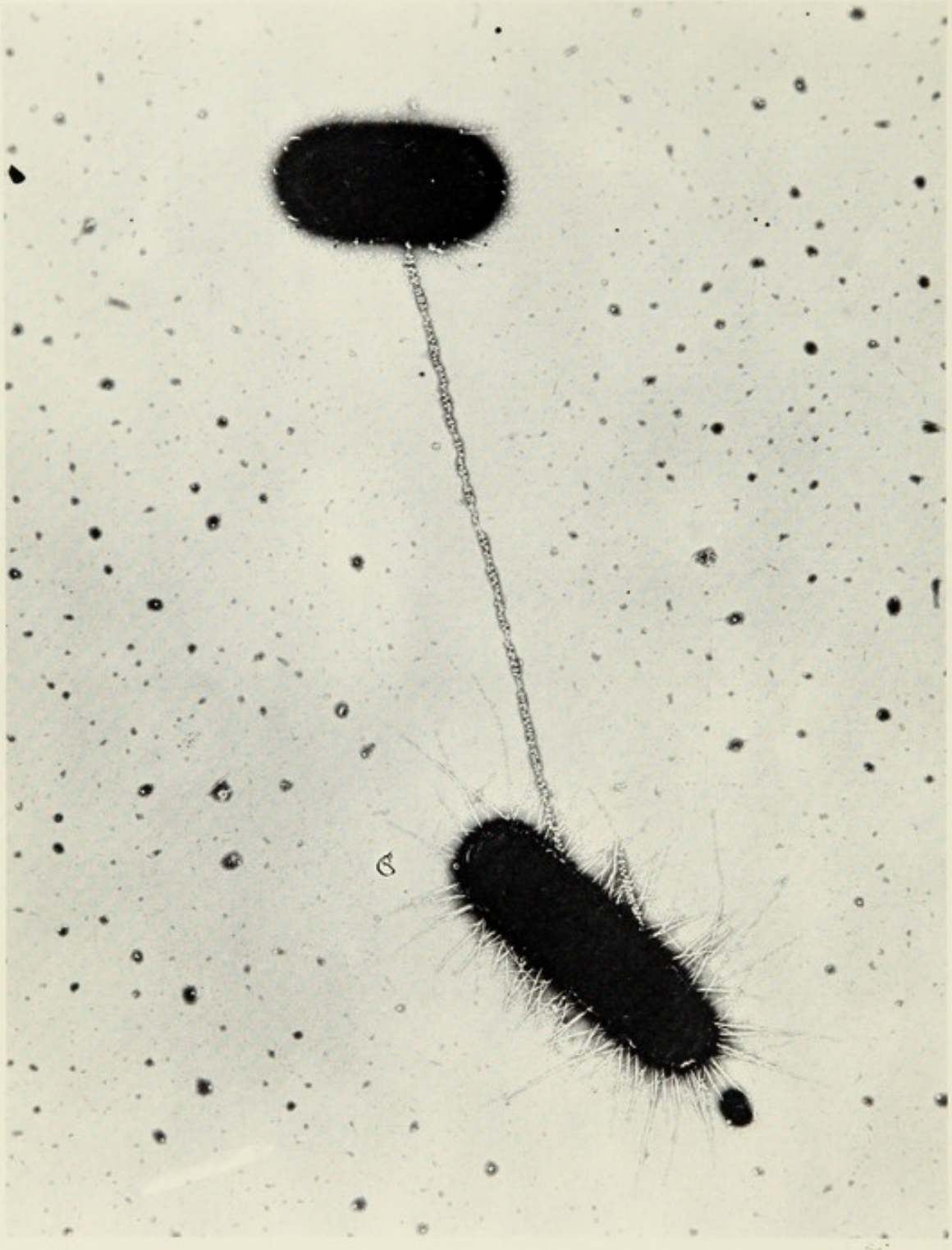
a generous scattering of personal speculation that is often based on fairly skimpy experimental evidence (or wishful thinking).

A number of people have been very patient and helpful to me during the time that it has taken to complete this effort. I wish particularly to thank Naomi Datta, Stanley Cohen, and John Lagnado for reading the entire manuscript and for their constructive comments. I particularly appreciated their gentle way of saying that something was missing, badly written or just plain wrong. Of course, any errors remaining are my own. I am also deeply grateful to my students and associates at Georgetown University and the University of Washington for their help. Patricia Guerry, Donald J. LeBlanc, Mary Vickers Hershfield and Lucy Stuart Tompkins were very helpful in their comments and suggestions about an interminable number of rough drafts. I share the joy of my secretary, Caryl-Sue Johnson, that this effort is finally completed, and I pay homage to Judith Anne Smith for typing the final draft of the manuscript. My thanks go to John Ashby of Pion Ltd. for his tolerance of an author who failed to meet even a single deadline. Many of the previously unpublished experiments presented in this volume were performed under grants from the National Science Foundation, the Commission of Enteric Disease of the Armed Forces Epidemiology Board, and the National Institutes of Health. I wish to thank these bodies for their support.

Finally, I thank my wife Rhoda for her understanding of a husband who cluttered her house with papers, books, journals, reprints, preprints and unkept promises.

Stanley Falkow
University of Washington, School of Medicine, Washington

To Rhoda, Lynn and Jill
... and to the memory
of
Tsutomu Watanabe



Frontispiece. Specific pair formation between a donor and recipient *E. coli* K-12. (Electron micrograph through the courtesy of C. C. Brinton, Jr.)

Contents

Historical perspective – drug resistance and R-factors

The use of sulphonamides and antibiotics has had an enormous influence on the pattern of infectious diseases throughout the world. We owe chemotherapy the debt of reducing the high mortality rate of many bacterial infections; but in helping to solve some of the problems of infectious disease, chemotherapy has created some problems of its own. One of these problems, a central theme of this book, has been the appearance of strains of organisms resistant to certain drugs. Apart from its obvious medical importance, the study of the genetic and biochemical basis of microbial drug resistance provides an important, unexpected, and, I think, exciting avenue to several aspects of fundamental biology.

Two alternative theories were initially advanced to explain the origin of microbial resistance to drugs. One view held that the resistant cells developed by spontaneous mutation independent of the presence of the drug. The other view held that the resistant cells were the adaptive product of interaction between the drug and bacterial cell. In 1943 the fluctuation test of Luria and Delbrück provided the first critical evidence to distinguish between the mutational and adaptive theories. The evidence presented by these workers was statistical in nature and indicated that resistant variants were present in the bacterial population before exposure to drugs and were therefore spontaneous mutants. These statistical findings were strengthened by the technique of indirect selection, which permitted the isolation of resistant clones that had never been in contact with a drug and could not have arisen by adaptation. This application of fluctuation analysis, and of indirect selection to the development of microbial resistance to sulphonamides, penicillin, streptomycin, chloramphenicol, and other drugs was consistent with the mutation hypothesis. Thus it became a general assumption that antibiotic resistance in microorganisms had developed solely by the selection of random spontaneous mutants in response to widespread chemotherapy and had evolved almost entirely within the past thirty years.

It seemed logical to conclude that studies *in vitro* of mutation to drug resistance would serve as the basis of explanation for the behaviour of organisms under natural conditions. A single-step pattern of high resistance to streptomycin was demonstrated by fluctuation analysis. On this basis one would suspect that bacterial resistance *in vivo* to streptomycin would be a common event and, indeed, it is established that the rapid emergence of resistant strains to this drug forms the main obstacle to its successful use clinically. Other studies *in vitro* showed, however, that a single mutational event often resulted in only a slight increase in drug resistance and that a number of sequential mutations were usually required before a sensitive organism developed a high level of resistance. This multi-step pattern of accumulating resistance by successive increments was

characteristic for penicillin, tetracycline, and chloramphenicol. One would expect therefore that development of resistance to penicillin, for example, might be a prolonged, arduous process. Indeed, whilst the development of penicillin resistance in the pneumococcus and gonococcus can be accomplished in the laboratory by sequential growth in the presence of the drug, nonetheless, after thirty years of penicillin therapy, the pneumococcus has yet to show a significant degree of penicillin resistance and the gonococcus has only just recently begun to show increased resistance to this drug. The correlation between the studies *in vitro* and the resistance patterns characteristic of naturally occurring strains seemed most satisfactory at first view.

1.1 Mutation is not the only answer

Staphylococci, like the pneumococcus and gonococcus, show resistance *in vitro* to penicillin after sequential growth in the presence of increasing amounts of the drug. The staphylococci, however, unlike the pneumococcus and gonococcus, have been the cause of great clinical concern because of the prevalence of penicillin-resistant strains. What is different about the staphylococci? The answer is that clinical isolates of staphylococci have acquired a mechanism of penicillin resistance, the ability to produce penicillinase, which cannot be duplicated in the laboratory despite numerous attempts by many investigators. Moreover it was observed as early as 1949 by Mary Barber that staphylococci spontaneously lose the ability to produce penicillinase at a relatively high rate and that reverse mutations to penicillinase production did not occur. More will be said about drug resistance in staphylococci in later pages, but for the moment one can simply say that both of these observations suggested that mutation was not a satisfactory explanation of the mechanism of penicillin resistance in staphylococci. In the Enterobacteriaceae also, several observations were recorded which indicated that the origin of drug-resistant strains could be accounted for by a mechanism other than mutation and selection.

At the end of World War 2 the sulphonamides (Su) were found to be most effective against the *Shigella* causing outbreaks of dysentery in Japan. According to surveys, however, this effectiveness lasted for only a relatively few years and by 1952 more than 80% of the *Shigella* isolates were highly resistant to sulphonamides. The introduction of streptomycin (Sm), tetracycline (Tc), and chloramphenicol (Cm) to Japan in 1950 was subsequently followed by their extensive use and, as might be expected, some resistance to these agents was encountered. Initially, strains were resistant to either tetracycline or streptomycin alone but, in 1956, Kitamoto reported the isolation of a *Shigella flexneri* 4a strain resistant to streptomycin, tetracycline, chloramphenicol, and sulphonamides. By 1957, a significant number of *Shigella* strains showing multiple resistance were

being isolated on a regular basis (table 1.1). If one attempts to explain this appearance of multiple resistance solely on the basis of mutation and selection, the problem becomes rather difficult.

If a single mutational event usually results in only a slight increase in drug resistance, then one must consider that a multiply resistant strain has been subjected to many rounds of mutation and selection. It is also recognized that whereas a number of potential mutational changes can increase resistance to a drug, many such mutations have a concomitant deleterious effect on the organism. For example, many mutants of *Shigella* resistant to tetracycline or chloramphenicol selected in the laboratory grow much more slowly than the drug-sensitive parental strain. Such strains are easily maintained in the laboratory but one can question whether such mutants, in the absence of drugs, would have much evolutionary potential (or still be highly virulent for that matter). Given a 'healthy' mutant, we must also note that it must be provided sequentially with just the proper selection conditions (for example, a proper drug concentration) to grow into a population of sufficient size to provide a reasonable probability for the occurrence of the next mutational step. Since genetic analysis has provided evidence that separate genes are responsible for resistance to streptomycin, chloramphenicol, tetracycline, and sulphonamides, and that the frequency of a single mutation to drug resistance is of the order of 10^{-7}–10^{-10} per bacterium per generation, it can be seen that we are dealing with an infinitesimally small probability. We can possibly conceive of one or two strains of *Shigella* that could meet the very stringent conditions that have been outlined. But the experience in Japan was that a number of different serotypes isolated during different outbreaks exhibited multiple resistance. In addition, the data from surveys (table 1.1) showed that the occurrence of multiple resistance was unreasonably higher than that of singly or

Table 1.1. Statistics of occurrence of antibiotic-resistant *Shigella* strains in Japan as reported in surveys by Mitsuhashi and Watanabe. Sm, streptomycin; Tc, tetracycline; Cm, chloramphenicol.

Year	No. of strains tested	Sm	Tc	Cm	Sm, Cm	Sm, Tc	Cm Tc	Sm, Cm and Tc
1953	4900	5	2	0	0	0	0	0
1954	4876	11	0	0	0	0	0	0
1955	5327	4	0	0	0	0	0	1
1956	4399	8	4	0	0	0	1	0
1957	4873	13	46	0	2	2	0	37
1958	6563	18	20	0	7	2	0	193
1959	4071	16	32	0	71	0	0	74
1960	3396	29	36	0	61	9	7	308

doubly resistant strains. Resistance to chloramphenicol alone, for example, was never observed but was usually present in the multiply resistant isolates. Thus, although there was no doubt that the extensive use of antibiotics provided ample selective pressure to ensure the survival and spread of resistant strains, the facts argued against any simple mechanism of random mutation and selection as the origin of the multiply resistant *Shigella* strains isolated in Japan.

1.2 Drug resistance is found to be transmissible

Careful epidemiological observations on the changing pattern of antibiotic-resistant strains of *Shigella* in Japan disclosed several unusual clinical features during some epidemics. It was observed repeatedly that some patients excreted *Shigella* strains completely sensitive to antimicrobial agents whereas other patients excreted multiple-drug-resistant strains of the same serological type. Indeed individual patients excreting both sensitive and multiple-drug-resistant *Shigella* strains of the same serotype were found. Sometimes multiply resistant strains were unexpectedly isolated from a patient who had excreted a drug-sensitive strain at the onset of dysentery and had been treated with only one drug—again a clear 'violation' of the mutation hypothesis. Moreover it was found that multiple-drug resistance was not restricted to *Shigella*. In one epidemic *Escherichia coli* strains were isolated that had the same pattern of resistance to chloramphenicol, streptomycin, tetracycline, and sulphonamides as the causative *Shigella* serotype.

No single acceptable explanation of these observations was available until Tomoichiro Akiba suggested that multiple resistance might be transferred from the drug-resistant *E. coli* to *Shigella* in the intestinal tracts of patients. This hypothesis and experiments supporting this hypothesis were presented by Akiba and his associates at the meeting of the Japan Bacteriological Society held on the 13th November, 1959, and were reported in 1960 in the *Japanese Journal of Microbiology*. Independently, Kunitaro Ochiai and his colleagues reported similar findings at a meeting of the Society of Chemotherapy of Japan held one day later, and subsequently also published (in 1959) their results in the *Japanese Medical Journal*. The experiments performed by Akiba and Ochiai were quite similar and straightforward.

In these experiments cultures of resistant *E. coli* were mixed in broth with a sensitive *Shigella* cell; after incubation and selective plating, multiple-drug-resistant *Shigella* with the same resistance pattern as the donor *E. coli* could be isolated. Further clinical observations to support the transfer hypothesis were made by Kagiwada and his coworkers. Initially they found a correlation between the presence of both resistant *E. coli* and *Shigella* in the same patient. Among 242 cases of shigellosis caused by drug-sensitive strains there were only 17 instances (7%) where

multiply resistant *E. coli* were also found. In marked contrast, among 35 cases caused by multiply resistant *Shigella*, there were 24 instances (69%) where multiply resistant *E. coli* were isolated. Kagiwada and his associates also made the significant observation that in seven patients who were excreting resistant *E. coli* and sensitive *Shigella* at the beginning of hospitalization, six showed multiply resistant *Shigella* in their stools two to five days after treatment with a single antibiotic. In further experiments, patients excreting drug-sensitive *Shigella* were infected peranally with broth cultures of drug-resistant *E. coli*. From three of 24 patients treated in this way, multiply resistant *Shigella* were obtained. These results then supported those of the experiments *in vitro* by Akiba and Ochiai and showed that the transfer of resistance could occur between bacteria in the gut.

The mechanism by which drug resistance could be transferred between bacteria was considered by both Akiba and Ochiai to be one of the three modes of gene transfer, which had been well established in bacteria only about ten years before the first multiply resistant culture was noted in Japan.

The modes of gene transfer in bacteria are: (1) transformation, in which the deoxyribonucleic acid (DNA) extracted from cells of one strain can induce heritable changes in cells of another; (2) transduction, in which a bacterial virus acts as a vector of bacterial genetic material; (3) conjugation, the transfer of genetic material involving direct cell to cell contact. Presumably it was the awareness of the Japanese workers of these gene-transfer mechanisms which led to their bold proposal that some mechanism other than mutation could explain the epidemiological and clinical findings. Both Akiba and Ochiai demonstrated that the mechanism responsible for the transfer of multiple drug resistance by mixed cultivation was neither transformation nor transduction.

Susumu Mitsuhashi, Tsutomu Watanabe, Rintaro Nakaya and their collaborators quickly confirmed and extended the initial observations on transmissible drug resistance. These workers clearly demonstrated that transfer was dependent upon direct cellular contact and was therefore a form of bacterial conjugation. Mitsuhashi proposed the term resistance factor (R-factor) for the transmissible resistance property—a term which has enjoyed general acceptance and will also be employed here. It was recognized that the drug resistance associated with R-factors was distinct. Mitsuhashi, for example, demonstrated that sensitive *E. coli* and *Shigella* isolated from epidemics did not give rise to multiple-drug-resistant mutants on exposure to a single drug. Single drug-resistant mutants isolated from these strains were not transmissible. Nor could transfer be demonstrated even after the strains were subjected to laborious multistep selection *in vitro* to give the same resistant properties as their natural counterparts.

The first comprehensive introduction of R-factors to the Western world was the review of Watanabe, which appeared in 1963. While this paper was in the press, Naomi Datta reported in 1962 that R-factors were present in *Salmonella typhimurium* strains isolated in England. In Germany, Gerhard Lebek discovered that R-factors were quite prevalent among enteropathogenic *E. coli* strains. Lebek also found that R-factors could include resistance to the antibiotics of the kanamycin–neomycin (Km) group. Thus within eight years of the first detection of a multiply resistant strain in Japan, the problem of infectious multiple-drug resistance was recognized as a world-wide problem with broad implications to medicine and of considerable genetic interest. Not only do R-factors render their hosts resistant to a number of antimicrobial agents but, at the same time, they give their hosts the ability to transmit this resistance to other bacteria. From the time of the discovery of transferable drug resistance, it was recognized that this phenomenon bore a similarity to the previously described transfer of chromosomal genes by the F-factor of *E. coli* and to the genetic elements determining the transfer of colicinogeny. As we shall see, the R-factors do indeed closely resemble these elements, so before one can consider, in detail, the particular genetic properties of R-factors, it is necessary to examine the general biology of genetic elements in bacteria that promote conjugation, and some cleverly disguised analogues which do not.

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The sex factor, F, and temperate phages

2.1 Discovery of bacterial conjugation

R-factors have become such a world-wide problem that many scientists have become rather blasé about the conjugal transfer of genetic material in microorganisms. Yet the discovery in 1946 by Lederberg and Tatum that genetic material could be transferred in *E. coli* K-12 by direct cell-to-cell contact preceded the discovery of R-factors by only twelve years. Although the bacteriological literature before 1946 contained purported morphological descriptions of cell fusion, the central problem facing Lederberg and Tatum was how actually to detect a small fraction of recombinant bacterial cells within a total bacterial population. They solved this problem by using a selective medium in which only the recombinant cells could multiply and the parental strains could not. *E. coli* K-12 ordinarily grows well on a minimal medium, in which the only carbon source is glucose and the only nitrogen source is ammonium salts. In the original recombination experiments, two mutant sublines of *E. coli* K-12 were employed, each possessing different specific amino acid requirements. Neither subline could grow individually upon the minimal medium. Yet when the two strains were mixed a small number of cells (about one per 10^7 cells plated) were found to grow. This was interpreted to mean that a recombinational event had occurred to reconstitute the 'wild-type' *E. coli*, and, of course, this interpretation has been borne out. The years following the discovery of conjugation have naturally led to the development of more sophisticated procedures in performing genetic 'crosses'. The fundamental principle of the selective medium remains, however, as the cornerstone of microbial genetic analysis. For example, the demonstration of R-factor transfer commonly employed today is only a modification of the original technique, with drugs rather than growth requirements being used as the selective agents. If one has a strain resistant to tetracycline and streptomycin, thought to harbour an R-factor, it is mixed with a strain which bears a chromosomal mutation to nalidixic acid (Nx). The nalidixic acid marker is usually chosen since resistance is not commonly found on R-factors. If the mixture is plated on a medium containing nalidixic acid + streptomycin + tetracycline, neither parental type can grow, but a Nx, Sm-Tc 'recombinant' strain can. In early years such recombinant types were assumed, by analogy with more highly evolved organisms, to be the fusion of two haploid cells to form a diploid zygote. But as conjugation in *E. coli* K-12 was studied more thoroughly, it became apparent that the results diverged markedly from such a classical genetic interpretation[1].

[1] The historical development of thought and experimentation concerning conjugation is surely fascinating, but beyond the scope of this monograph. Excellent narrative presentations of the historical evolution of microbial genetics can be found in the books by Hayes and by Stent.

2.2 Discovery of the F-factor

Experiments by William Hayes and by L. L. Cavalli and the Lederbergs demonstrated that genetic transfer by conjugation was a unidirectional transfer of genetic material from a donor cell to a recipient cell. Donor strains of *E. coli* K-12 were called F^+ (for fertility) and recipient strains F^-. $F^- \times F^-$ crosses were uniformly sterile whereas $F^+ \times F^-$ crosses were fertile, albeit at the low frequency of 10^{-5}–10^{-7} recombinant cells per plated donor cell. $F^+ \times F^+$ crosses were also fertile, but at an even lower frequency. The most exciting finding was that the F^+ character was transmissible. When differentially marked F^+ and F^- cells were grown together for only a short time, the F^- cells were converted into F^+ with an efficiency often approaching 100%. Thus the rate of F^+ conversion was far greater than the rate of recombination for chromosomal genes. The F^+ character, or 'sex factor', acquired by 'infection' was shown to be an inheritable and stable property. Yet all genetic recombinant types from $F^+ \times F^-$ crosses were also F^+, so that the sex factor could not be assigned any locus on the bacterial chromosome. Rarely, F^+ cells lost their fertility and became F^-. No known treatment could change these F^- cells back to F^+ except for a new mating experience with an F^+ cell. Nor has the F^+ state ever been observed to arise by mutation in an F^- culture. It seemed clear therefore that F-factor was a distinct, independent genetic entity, which possessed a high transfer frequency and whose presence was associated, as well, with a low probability of chromosomal transfer.

Soon after the discovery of F-factor, two mutants of the same F^+ strain were independently isolated, which showed a high frequency of chromosomal transfer in crosses with F^- strains. These strains were called Hfr (for high frequency of recombination) and possessed some properties that were strikingly different from those of F^+ strains, namely:

(1) The higher frequency of recombination was not a trivial increase, but was at least a thousand times greater than that of an equivalent $F^+ \times F^-$ cross.

(2) Hfr, unlike F^+, strains were not infectious and could not ordinarily convert F^- cells to the F^+ or Hfr donor type by simple mixed cultivation. The recombinant cells from $F^+ \times F^-$ crosses were invariably F^+, whereas those from Hfr $\times$ F^- crosses were almost invariably F^-.

(3) In $F^+ \times F^-$ crosses most genes for which selection is made are transferred at about the same low frequency. In Hfr $\times$ F^- crosses, the very high frequency of recombination was manifest for only certain genes of the donor cell, whereas others were transferred at a frequency only slightly greater than in an $F^+ \times F^-$ mating. Thus the term Hfr was meaningful only when the selective conditions of the cross were specified.

(4) Hfr strains could revert at a low rate to an F^+ state indistinguishable from the parent F^+ strain.

(5) It had been discovered that treatment of F^+ cells with certain acridine dyes or by irradiation could cause a significant fraction of cells to become

F^-. Similar 'curing' experiments had no such effect in converting Hfr cells into F^-.

The differences between F^+ and Hfr cells are summarized in table 2.1. The impression gained from this comparison was that the $F^+ \rightleftharpoons$ Hfr transition represented some physical alteration of the sex factor, which was associated with a loss in infectivity and a concomitant increase in chromosomal transfer.

Table 2.1. A comparison of properties of F^+ and Hfr donor types.

Property	F^+ cells	Hfr cells
F-factor infectivity	Infectious	Not infectious
Recombinant cells	All F^+	Mostly F^-
Recombination frequency	Uniformly low for all genes	Very high for some genes, low for others
Convertability with acridine dyes	Can be converted into F^-	Cannot be converted into F^-

2.3 An alteration of F-factor within the bacterial cell

The general organization of the *E. coli* K-12 chromosome and the nature of F within different donor cells was revealed in 1956 by the simple, yet ingenious, studies of Elie Wollman, Francois Jacob and William Hayes, who began to study conjugation at the cellular level. They mixed cells of an Hfr strain with cells of a multiply mutant F^- derivative and, at varying times, they deliberately 'interrupted' the mating in a mechanical blender. Treatment in a blender affects the viability of neither the parental nor the recombinant strains, but does effectively halt the conjugal process. The mating mixture was then plated on different media, which selected for recombinant cells that might have received donor chromosomal genes. It was found that the cells in the Hfr population transfer their genes in an oriented manner, such that genes enter the F^- cell in a sequential order, as shown in figure 2.1. The frequency of inheritance of donor genes in recombinant strains was highest for markers near the beginning of the sequence, and lowest for genes which were transferred late in the sequence, indicating that the chromosome is not usually transferred completely from a donor to a recipient. Indeed, if Hfr and F^- cells are simply permitted to conjugate without interruption, one observes a gradient of recombination frequencies that is completely equivalent to the sequential entry of genes. In other words, during a mating there is a fixed probability of spontaneous interruption, which explains why some genes were found in recombinant cells of Hfr x F^- crosses so much more frequently than others. The high frequency of recombination was for genes transferred early, and the low frequency of recombination for those genes transferred late in the oriented sequence. The original idea that bacterial recombination represented a

fusion of two complete bacterial chromosomes was therefore found to be incorrect. The resulting zygotes from an Hfr x F^- mating were usually not complete, but represented instead partial zygotes (merozygotes). Of course, it was possible for an F^- cell to receive the entire Hfr chromosome if spontaneous interruption did not occur. On these rather rare occasions it was found that these recombinant cells had become, in turn, Hfr cells. Thus F, in an Hfr cell, appeared to have a fixed chromosomal location near the end of the chromosome.

These basic observations were repeated in 1958 by Jacob and Wollman with a series of independent Hfr isolates, but a curious discrepancy was observed. Different Hfr strains of *E. coli* K-12 gave different sequences of donor marker transfer to the same F^- strains. Table 2.2 shows that the origin of transfer, *Or*, seems to vary widely in different strains. Yet, after looking at this table it becomes clear that it was not the total gene sequence that varied, but rather the position of *Or* and the direction of gene transfer which was dynamic. It can be seen, for example, that the genes *thr-leu-pro-lac* are invariant neighbours in all of the Hfr strains except P4X6. In some strains, like Hayes, however, the transfer order is *thr-leu-pro-lac*; in others, like Cavalli, the transfer order is *lac-pro-leu-thr*, which is just the reverse. The one exception, P4X6, has the *pro-lac*

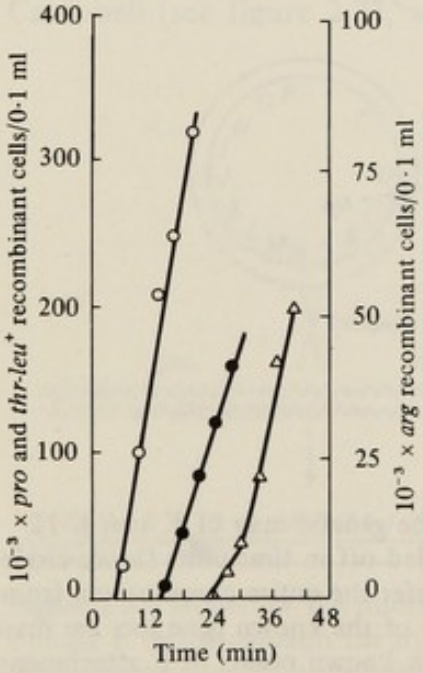

Figure 2.1. Kinetics of gene transfer by an *E. coli* Hfr strain. A mating was performed between an Hfr strain, sensitive to streptomycin, and a recipient cell which required proline (*pro*), threonine + leucine (*thr* + *leu*), and arginine (*arg*), and was resistant to streptomycin. At timed intervals samples were removed and placed in a blender for 30 seconds. After interruption of the mating, samples were diluted, and plated on media containing streptomycin but lacking one of the amino acids required by the recipients. The recombinant cells selected on these selective media are plotted as a function of time-sampling. ○, *pro*; ●, *thr-leu*; △, *arg*.

relationship apparently disrupted somehow by the origin. A model accommodating these features was proposed by Jacob and Wollman, namely that the chromosome of *E. coli* K-12 was circular, as shown diagrammatically in figure 2.2. This first proposal of a circular

Table 2.2. Gene transfer order by different Hfr strains of *E. coli* K-12.

Hfr strain	Order of gene transfer
Hayes	*Or-thr-leu-pro-lac-gal-trp-his-str-xyl-ile-met*
Cavalli	*Or-lac-pro-leu-thr-met-ile-xyl-str-his-trp-gal*
P4X6	*Or-pro-leu-thr-met-ile-xyl-str-his-trp-gal-lac*
5	*Or-met-thr-leu-pro-lac-gal-trp-his-str-xyl-ile*
6	*Or-ile-met-thr-leu-pro-lac-gal-trp-his-str-xyl*
AB311	*Or-his-trp-gal-lac-pro-leu-thr-met-ile-xyl-str*
AB313	*Or-xyl-str-his-trp-gal-lac-pro-leu-thr-met-ile*

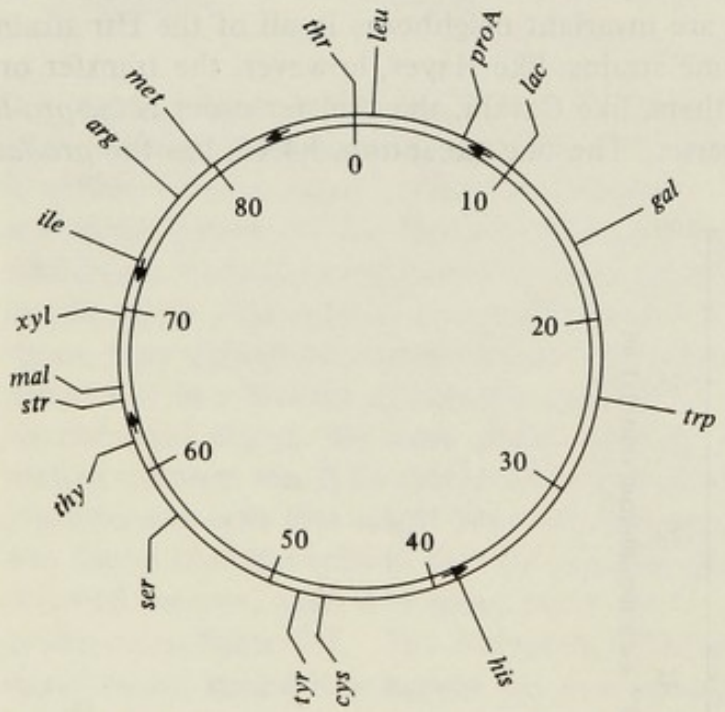

Figure 2.2. Diagrammatic representation of the genetic map of *E. coli* K-12. The circular linkage map of *E. coli* K-12 is ruled off in time-units (inner circle) based on the time of 90 min required to transfer the entire chromosome from an Hfr to a recipient cell. The position of a few of the known gene loci are marked off on the outer circle. The arrow heads show known points of F attachment on the chromosome and show the origin and direction of transfer of the Hfr strain. The markers shown are: *lac*, lactose utilization; *pro*A, proline synthesis; *leu*, leucine synthesis; *thr*, threonine synthesis; *met*, methionine synthesis; *arg*, arginine synthesis; *ile*, isoleucine synthesis; *xyl*, xylose utilization; *mal*, maltose utilization; *str*, streptomycin resistance, sensitivity and dependence; *thy*, thymidylate synthetase; *ser*, serine synthesis; *tyr*, tyrosine synthesis; *cys*, cysteine synthesis; *his*, histidine synthesis; *trp*, tryptopan synthesis; *gal*, utilization of galactose.

chromosome for *E. coli* K-12 was directly confirmed some years later by radioautographic visualization of a single circular DNA molecule with a molecular weight of about $2{\cdot}5 \times 10^9$. In fact the circularity of bacterial and viral chromosomes has become such a generalization that it has been called the 'rule of the ring'.

Given a circular chromosome, it is readily seen that any Hfr type could easily be produced by opening the circle and establishing one end as the origin and the other as the terminus. The transition $F^+ \rightarrow$ Hfr was assumed to be related to the location of *Or* and the 'breakage' of the chromosome. Just how the F-factor managed to do this was, according to Jacob and Wollman, dependent upon the different location of F in an Hfr cell to that in an F^+ cell. Since, in an F^+ cell, the sex factor was infectious and could be irreversibly eliminated by acridine dyes, it was assumed (and again later confirmed) that F was an autonomous cytoplasmic genetic element composed of DNA that was distinct from the chromosome. The $F^+ \rightarrow$ Hfr transition, on the other hand, was assumed to represent the direct attachment of the F genome to the bacterial chromosome. It is now established that the attachment of F to the bacterial chromosome does not represent a permanent breakage of the chromosome, but rather this event is visualized as a recombinational coalescence of two circular genomes into a single circular structure. According to the model proposed by Alan Campbell (see figure 2.3), a small circular F chromosome pairs with a

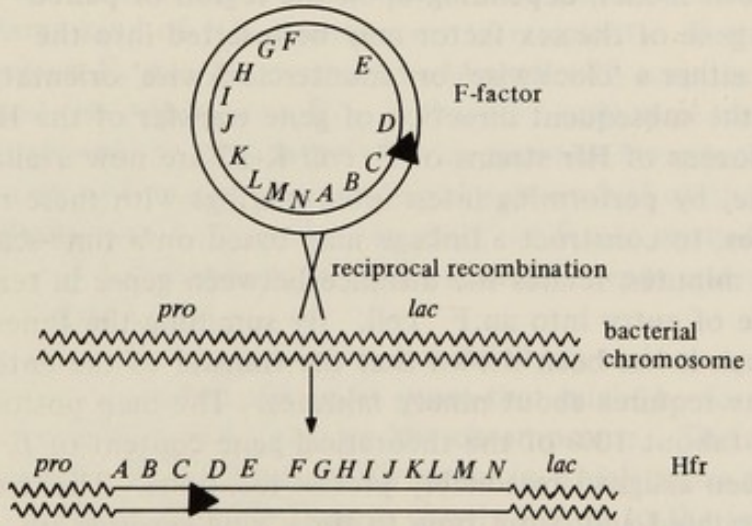

Figure 2.3. Model for the insertion of F into the bacterial chromosome. The circular F factor contains the hypothetical genes *A*–*N* with an origin of transfer of DNA depicted by an arrow between *C* and *D*. The jagged line represents a part of the much larger bacterial chromosome containing the bacterial genes *pro* and *lac*. After a pairing between homologous regions, a reciprocal crossover between F and the chromosome results in the linear insertion of F into the host genome to form an Hfr cell. Since F shares a number of different homologous regions with the bacterial chromosome, the gene order and the origin of replication of F may vary from Hfr to Hfr. (Redrawn from Sharp, P. A., Hsu, H. T., Ohtsubo, E., Davidson, N., 1972, "Electron microscope heteroduplex studies of sequence relationships among plasmids of *Escherichia coli*. 1. Structure of F prime factors", *J. Mol. Biol.*, **71**, 471.)

homologous region on the circular bacterial chromosome. Since there are a number of different Hfr types, it can be assumed that F shares a number of homologous regions with the bacterial chromosome (see also pp.196–197) for a recent view of these sequences held in common). After the pairing step, a reciprocal crossover between F and the bacterial chromosome, by breakage and reunion, results in the insertion of F into the continuity of the host genome. In this recombinational step no bacterial genes or F genes are lost; the F chromosome is added to the host chromosome, which becomes slightly larger in size. It is, furthermore, not difficult to see that a reversal of this process could lead to the reconstitution of a circular autonomous F factor.

The available evidence suggests therefore that upon integration of F to produce an Hfr donor, the chromosome maintains its closed circular form. Under the stimulus of conjugation, however, the chromosome does open at the site of F attachment. One end becomes the origin, which begins to enter the F^- cell in a linearly oriented fashion. It now seems likely that the *Or* end contains a small piece of F genetic material, but that the bulk of the F DNA remains at the terminus of the chromosome. As mentioned earlier, although most recombinant types issuing from an Hfr × F^- mating remain F^-, should a recombinant cell receive the terminal part of the chromosome and the origin, it is converted into an Hfr cell. The origin therefore seems to be a specific locus on the sex factor chromosome. Thus in the Campbell model, depending upon the region of paired homology, the *Or* gene of the sex factor may be inserted into the chromosome with either a 'clockwise' or 'counterclockwise' orientation, which determines the subsequent direction of gene transfer of the Hfr strain. Literally dozens of Hfr strains of *E. coli* K-12 are now available. It has been possible, by performing interrupted matings with these many independent isolates, to construct a linkage map based on a time-scale. This time-scale, in minutes, relates the distance between genes in terms of their different time of entry into an F^- cell. By summing the times between various loci, it has been shown that the transfer of the entire *E. coli* chromosome requires about ninety minutes. The map positions of roughly 450 genes (about 10% of the theoretical gene content of *E. coli* K-12) have now been assigned reasonably precise locations. The map is being constantly updated and, from time to time, new versions are published under the coordination of A. L. Taylor.

2.4 Sexduction

The sex factor F emerges as a genetic agent capable of two alternative modes of existence within the cell. It is autonomous in F^+ cells and promotes its own transfer at a very high frequency. It is now generally assumed that autonomous F probably does not initiate significant chromosome transfer, but that the low-level chromosomal transfer observed in F^+ × F^- crosses is the sole result of Hfr mutants in the F^+

population[2]. In Hfr cells, the F-factor is integrated and behaves like a chromosomal marker most of the time. At conjugation, the integrated sex factor does promote its own transfer but, as a result of its attachment, the chromosome is also mobilized. It soon became clear, moreover, that the alteration of F from the autonomous to the integrated state and back again was not without further genetic consequences—both to F and the host bacterium.

The earliest evidence of genetic interaction between F and the host chromosome (and, incidentally, direct support for the Campbell model) was the isolation in 1960 by Edward A. Adelberg and S. N. Burns of a mutant from an Hfr strain which possessed properties intermediate between F^+ and Hfr. This mutant, called F_2, transferred the host chromosome only one-tenth as frequently as the Hfr parental type, but with the same oriented sequence. Unlike the Hfr parent, F_2 converted all recipients into the donor state; converted cell lines showed similar F_2 properties of *both* infectivity and oriented gene transfer. These properties led Adelberg to suggest that initially an integrated F had incorporated an adjacent segment of the host chromosome during the process of being detached from an Hfr strain to return to the autonomous state. Because of the strong homology of the resulting autonomous agent, it was reasoned that the F_2 mutant frequently alternated between the autonomous and integrated state, and because of the molecular 'memory' of the F_2 structure for a particular site on the chromosome, it always formed the same kind of Hfr chromosome in recipients. If the original cell line in which F_2 was discovered was treated with acridine dyes, F_2 was eliminated and the cell became F^-. If these F^- cells were now reinfected with an 'ordinary' F, they behaved once again as intermediate gene donors. In other words, just as F_2 retained a 'memory' of the chromosome, the chromosome retained a 'memory' of F. It seemed logical to conclude that F and the chromosome had exchanged genes and were altered in a reciprocal way.

These results led Jacob and Adelberg to look specifically for an F-factor carrying recognizable host genes that might be formed during the detachment of F from an Hfr chromosome. Their method was ingeniously simple. They chose an Hfr strain in which genes governing the ability to ferment lactose (*lac*$^+$) were closely linked to the integrated sex factor, and were transferred ordinarily as a very late marker in an interrupted mating. They then mated this Hfr strain with a *lac*$^-$ F^- recipient and selected for recombinant cells which received *lac*$^+$ too early in the mating to have been transferred in the usual way by the Hfr population. Indeed they found

[2] It has been pointed out, however, that autonomous F (and other sex factors) may possess an active alternative mechanism of chromosome mobilization not involving integration of the sex factor. Usually, the alternative mechanisms occur with only a small probability and it is still fair to say that most recombinants in an $F^+ \times F^-$ cross result from the Hfr mutants in the F^+ population.

rare *lac*$^+$ colonies which differed from usual recombinant populations in several respects. Ordinarily one thinks of a bacterial recombinant cell as one in which the donor gene has replaced its recipient counterpart by recombination. These rare *lac*$^+$ recombinant colonies, however, were unstable and constantly segregated *lac*$^-$ cells at a frequency of about 10^{-3} per cell per generation. They appeared, therefore, to be relatively stable partial diploids for the *lac*$^+$ region. The *lac*$^+$ cells were also intermediate donors of the chromosome and, in fact, transferred the chromosome with the same polarity as the original Hfr strain. The sex factor and *lac*$^+$ genes, however, were transferred together with the very high frequency of an autonomous F$^+$ agent. In short, F and the *lac*$^+$ genes appeared to behave as a single unit of replication and transfer! Hybrid or substituted sex factors such as F_2 and F–*lac*$^+$ are called F′-factors (F-prime factors), and the general process whereby the sex factor acquires host genetic material has been given the name sexduction or F-duction. Since 1959–1960, when this process was first described, a large number of independently isolated F′-factors have been derived from different Hfr strains. Some F′-factors are known which carry over 10% of the entire *E. coli* chromosome. I should stress that sexduction makes it possible to isolate virtually any gene on an F-factor and, by virtue of its associated infectivity, to create bacterial strains which are diploid for virtually any desired gene. These strains have been of enormous value in establishing the dominance and recessiveness of different alleles of the same gene and have proved especially valuable in studying the regulatory interactions of genes.

F′-factors probably arise by (1) a reciprocal crossover between chromosomal sites on either side of an integrated sex factor, or (2) a reciprocal crossover between an adjacent chromosomal site and a site in the integrated F. The products of the first type of exchange would contain both an early chromosomal gene and a terminal chromosomal gene of the ancestral Hfr chromosome (figure 2.4: type II F′ excision). Such an F′-factor would contain the entire F genome plus the acquired chromosomal genes and therefore would always be physically larger than the autonomous wild-type F. Interestingly enough the bacterial cell in which this event occurred would suffer a deletion in its chromosome corresponding to the material present in the F′-factor. The products of the second type of exchange would contain either a late gene or an early gene (figure 2.4: type I F′excision). Moreover since the crossover would occur within F it is possible that a piece of F would be left behind in the chromosome, as was the case for F_2. Also the host chromosome would suffer a deletion of the material present in the F′-factor. Both general types of F′-factors have, in fact, been isolated, and it has been confirmed that the host chromosome remaining after F′ formation does contain a deletion corresponding to the material now residing with F in the autonomous state. The deletion does not result in a broken chromosome,

but rather it is resealed to again form a closed, albeit slightly smaller, structure. The genetic data obtained with F′-factors therefore readily confirm the notion that F is inserted into the chromosome of Hfr bacteria.

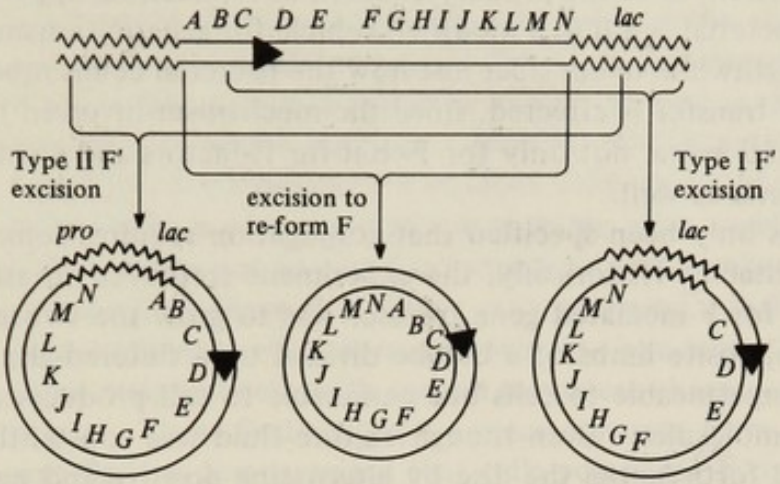

Figure 2.4. Excision of F from an Hfr chromosome and the formation of F′-factor chromosomes. An F-factor inserted into a bacterial chromosome can, by excision, revert to the autonomous F^+ state. Occasionally, because of an aberrant excision process, a circular F′-factor carrying F genes and some bacterial genes is formed. In a type I excision, a cut is made within F and on the bacterial chromosome. In the example, this leads to an F′ which has lost the F genes *AB* and has gained the bacterial gene *lac*. In a type II excision, the cuts are made within the bacterial sequences on *both* sides of F. In the example, this leads to an F′ carrying all the F genes and both the bacterial genes *pro* and *lac*. (Redrawn from Sharp, P. A., Hsu, H. T., Ohtsubo, E., Davidson, N., 1972, "Electron microscope heteroduplex studies of sequence relationships among plasmids of *Escherichia coli*. 1. Structure of F prime factors", *J. Mol. Biol.*, 71, 471.)

2.5 Conjugation at the cellular level

The volume of experimental data on bacterial conjugation has become so extensive that it is often overwhelming. The conjugal act in *E. coli* K-12 is complex. After all, as noted by Hayes, *E. coli* is an organism where the duration of the sexual act can occasionally exceed four times the normal life-span of the cell. The F-factor is the essence of conjugation and it may be seen that, although host cell cooperation is essential, F is really concerned primarily with bringing about its own transmission rather than with chromosome transfer. There is a good deal of emphasis in the literature on Hfr cells and genetic mapping. So much so, in fact, that it can sometimes be forgotten that the wild-type *E. coli* K-12 was F^+, and that Hfr cells are relatively rare individuals in the total bacterial population. Obviously occasional chromosomal transfer is important in the evolutionary sense but, in the world outside the microbial geneticist's laboratory, analogues of F, such as R-factors, are found very frequently in microorganisms but Hfr cells are not. Many of the F analogues also have the potential to bring about chromosome transfer. In fact it is quite clear

that sex factors in Nature do interact with bacterial chromosomes since one can readily isolate F′-factor analogues in natural bacterial populations. The F-factor therefore seems to be a finely honed evolutionary product whose major function, after the primary concern of replication, appears to be to change a bacterial cell into a modified vehicle for genetic transmission. This makes it worthwhile to consider just how the bacterial cell is modified and how genetic transfer is effected, since the mechanism involved holds true, in the general sense, not only for F- but for R-factors and most other transmissible agents as well.

Thus far it has only been specified that conjugation requires some form of cell-to-cell contact. Historically, the experiment employed to establish this prerequisite for F-mediated gene transfer was to grow the two genetic partners in the opposite limbs of a U-tube divided by a sintered-glass disc, this disc being impermeable to cells but permeable to cell products and biological macromolecules. Even though culture fluid was constantly flushed back and forth across the disc by alternating positive and negative pressure, no recombinant cells were found. This experimental implication of the necessity for some form of direct cellular contact was reinforced by early phase-contrast and electron microscopy, which showed what appeared to be specific donor–recipient unions. The process of bacterial conjugation has now been divided into several distinct steps, which are convenient for discussion but are not really so clearly distinguishable by experiment (figure 2.5).

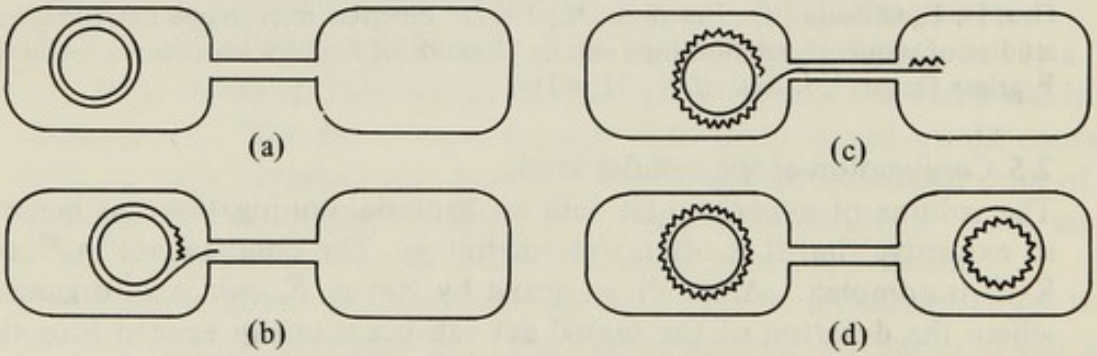

Figure 2.5. Diagrammatic representation of the conjugal steps involved in F-factor transfer. (a) *Specific pair formation.* This step largely follows the kinetics of random collision between two bacteria. The proteinaceous F-pilus is essential for this step. Specific pairs do not necessarily conjugate. (b) *Effective pair formation.* This step is presumed to involve the formation of a specific conjugation tube between the donor and the recipient cells. It is the first step which ensures that genetic material can pass from the donor to the recipient. (c) *Sex-factor mobilization and transfer.* The mobilization and transfer of a sex factor from a donor to a recipient is a specialized form of DNA replication. An early mobilization step is an enzymatic 'nick' of a circular sex-factor molecule; this exposes a -5′phosphate end of a *single* DNA strand which then enters the recipient. (d) *Establishment.* The entry of the single strand of sex-factor DNA is accomplished by the synthesis of a complementary strand within the recipient cell. Once synthesis and replication have been completed, the recipient, in turn, becomes a genetic vehicle for sex-factor transmission.

2.5.1 Specific pair formation

The first step in the conjugal act between two microorganisms is the formation of a specific pair. Experimentally, specific pair formation has been defined as the formation of a cell union which is stable enough to resist gentle dilution. Specific pairs form in the absence of energy metabolism in either parent, and the rate and frequency of pair formation is a function of the population density as well as the viscosity and temperature of the medium. Specific pair formation largely follows therefore, the kinetics of a random collision between two particles. The reason why a donor and recipient might stick together after a random collision was not known for some time. A number of investigators had presented evidence that donor cells possessed a different cellular surface from recipient cells. It was known, for example, that F^+ cells differed from recipient cells with respect to surface charge and motility in semisolid media. These findings took a more concrete form in 1960 when Ørskov and Ørskov demonstrated that cells possessing F produced a distinct antigen, f^+, which was totally absent from F^- cells. The basis for understanding these phenotypic differences became clear after the isolation of bacteriophages (phages) that grew only on cells harbouring F. These donor-specific phages were of two types: isometric phages, which contained RNA (indeed these were the first examples of RNA-containing bacterial viruses), and filamentous phages, which contained a single strand of DNA. When these phages were added to a donor cell population, it was found that they adsorbed to a specific cell structure, which was called the F-pilus by C. C. Brinton Jr. and his associates. The F-pilus appears in the electron microscope as a filament up to 20 μm in length and about 8·5 nm (85 Å) wide with a central axial hole about 3·5 nm (35 Å) in diameter. The isometric donor-specific phages adsorb to the sides of the pilus whereas the filamentous phages adsorb to the pilus tip.

F-pili are an assemblage of a single kind of phosphoglycoprotein molecule, F-pilin. F-pilin is unusual in that four of the commonly occurring amino acids, cysteine, proline, histidine, and arginine, are missing. The functions of the phosphate and glucose molecules are unknown at present. Many enteric bacteria are literally covered with superficially analogous structures, which are generally much shorter (about 1·5 μm in length) and are called common pili, Type 1 pili or fimbriae. Type 1 pili are not concerned with genetic transfer; indeed, their primary function still remains obscure. It should further be noted that F-pili are generally present at only one to eight per donor cell, whereas Type 1 pili are produced all over the cell surface in large numbers.

The frontispiece shows a specific pair. The donor cell appears to be hirsute by virtue of a covering of short Type 1 pili. The prominent F-pilus, covered with adsorbed male-specific phage as a morphological marker, is found to be in direct contact with the recipient cell. In this example, the recipient was deliberately chosen to be morphologically

shorter and plumper and has become 'bald' via a chromosomal mutation in one of the Type 1 pili genes. The experiments of Brinton and his associates, and numerous subsequent studies by others, have clearly established that F-pili are essential for specific pair formation. For example, if F-pili are removed from donor cells by blending, the bacteria can no longer form specific pairs with recipients until the pili are resynthesized. In terms of specific pair formation, donor pili have been likened to grappling hooks. It is implicit, of course, that the tip of the donor pilus and the recipient cell surface must undergo some interaction. As yet, it has not been possible to describe this interaction in any definite morphological or biochemical terms. One can only state that upon mixing donor and recipient cells, pairs form which are bound together over a distance by F-pili, and that these pairs are stable enough to resist gentle pipetting and dilution.

2.5.2 Effective pair formation

Just because two cells form a specific pair does not necessarily mean that a successful conjugal event will follow. Indeed, specific pairs can form even between cells that are dead. A step must follow specific pair formation that initiates a series of biochemical sequences which ensure that genetic material can pass from the donor to the recipient cell. This second step, effective pair formation, is the least-well-understood stage of bacterial conjugation because it is difficult to study experimentally. The central question concerning effective pair formation revolves around the nature of the actual bridge between cells used to conduct the DNA from the donor to the recipient cell. Since the discovery of donor pili, Brinton and others have asserted that the F-pili, with their central axial hole, serve as both the organelle for specific pair formation and as a presynthesized conduit by which the DNA subsequently enters the recipient. This attractive hypothesis has not been confirmed, because no investigator has yet been able to find DNA present within the pilus channel during conjugation. This is by no means a telling argument against the model, since it seems likely that DNA within a donor pilus might easily be lost in isolation of the pili.

Roy Curtiss III, in an excellent review (1969) of bacterial conjugation, has some substantial doubts about the pilus conduction model. He notes, for example, that a feature of the pilus model is that genetic transfer can occur even though mating pairs are separated by several microns from each other. Curtiss cites several lines of evidence to suggest that actual wall-to-wall contact is required for efficient genetic transfer to occur. He proposes therefore that the pilus conduction model must at least be modified to bring about wall-to-wall contact, either by insertion of the pilus into the female or withdrawal of the pilus into the donor. Curtiss' own model does not involve transfer of DNA through the pilus. He visualizes that the formation of effective pairs involves the two-step establishment of a conjugal bridge through which genetic material can be transferred. In the

first step the donor pilus is withdrawn into the donor cell, with expenditure of energy, to bring the donor and recipient cells into direct wall-to-wall contact. The second step is seen as the synthesis of a substance which acts to join the donor and recipient cell membranes between the sites of donor pilus tip attachment to the F^- and the 'hole' remaining in the donor after complete pilus withdrawal[3]. Just as no one has demonstrated DNA within pili, neither has pilus withdrawal into the donor been demonstrated. It seems to me, however, that the weight of the experimental evidence is consistent with F-pili being the critical (and possibly the only) surface structure involved in DNA transfer. As will be pointed out in a later chapter (chapter 8) some nine to ten of the twelve known sex-factor cistrons required for F transfer are concerned in some way with F-pili synthesis—a point which Brinton often cites in his argument that the F-pilus is *the* organelle of DNA transfer.

Whatever may eventually prove to be the conjugation bridge, the specific pairing and effective pair formation steps are highly specific and short-lived. I say specific in the sense that they are solely mediated by sex-factor products and that the conjugation bridge is not simply a general tube between the donor and recipient. During conjugation there is virtually no cytoplasmic mixing between the donor and recipient; only DNA is transferred. The steps are termed short-lived since specific pairs are normally formed within three to five minutes after cell mixing and DNA begins to appear in recipients shortly thereafter. Obviously the cells necessarily undergo surface interaction during this short period, but in addition the sex factor or donor chromosomal DNA must undergo some change preparatory to transfer.

2.5.3 Sex-factor mobilization and transfer

The mobilization and transfer of a sex factor from a donor to a recipient appears to be a special form of DNA replication. The details of the molecular biology of sex factors are given in chapter 6, but the process can be sketched broadly as follows. F and all other sex factors described thus far exist as circular molecules within the bacterial cell. Even when F is present in an Hfr cell, it is still part of one large circular bacterial chromosome. The initial interaction between a donor and a recipient cell presumably 'triggers', via an unspecified biochemical signal, a series of enzymatic events. These prepare (mobilize) the closed molecule to a state permitting linear sequential transfer of the sex factor or attached chromosome to the recipient. An early mobilization step presumably is the action of a specific endonuclease, which 'nicks' the circular molecule

[3] The idea that there can be withdrawal of donor material is not a new concept. It had been noted in interrupted matings that transfer frequency of an early gene quickly reached a peak and, after a plateau, began actually to decrease quite dramatically. This was interpreted, mostly on mathematical grounds, to mean the donor could withdraw its pilus, which still contained chromosomal material.

and exposes a 5′-phosphate end of a single DNA strand. The actual passage of DNA involves the transfer of a single strand, with the exposed 5′-phosphate at the origin, from the donor to the recipient. The complementary strand is synthesized within the recipient. The single strand remaining in the donor is employed as a template; a complementary strand is synthesized and, in this way, the sex-factor genome is preserved within the donor cell. In Hfr transfer, one assumes that the 'nicking' of the chromosome occurs at the site of F attachment and DNA transfer proceeds in a similar manner. It should be noted that in both sex-factor and chromosome transfer the actual DNA strand transferred was one synthesized before mating. The conjugal transfer of DNA therefore seems to employ the energy of DNA replication as its driving force and represents a remarkable spatial and enzymatic separation of the normal replicative process.

2.5.4 Establishment and recombination

The entry of a single strand of sex-factor DNA into a recipient cell requires that a complementary strand be synthesized. The resulting linear double-stranded molecule must be refractory to nucleases within the cell, be able to circularize and eventually to replicate. This is not an automatic sequence of events. All of these steps are complicated and infer that the host-cell synthesizing machinery is cooperative, and that the sex-factor DNA can express a number of enzymes and biochemical control mechanisms. Some of the properties enabling a cell to resist sex-factor establishment will be considered in chapters 7 and 8. On the assumption that conditions are correct, however, successful replication of the sex factor in the recipient is synonomous with establishment, and the cell in turn becomes a genetic vehicle for sex-factor transmission.

A sex factor must satisfy less-stringent requirements for survival than a transferred fragment of bacterial chromosome. Whereas a sex factor inherently possesses all of the necessary genetic information to replicate as a whole, a fragment of chromosome ordinarily does not. Not only must the chromosomal fragment meet most of the criteria for sex-factor establishment but, to be perpetuated, must gain entry into the host chromosome, thereby forming a recombinant chromosome. This event requires synapsis between the transferred fragment and its homologous segment on the recipient genome. It is presumed that the synaptic alignment of homologous regions involves effective recognition and hydrogen bonding between complementary nucleotide base sequences in the two DNA strands. There is experimental evidence favouring physical incorporation of donor DNA by covalent bonds into a recipient genome by substitution of a single donor DNA strand for a single recipient DNA strand, or by replacement of both recipient DNA strands by donor DNA. Most investigators presently favour the view that the complement of the transferred strand is synthesized before recombination. Despite the uncertainty about the

precise physical form of the interacting molecules, the evidence clearly supports a model for recombination which involves the breakage of the recipient chromosomes and the insertion and rejoining of the donor fragment—whether double- or single-stranded. This exchange results in integration of part (or all) of the transferred donor fragment and expulsion of the corresponding part of the F^- chromosome. Once integrated into the recipient genome, the donor DNA fragments are replicated as part of the recombinant chromosome and are transmitted to subsequent daughter cells.

The precise mechanisms by which a sex factor accomplishes the genetic feats of alternative modes of replication and genetic transfer remain largely unknown. One can, however, look to the temperate bacterial virus, phage λ, to see some striking analogies with F and gain a clearer view of the functional controls employed by an element which ordinarily replicates autonomously from the bacterial chromosome.

2.6 Temperate bacteriophages

Bacteriophages (phages) are a unique class of viruses which multiply in bacterial cells. A phage particle is metabolically inert and consists of a single type of nucleic acid encapsulated in a protein coat from which (usually) projects a tubular tail-like structure. A 'typical' phage particle adsorbs to a bacterial cell by its tail and forcibly injects its nucleic acid into the cell. The injected nucleic acid replicates extensively, and directs the intracellular synthesis and assembly of many new phage particles, which are then liberated by phage-directed lysis of the host cell. The T viruses of *E. coli* have been employed as a model system for studying this productive cycle of phage development. Phages, such as the T group, which invariably follow a productive cycle after infection are called 'virulent'. Not all bacteriophages are virulent. Some phages have two alternative paths after infection of a sensitive host. In some cells the phage follows the productive cycle of phage multiplication. In other cells the phage is carried in a latent state called prophage. Phages possessing this added potentiality of 'peaceful coexistence' with the cell are called temperate.

A bacterial cell carrying a prophage is called a lysogen. Lysogenic cells regularly release mature phage particles (at a frequency of about 10^{-3} per cell division) into the medium in which they are grown. In the early 1950s André Lwoff and his associates recognized, by single cell microculture, that every cell in a lysogenic culture has the genetic capacity to produce phage, even though the probability of spontaneous phage release by individual cells is low. It was suggested, therefore, that phage production by lysogenic bacterial cells might be inducible by alteration of growth conditions. Shortly after this suggestion was put forth, Lwoff, Siminovitch and Kjeldgaard found that the exposure of lysogenic cultures to relatively low doses of ultraviolet light was followed, within a short time, by massive cellular lysis and the liberation of very large numbers of virus particles.

Many chemical and physical agents are now known which can lead to the induction of lysogenic bacteria. Most of these inducing agents inhibit host DNA synthesis, and include X-rays, nitrogen mustards, the drug mitomycin C, or simply thymine deprivation. The study of phage maturation in induced lysogenic cultures shows that, in all respects, the major steps are the same as when the virus follows a productive cycle in a sensitive cell. The phenomenon of lysogeny within a substantial fraction of bacterial cells after infection by a temperate virus posed several questions. What was the nature and mode of inheritance of the latent, noninfectious prophage? How is prophage maintained and what factors contribute to the removal of maintenance by induction? What mechanisms control the biochemical decision of a virus to lyse or lysogenize?

2.7 The phage λ of *E. coli* K-12

The *E. coli* K-12 strain used by Lederberg and Tatum (section 2.1) carries a prophage called λ. The fact that *E. coli* K-12 was a lysogen was not detected, however, until 1951. At that time a nonlysogenic, and hence phage-sensitive, derivative was accidentally discovered among survivors of an ultraviolet treatment of the strain during a search for mutants. Phage λ has become, without question, the best-studied virus in the world. The technical advantages of phage λ as an experimental organism, and the scientific achievements realized with this virus, can be appreciated in part by looking through the book *The Bacteriophage Lambda* written by the principal disciples of 'lambdology'. In the following sections I shall attempt to give a broad overview of this phage in order to show how the phage λ's way of perpetuating lysogeny, together with a knowledge of its molecular features, can contribute to an understanding of the operation of sex factors.

2.7.1 The DNA of phage λ

The DNA extracted from a phage λ particle is a double-stranded linear molecule with a molecular weight of about 31×10^6, which is sufficient to accommodate some 40–50 average-sized genes. Phage λ DNA contains about 0·50 mol fraction of guanine + cytosine (G+C) base pairs, and 0·50 mol fraction of adenine + thymine (A + T) base pairs. Despite this seeming monotony, the distribution of nucleotides along the linear DNA chain is far from random. The phage λ DNA molecule can be divided into several segments, which differ by as much as 0·20 mol fraction of G+C. The biological significance of this nonuniform distribution is unknown, although it may have both structural and evolutionary significance. The point is raised only to mention that this is a common feature of both sex-factor and temperate phage DNA, but is not generally a feature of the chromosomes of virulent phages or bacteria. The DNA of phage λ has cohesive ('sticky') ends, hence it can cyclize or form end-to-end aggregates. The 'right' and 'left' ends of the molecule are different so that 'rights' can only join with 'lefts'. This phenomenon is now understood in terms of the

ends of the phage λ DNA molecule actually being short complementary single-stranded polynucleotides on each of the 5′-phosphate ends of the double-stranded molecule (figure 2.6). The reversible joining of ends can proceed spontaneously *in vitro* at elevated concentrations of salt together with gentle heating. This phenomenon occurs more rapidly *in vivo*.

When a particle of phage λ infects a bacterium, it injects a linear DNA molecule into the host cell. The sticky ends of the molecule permit the two ends to close spontaneously to form a hydrogen-bonded ring, which can, in turn, be sealed covalently by a preformed host polynucleotide ligase. Thus within five minutes after infection most phage λ DNA molecules are converted into covalently closed circular molecules. R-factors and other sex factors also enter cells as linear molecules and are rapidly converted into circular molecules (chapter 6). It is not known whether the sex factors have cohesive ends analogous to phage λ DNA. Cohesive ends certainly provide a simple way to make DNA rings, but rings can also be formed by recombination between repeated nucleotide sequences (redundant) regions at the DNA ends. It has also been supposed that cohesive ends may play an important role in promoting recombination between phages. This supposition is based on the observation that phages can recombine with phage λ have similar, if not identical, 'sticky' ends even though their individual overall fine structure may be largely nonhomologous.

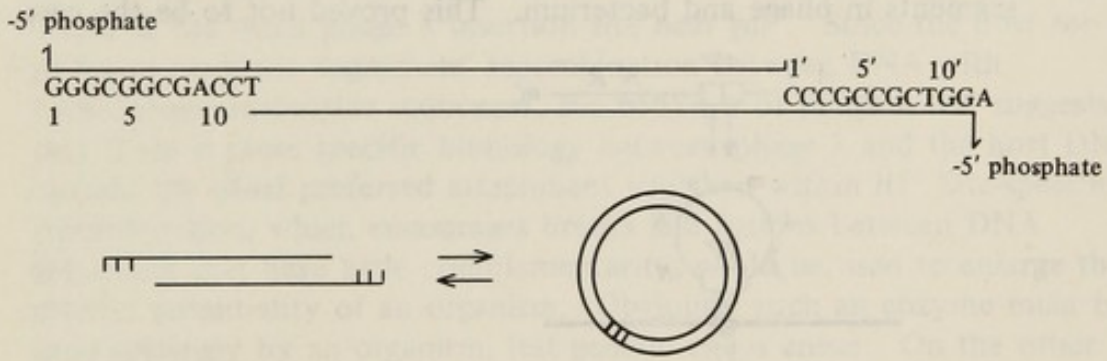

Figure 2.6. The fine structure of the cohesive ends of phage λ has been determined by Wu and Taylor (1971). Each of the twelve nucleotides at the left cohesive end is exactly matched by a complementary sequence at the right end. The diagram shows how these cohesive ends can interact to form a circular structure. The 'gaps' in the circle can be sealed by a host polynucleotide ligase to yield a covalently closed molecule. The end-to-end joining of left and right ends can also occur to generate linear molecules longer than unit length.

2.7.2 Nature of prophage

After the discovery of F it was immediately recognized that it was possible to lysogenize both donor and recipient bacteria, and determine the exact intracellular location of prophage λ by performing bacterial crosses. The answer to the problem of the nature of prophage λ became apparent by mating Hfr nonlysogenic strains (λ^-) with F^- lysogens (λ^+). Prophage λ behaved as if it were attached to the bacterial chromosome adjacent to the

bacterial genes determining the utilization of galactose (*gal*). The isolation of other temperate phages of *E. coli* K-12 from Nature, and the subsequent analysis of the behaviour of their prophage in bacterial matings, confirmed that most prophages were attached to the bacterial chromosome and could be mapped at a particular locus in the same way as any other bacterial determinant. The particular locus for each prophage type was, however, different. That is, attachment of any particular prophage type was site-specific. Thus, after a good deal of study, it could be concluded that prophage λ was formed by insertion of phage λ DNA into the bacterial chromosome at a specific site where it is, thereafter, passively replicated as part of the bacterial chromosome, once each replication cycle. This host–parasite relationship could only be disrupted by the induction phenomenon.

The insertion of prophage DNA was imagined to occur in much the same way as already described for the insertion of F into the bacterial chromosome to form an Hfr cell (figure 2.7). As applied to phage λ, the essential features of this model are (1) the conversion of the infecting DNA from a linear into a circular form by joining ends, and (2) its insertion into the bacterial chromosome by a single reciprocal recombination at specific loci in each DNA molecule. It was originally thought that insertion of the viral chromosome into the bacterial chromosome involved ordinary recombination between homologous DNA segments in phage and bacterium. This proved not to be the case,

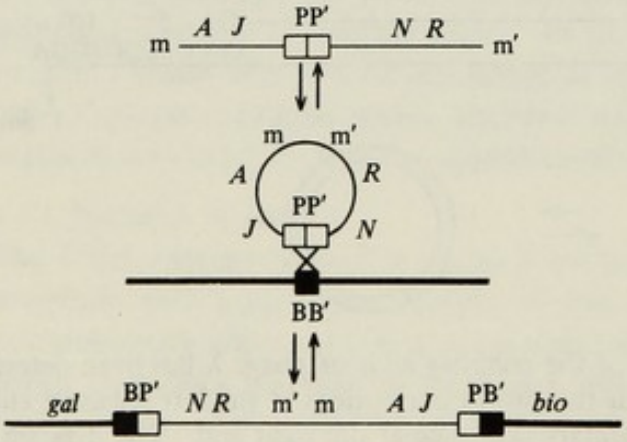

Figure 2.7. Insertion of phage λ into the bacterial chromosome. The light line represents the phage chromosome, and the heavy line represents the bacterial chromosome. The rectangles PP′ and BB′ represent the phage and bacterial sites of insertional recombination respectively. The symbols m and m′ refer to the ends of the linear phage λ DNA molecule; *A*, *J*, *R*, and *N* refer to phage structural genes. The symbols *gal* and *bio* refer to the bacterial genes determining utilization of galactose and the biosynthesis of biotin respectively.

The linear phage molecule (top diagram) is seen to circularize by end-joining followed by a site-specific insertional crossover between the BB′ and PP′ sites (centre of diagram). It can be seen that the order of genetic markers on the resulting prophage has become a cyclic permutation of the order on the free molecules (lower diagram). It should be noted that the process is reversible. All of the features of this proposed mechanism have been confirmed experimentally. (After Campbell, 1971.)

however. Certain bacterial mutants called *rec*$^-$ (recombination-less) are very deficient in carrying out genetic recombination. The introduction of an F factor into such a strain does not affect its ability to replicate, but the F → Hfr transition is virtually abolished. On the other hand, the insertion of λ prophage in *rec*$^-$ bacterial hosts is normal. It is now recognized, from these studies and others with phage λ mutants, that the phage determines a 'site-specific' recombination enzyme (*int*). A separate phage-directed enzyme (*xis*) is additionally employed when the prophage is excised from the chromosome upon induction. The presence of these phage-directed functions controlling insertion and excision probably explains why virtually all cells that survive infection by phage λ are lysogenic, and why practically all lysogenic cells can be induced to release phage. They are highly efficient recombinational processes mediated by specific enzymes. Interestingly enough, the recombination mediated by *int* and *xis* does not require that base pairs involved in the crossover be related by base-pair complementarity. Rather, it is the *int* enzyme which 'recognizes' a specific sequence of nucleotide pairs on both the host and the phage to provide a specific integration pathway. Phage λ *int*$^-$ mutants have almost entirely lost the ability to lysogenize a host. On the rare occasion when a phage λ *int*$^-$ does lysogenize a cell, it probably does so by the bacterial *rec* pathway. Yet these rare λ *int*$^-$ prophages are not found at the usual phage λ insertion site near *gal*$^+$. Since the host *rec* pathway mediates 'legitimate' recombination between DNA with homologous nucleotide sequences, the behavior of phage λ *int*$^-$ suggests that there is more specific homology between phage λ and the host DNA *outside* the usual preferred attachment site than within it! Site-specific recombination, which encourages breaks and fusions between DNA sequences that have little complementarity, could be used to enlarge the genetic potentiality of an organism. Obviously such an enzyme must be used sparingly by an organism, lest genetic chaos ensue. On the other hand it does provide a useful way of thinking about 'illegitimate' recombination between apparently diverse genetic elements. Such illegitimate possibilities have to be considered later on, for example, when R-factor behaviour is examined. A more immediate example of rare 'illegitimate' recombination is the origin of transducing lines of phage λ.

Excision of prophage from the chromosome is generally assumed to be the reverse process of insertion. As already mentioned, excision is an efficient process requiring both *int* and *xis* gene products. Yet a small fraction (about one in 10^5) of prophages excised from the chromosome are deviant. These prophages carry a substitution of bacterial genetic material for a portion of the phage chromosome. The bacterial genes which are substituted are limited to those immediately flanking the prophage. One of these, mentioned already, is *gal*, the other is the bacterial gene determining the synthesis of biotin (*bio*). Since these deviant prophages lack a full complement of phage λ genes, they are

deficient in several viral specific functions. However, more often than not, the deviant phage λ *gal* or λ *bio* DNA is structurally capable of going through all stages of the productive cycle, provided that a normal phage λ (helper virus) is present in the same cell to provide the missing functions. Upon infection of a sensitive host with phage particles containing λ *gal* (λdg) or λ *bio* DNA, host cells may acquire the carried bacterial genes from the original lysogen. This process is called specialized transduction. Of course a λ *gal* molecule entering a host cannot follow a complete productive cycle without a helper, but it can either lysogenize the new host or give up the bacterial gene by legitimate recombination with the chromosome. The origin of transducing lines of phage λ is generally seen as a consequence of abnormal prophage excision, which deletes phage DNA from one end of the prophage and adds bacterial DNA to the other (figure 2.8).

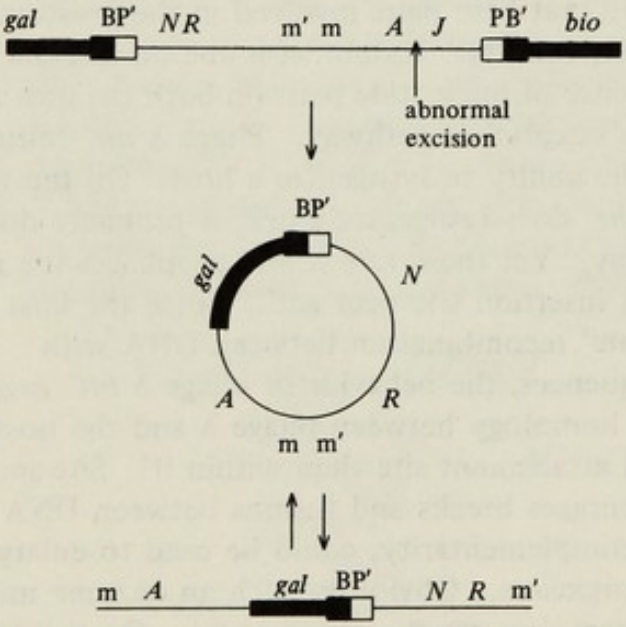

Figure 2.8. Prophage excision and the origin of transducing lines of phage λ. The light line represents the phage chromosome and the heavy line the bacterial chromosome. The rectangles PP′ and BB′ represent the attachment sites on the phage and bacterial chromosome respectively. The symbols m and m′ refer to ends of the linear phage chromosome; *A*, *J*, *N*, and *R* refer to phage structural genes. The symbols *gal* and *bio* refer to bacterial genes determining the utilization of galactose and the biosynthesis of biotin respectively. Ordinarily the prophage (top diagram) is excised by a site-specific crossover between the ends containing the attachment sites. This exchange is mediated by the phage genes *int* and *xis* as described in the text. Normal detachment follows the process seen in figure 2.7, which reconstructs the normal phage molecule and which enters the usual productive cycle. Abnormal excision occurs about one in 10^6 times from an illegitimate recombination between a point within the prophage and a point on the bacterial chromosome. In the example depicted here, an abnormal excision occurs between the *A* and *J* genes, and the subsequent recombinational event deletes the *J* gene from the prophage chromosome, which is replaced by the bacterial *gal* genes. It is also possible for the bacterial *bio* genes to replace part of the prophage by an analogous abnormal excision. The predictions of this model have been confirmed experimentally. (After Campbell, 1971.)

2.7.3 Maintenance of prophage

For phage λ DNA to be inserted into the chromosome as a prophage, something other than just an integration pathway is required in order to repress autonomous phage λ DNA replication, and most other phage functions. This can be dramatically seen in bacterial crosses. In Hfr (λ^-) × F^- (λ^+)crosses, the prophage λ behaves as if it were just another bacterial gene. On the other hand, if the mating is performed between Hfr (λ^+) and F^- (λ^-) parental cells, the outcome is quite different. Lambda is not found on a recombinant chromosome, rather the transfer of the prophage λ is invariably followed by the immediate induction of phage λ and subsequent lysis of the F^- cell. This phenomenon, called zygotic induction by Jacob and Wollman, argues that something is missing from the cytoplasm of a λ^- cell, which leads to the disruption of the prophage–chromosome relationship.

Jacob and Monod saw lysogeny, and the maintenance of the prophage, as but a typical example of gene regulation by a repressor, as elaborated in their 'operon concept'. The operon was viewed as a coordinated functional unit under negative regulation. Based on genetic data with both phage λ and the *lac* genes of *E. coli*, the components of the operon were seen as (1) a regulatory gene, which was responsible for the synthesis of a freely diffusible protein called the repressor; the repressor served as a chemical link between the regulatory gene and its site of action (2), the operator, which, in turn, controlled at the transcriptional level the expression of (3), adjacent structural genes, which carried the genetic information for one or more linked functional activities. The presumption was that the binding of the repressor to its specific site on the operator obstructed transcription of the entire block of adjacent structural genes. In the absence of repressor, the transcription of the operon proceeded normally. An important attribute of a freely diffusible repressor is that it can interact with its specific operator region on a separate chromosome (*trans* effect) as well as its own chromosome (*cis* effect). The operator gene, however, affects the structural genes of only its own chromosome, but not of a homologous chromosome in the same cytoplasm.

The application of the operon concept to lysogeny accounts for many of the known facts about this phenomenon. A repressor of phage λ lytic development is specified by a phage gene called *c*I. This gene was initially discovered because phage λ mutants defective in repressor synthesis were virulent and could not lysogenize sensitive cells. Subsequently the product of the *c*I gene was isolated as a protein with a monomeric molecular weight of about 27000. In its active form the repressor is a tetramer, which binds directly to two operator genes on either side of the *c*I genetic locus. The binding of repressor to phage λ DNA blocks early phage functions required for DNA synthesis. The only major gene products expressed by a prophage is the *c*I repressor protein and a closely linked

gene *rex* whose specific function is not known. Not only does continued synthesis of repressor maintain the lysogenic state, but additionally one can see that it confers immunity against superinfecting phages of the same type as the prophage. That is, a phage λ lysogenic cell can continue to adsorb phage λ particles, and the DNA of the phage can still be injected into the cell. The vegetative functions of the incoming phage DNA are switched off, however, by the *trans* effect of the preformed repressor in the cytoplasm. Thus lysogeny makes the host cell phage-resistant; this may be the major benefit that the host derives from lysogenization.

Induction of the prophage follows inactivation of the *c*I repressor, or, as with zygotic induction, the introduction of a prophage into a repressorless cell. It is presumed that inducing agents, such as ultraviolet light, indirectly inactivate repressor biosynthesis. The suggestion has been made that the release of repression, and efficient replication after induction, may be a response to a signal which seems to indicate to the prophage that the host cell is in imminent danger.

Negative control by the single *c*I repressor protein effectively permits one to understand prophage maintenance, the immunity of lysogenic bacteria to superinfection, and the phenomenon of prophage induction. Lysogeny can be seen as the combined effect of a specific integration pathway coupled with the action of regulatory genes which provide for repression of lytic functions. Generally, the newcomer to phage biology wishes to know what precisely determines the 'decision' of the infecting phage to lyse or lysogenize. Despite the fact that phage λ is the best-studied virus in the world, no one really knows. It is recognized that the frequency with which an infecting phage λ particle initiates prophage replication instead of productive growth depends upon the temperature, the growth state of the bacteria, and the genotypes of both the host and the phage. This is not very revealing since, in a general sense, the same parameters affect most biological phenomena. One recognizes that there must be a very fine biochemical balance between *c*I repressor and other phage functions. For example, the gene that codes for the *int* product necessary for prophage insertion is repressed by the *c*I product. Thus, from the standpoint of efficient lysogeny, *c*I cannot be expressed so quickly as to hinder expression of *int*. To better understand the alternative modes of replication of phage λ, it is necessary to consider in more detail its genetic organization and regulation of development.

2.8 Regulation of phage λ development

2.8.1 Genetic structure of phage λ

Figure 2.9 shows the arrangement of genes on phage λ DNA as deduced from genetic mapping experiments on both vegetative phage and prophage. A special feature of phage λ, and by analogy probably the F factor, is that two different linkage maps can be constructed for the genome, depending upon the stage of its life cycle, that is, autonomous or

integrated. This permutation of gene order is cyclic and reflects the nature of the site-specific insertional recombination between two circular structures. It is apparent from the genetic map that genes with related functions are clustered together on the chromosome. Although such clustering appears to be a 'logical' organization, the full biological significance is unknown because there is obviously no fossil record of phage λ with which one could follow the evolutionary history of this virus. We can only infer that the present phage λ organization represents untold numbers of legitimate and illegitimate recombinational events, coupled with a need to coordinate its physiological activities in order to sustain its dual mode of existence.

Phage λ has sufficient DNA to encode about 50 proteins of molecular weight 33000, and about 35 genes are known. The following is a brief description of some of the more important genes in each functional region.
(1) Head genes. The phage head of λ, which is the icosahedral protein surrounding the DNA, is determined by seven genes (*AWBCDEF*). Mutants defective in any of these genes fail to make functionally active heads, but produce normal amounts of tails.
(2) Tail genes. The tail of phage λ is relatively simple. It is a flexible tube terminating in a fibre, which presumably makes initial effective contact with a sensitive cell. This hollow tube serves as the conduit for injection of DNA. Its structure and synthesis are determined by eleven genes (*ZUVGTHMLKIJ*) just adjacent to the head genes. Mutations in any of these genes abolish the appearance of normal tails in lysates.

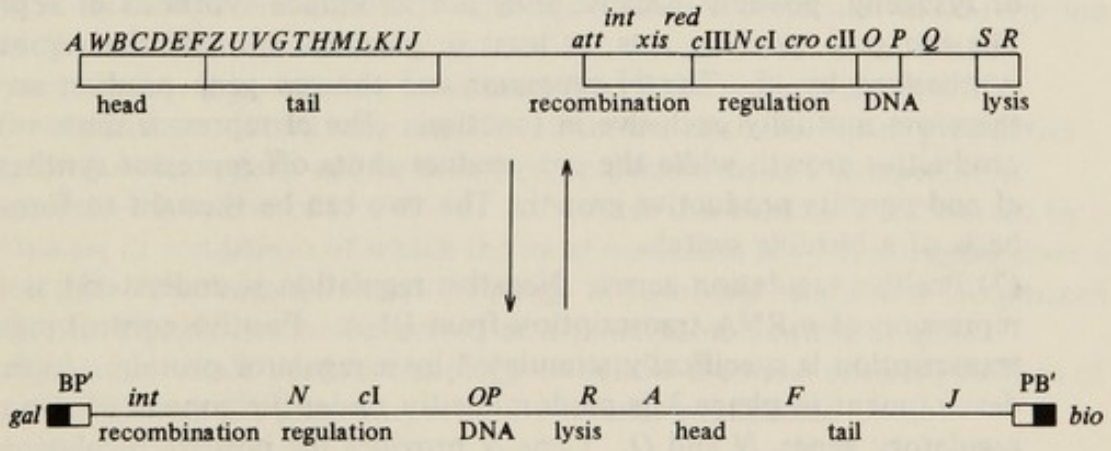

Figure 2.9. A genetic and functional map of phage λ DNA. The gene order in the linear DNA molecules extracted from phage molecules is shown at the top. The gene distances are arbitrary. Genes essential for a normal productive cycle are designated in capital letters (*A*-*Z*). Genes not essential for lytic growth are the *c*I, *c*II, *c*III and *cro* genes involved in regulation, the *int* and *xis* genes concerned with site-specific recombination in the *att* locus during prophage insertion and excision, and the *red* genes, which specify proteins involved in general genetic recombination. The lower sequence is the gene order of the prophage generated by a site-specific recombination event. It can be seen that the order of markers on the prophage is a cyclic permutation of the order on the free phage molecule. (See also figures 2.7 and 2.8.)

(3) Recombination genes. The role of *int* and *xis* genes in bringing about site-specific recombination at a point *att* on the phage and bacterial chromosome has already been discussed. In addition, phage λ possesses genes called *red*, which determine an exonuclease (*red* α), and another protein (*red* β), which is physically attached to the exonuclease but devoid of any known enzymatic activity. The *red* genes are active in bringing about general genetic recombination of two molecules, as distinct from the site-specific recombination mediated by *int* and *xis*. Another gene called gamma (γ) is also involved in general recombination.
(4) DNA synthesis genes. Two genes, *O* and *P*, are needed for phage λ DNA replication. The actual function of these genes is unknown. Some recent experiments suggest that O protein, P protein and the product of a bacterial gene interact, directly or indirectly to initiate replication of phage λ DNA. The host supplies the biosynthetic machinery for DNA precursors of phage λ DNA synthesis. The mode of phage λ replication, which is bidirectional, will be discussed in a later chapter (chapter 6).
(5) Lysis genes. Two genes, *S* and *R*, control the synthesis of enzymes involved in lysis of the host. The gene *R* codes for a lysozyme, which digests the cell-wall substance by splitting a bond between D-amino acids. The precise role of gene *S* is not known. The most recent data indicate that the *S* gene product somehow damages the host cell membrane.
(6) Negative regulation genes. The repressor protein, specified by gene *c*I (responsible for maintenance of prophage), has already been discussed. The products of genes *c*II and *c*III are necessary for the establishment of lysogeny, possibly because they act to induce synthesis of repressor. The *cro* gene product acts, at least in part, to shut off the repressor synthesized by *c*I. The *c*I repressor and the *cro* gene product are therefore mutually exclusive in function. The *c*I repressor shuts off productive growth while the *cro* product shuts off repressor synthesized by *c*I and permits productive growth. The two can be thought to form the basis of a bistable switch.
(7) Positive regulation genes. Negative regulation is understood as the repression of mRNA transcription from DNA. Positive control means that transcription is specifically stimulated by a regulator protein. Lytic development in phage λ is predominantly under the control of two positive regulatory genes, *N* and *Q*. Gene *N* provides for positive regulation of three regions of phage λ DNA: the recombination genes, DNA genes, and *Q* genes. Gene *Q* is a positive regulatory gene directing the synthesis of a protein that markedly activates mRNA and, subsequently, protein synthesis from the lysis genes; head genes; and tail genes. It can be seen that regulation is sequential. Synthesis of the *Q* gene product depends upon direct or indirect activation by the *N* gene product.

2.8.2 Lysis or lysogeny?

The productive growth of phage λ proceeds in stages. Initially in a complete medium at 37°C, there is a slow accumulation of phage λ circular DNA for about 15 minutes, followed by a rapid burst of DNA synthesis to amounts equivalent to sixty linear molecules or more by 20 minutes after infection. Between 20 and 30 minutes, transcription of head- and tail-specific mRNA increases enormously, and mature phage particles begin to accumulate in the cells by about 30 minutes. Host lysis occurs within 50 minutes. It seems likely therefore that the decision for lysis or lysogeny is made sometime before the twentieth minute after infection and is related to the interaction of the regulatory proteins during an early phase of development. Experiments performed to measure the rate of transcription from various segments indicate that an 'immediate early' period after infection involves the *N* gene. The N product formed then positively regulates transcription of N-dependent genes during a 'delayed early' period. The transcription stimulated by N proceeds in two directions. To the left of *N*, the *c*III and recombination gene products are transcribed. To the right of *N*, the *cro*, *c*II, and DNA segments are transcribed. At the early stage after infection (within 5 minutes) biochemical machinery for both lysis and lysogeny are present. The *c*II and *c*III genes induce *c*I repressor synthesis, and the recombination enzymes favouring lysogeny are synthesized. In contrast the *cro* product negatively regulates repressor, while the DNA and *Q* genes set in motion favour lysis. It should be noted that DNA replication is a good deal faster than transcription. It only takes a little more than one minute to replicate a phage λ molecule. Transcription of the entire molecule would take about 10 minutes. Thus, while transcription is proceeding from the DNA, DNA replicas are already accumulating. In the long run, therefore, the choice between productive growth and lysogeny can be seen in simplistic terms as a competition between the action of *c*I and *cro*. Of course this balance is influenced by a number of conditions of which the most notable is the physiological state of the cell. The bistable switch of phage λ, involving *c*I and *cro* temporized by the *N* gene, can be conceived as a remarkable control of gene expression, which permits the phage to follow the most efficient pattern of replication for the prevailing selective cellular conditions.

2.9 Episomes, plasmids, and replicons

This chapter has been concerned with two genetic elements, F and λ, which share the common host, *E. coli*. One is a typical temperate virus. It is potentially lethal and leads a kind of Jekyl–Hyde existence based on a set of complicated controls. The F-factor is, by all criteria, benign. It most often behaves as a supernumerary chromosome, but has modified the bacterial cell to act as a vehicle for genetic transmission. It seems reasonable to assume that F and phage λ have a long evolutionary history of association with their host, and each has adapted to this association in a

unique way. Although it is clear enough that F and phage λ are distinct biological 'species', it is recognized that, at the genetic level, they share some quite basic common properties. Jacob and Wollman, whose work was so important in determining the properties of both F and phage λ, were so struck by these basic similarities, that they suggested them as forming the basis for recognizing a new class of genetic elements, which they named 'episomes'. The episomes were visualized as possessing a constellation of unique properties typified by F and phage λ. In each case the genetic element was additional to the normal genome of the cell. Moreover the presence of an episome was not essential, under most circumstances, for successful growth and metabolism of a host bacterial cell. Episomes were seen to be often infectious, either by promoting their own transfer or by the production of infectious particles. The most important and novel feature of episomes was that they could *replicate in one or the other of two alternative states*—either independently in the cytoplasm or, after insertion, as an integral part of the bacterial chromosome. It can be seen that this definition of episomes excluded both chromosomal genes, for which a state of autonomous replication does not exist, and exclusively cytoplasmic determinants such as virulent phages.

The episome concept had a remarkable impact. The fact that two such seemingly diverse elements as a phage and a sex factor shared these common properties led investigators to seek further examples in Nature. Moreover the episome concept was a useful model to consider with respect to understanding cellular differentiation and the relationship between nuclear and cytoplasmic heredity in animals and plants. Jacob and Wollman prophetically pointed out in 1958 that, while the episome concept was a useful operational definition, one should expect that, through appropriate mutations, a whole series of elements may arise ranging from purely cytoplasmic determinants, some pathogenic, some not, to purely integrated elements.

The discovery of R-factors coincided very closely with the publication of the episome concept. Initially it was more or less simply assumed that R-factors were episomes. R-factors, like F, were transferred by cell-to-cell contact. They were clearly accessory independent genetic elements. The epithet episome, however, always implies some sort of permanent interaction between the host chromosome and the additional element. R-factors, as well as a variety of genetic elements isolated during the past few years, do not appear to have a stable chromosomal state. A number of investigators therefore suggested that the term episome is too restrictive because of its specific connotations concerning chromosomal integration. The term plasmid was defined by Joshua Lederberg to describe simply all extrachromosomal genetic structures that can reproduce autonomously, without regard to whether they could or could not integrate into the host chromosome. To avoid uncertainties and contradictions, I shall use the term plasmid for the remainder of the book.

The substitution of the term plasmid for episome in no way detracts from the basic thrust, that F- and R-factors share a number of common genetic properties. When we think of R-factors, the phage λ and F models are still a most useful way to account for the mechanistic basis for a number of experimental observations. Moreover the better-known F and phage λ systems suggest experiments to try, and analogous molecules to look for, in dealing with R-factors. That is not to say that one simply assumes similarity without trying to confirm or disprove it, but F and phage λ models provide a firm basis for comparison.

All plasmids in bacteria are units of replication or replicons. If a fragment of the bacterial chromosome is introduced into a recipient cell during conjugation between an Hfr cell and an F^-, the piece of chromosome may become integrated by recombination. If recombination does not occur, the fragments of chromosome cannot replicate and are, almost invariably, lost. Plasmids, which are generally less than one-twentieth the size of the bacterial chromosome, contain the information for self-replication, and often do so faster than the host chromosome. If a fragment of chromosome such as *gal* is introduced 'by accident' as a part of F or of phage λ, it then has gained the property of autonomous replication and replicates at the pace of F or phage λ.

Observations such as these led Jacob and Brenner, in 1963, to the following conclusions:

A genetic element such as a plasmid or a bacterial chromosome constitutes a replicon.

Such a unit can replicate only as a whole.

The capacity to behave as a replicon must depend upon the presence and activity of certain specific systems of signals allowing, or preventing, replication.

Jacob and Brenner viewed the replicon as a DNA molecule whose replication is coordinately controlled by the synthesis of a specific initiator, which acts on a unique localized initiation site or replicator. The initiator and replicator are assumed to be specific in that the initiator of an R-factor, for example, recognizes the R-factor replication locus but not that of the bacterial chromosome. In the simplest case a replicon is controlled by a single positive control mechanism in which a sequence-specific substance starts the replication process, and progressive polymerization follows the usual base-pairing rules of DNA structure. It must be recognized, however, that some replicons may have a more complicated series of both positive and negative controls, which control not only initiation, but also the progressive growth of the replicating structure.

The replicon model is a simplified way to account for many of the features of DNA replication in bacteria and their plasmids. In lysogenic bacteria, for example, the repressor synthesized by *c*I prevents the

prophage (replicon) from multiplying, because one presumes the production of its specific initiator is indirectly inhibited. The prophage then behaves as part of the bacterial replicon, and its replication is controlled by the bacterial initiator and replicator which are not repressed. The reverse may also be true. At conjugation it appears that an integrated sex factor takes over replication of the bacterial chromosome. Occasionally, when some genetic tragedy befalls the host replicating system, an integrated prophage or sex factor may take on the chores of replication control for all of the DNA in the cell on a more or less permanent basis. The point is that once the replicon has initiated replication, this continues to completion, and copies all DNA included in its continuity no matter what its origin. If two replicons should fuse into a single one by recombination, then one of the specific systems of replication will ordinarily prevail. An important corollary to the replicon model is that a plasmid (or bacterial chromosome) must coordinate its replication with cellular division so that replicas are properly distributed to daughter cells. It has been most often suggested that the coordination of replication and replicon distribution is accomplished by means of a site of DNA attachment (maintenance site) within the cell. A structural cell component such as the bacterial membrane seems the likely candidate.

In the following chapter the spectrum of plasmids found in bacteria will be considered. The F and phage λ models will serve as the prototypes for comparison, simply because they are the best-known examples of the class. It may be noted here that the only functions absolutely essential for plasmid survival are probably those involved to ensure autonomous replication and replica distribution. Such functions may occupy only a small proportion of the entire plasmid gene complement. The remaining 'nonessential' plasmid functions are those which may be assumed to confer 'transient' evolutional advantage on the plasmid.

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A 'simplified' guide to plasmids

The episome concept in its original context was to draw attention to the genetic properties shared by temperate phages and the prototype sex factor F. Lysogeny is common in bacteria; temperate phages abound. The sex factor of *E. coli* K-12 is not unique but, rather, is a special case of a very general phenomenon. For example, about one-third of freshly isolated *E. coli* strains harbour at least one identifiable transmissible agent. Moreover a growing number of plasmids have been described, some transmissible, some not, which specify drug resistance, the biosynthesis of bacterial antigens, haemolytic activity, enterotoxin production, and other functions. It might seem, at first view, that such a wide range and variety of plasmid-associated functions defy a simple classification. A good deal of this variety is superficial, however, and focuses on physiological function, *per se*, rather than on the genetic properties of the plasmids themselves. One may begin with the biological distinction between plasmids which promote conjugation and those which do not. This distinction is useful for discussion purposes, although on practical grounds it should be understood that plasmid-promoted genetic transfer may occur at a frequency of from 100% down to a level so low that its detection is largely a matter of luck.

3.1 Transmissible plasmids

3.1.1 F-factor

Under ideal conditions the efficiency of conjugation between F^+, Hfr, or F' strains, and a suitable recipient approaches 100%. Essentially all donor cells show F-pili on their cell surfaces. Thus the two principal features of F, production of F-pili and donor ability, are expressed by every cell or, to put it in other terms, the donor function of F is not repressed. The reason for considering donor ability and pilus biosynthesis in terms of repression requires a close examination of some other transmissible plasmids.

3.1.2 Col I plasmid

Many strains of *E. coli* produce diffusible proteins, termed colicins, which are bactericidal to other enteric organisms. Although only colicins will be discussed, it should be noted that production of analogous substances, bacteriocins, is a general phenomenon in many bacterial groups, The ability to produce a colicin is a hereditary potential of a bacterial cell, and bacteria which produce colicins are described as colicinogenic. The actual genetic determinants which specify colicin synthesis are called *col.* Many colicins have been described, primarily by Pierre Fredericq, but for the present purpose one need only be concerned with the genetic determinant of colicin I production, Col I.

If an *E. coli* strain carrying Col I is grown in mixed culture with an F^- strain which is Col^-, a proportion of the F^- cells are converted into Col I. Thus the genetic determinants of Col I are transmissible like F. In fact it can be shown that Col I even promotes transfer of the *E. coli* chromosome at a very low rate (about one in 10^8 cells).

There are two primary differences between F and Col I. First, in an $F^+ \times F^-$ mating, the F^- cells are virtually all infected with F^+ within an hour. In an analogous Col I x F^- mixture usually less than 1% of the F^- cells are converted into Col I after incubation for about five hours. Thus Col I transfer is a low-frequency transfer (LFT) system. If incubation is continued overnight, however, the proportion of Col I-infected cells increases up to 100%. Col I transfer therefore seems to become more efficient as time goes on. It is now well established that only about 10^{-4}–10^{-3} of the cells in an established Col I culture can transfer the plasmid at any given time. Newly infected cells, however, can transfer the Col I plasmid at a frequency of 100% and so constitute a high-frequency transfer (HFT) system, like F. The HFT state is maintained in a newly infected cell for only a few cell generations and finally the more characteristic LFT rate is attained. In other words it appeared that in most Col I cells, in contrast to F^+ cells, transfer was repressed, whereas in newly infected cells Col I was no longer repressed (de-repressed).

In 1967 it occurred to Guy G. Meynell and Alan Lawn that the appearance of conjugating ability in a Col I HFT culture might be associated with the transient appearance of a new structure on the cells, analogous to the sex pilus of F. It was assumed that the high-frequency transfer from newly infected cells occurred because they possessed no preformed repressor for pilus synthesis. When HFT cultures were examined, indeed a new type of pilus was formed, which they termed the I-pilus. The I-pilus was absent from Col^- cells and from LFT cells.

The I-pilus differs both morphologically and antigenically from the F-pilus. Nor does the I-pilus serve as a receptor for F-specific phages. Subsequently Meynell and Lawn isolated, from sewage, filamentious phages which specifically adsorb to the I-pilus but not the F-pilus. Table 3.1 permits a comparison of the properties of F- and I-pili. It can be seen that the F-pilus is considerably longer, with a more distinct axial hole.

Table 3.1. Some characteristics of F- and I-pili. Data from Meynell *et al.* (1968) and Meynell and Lawn (1967).

Pilus	Maximum length (μm)	Width (nm)	Axial canal	Phage adsorbed	
				F	I
F-like	20	6·0–12·0	Often seen	+	−
I-like	1·5	7·5–13·5	Rarely seen	−	+
Type 1	1·5	4·5–10·0	Prominent	−	−

Both types often terminate in knobs, the origin and function of which (if any) remain unknown. The Type I pili have a superficial resemblance to the I-pilus but are readily distinguished by their failure to adsorb either sex-specific phage. It should be noted that both F- and I-sex pili are generally present as only one to eight per donor cell, whereas Type 1 pili are produced all over the cell surface in large numbers.

3.1.3 R-factors

(a) *fi*$^+$ R-*factors.* When R-factors were first described in 1959, their analogy with F seemed apparent. They promoted conjugation and their own transfer, and some R-factors could promote low-frequency transfer of the bacterial chromosome. As they were studied in more detail, however, they seemed to behave more like Col I than F. R-factor transfer occurred far less often (about one in 10^5) than that seen with F. Cultures containing R-factors were, furthermore, not visibly lysed by F- or I-specific phages nor agglutinated by F- or I-specific antisera.

A surprising finding by several investigators was that many R-factors when introduced into an F$^+$, Hfr, or F′ cell actually inhibited the function of F. An R$^+$F$^+$ cell, for example, now conjugated at only the reduced rate of the R-factor. At the same time the R$^+$F$^+$ cell was no longer susceptible to lysis by the F-specific phages nor agglutinated in F-specific antiserum. In HfrR$^+$ strains, the frequency of chromosome transfer was reduced to less than 1% of its normal value. This inhibition of F function was not due to the elimination of the F-factor.

Segregants were isolated from such cultures which had lost R and had regained full F activity. Similarly cells harbouring both an R-factor and an F′, such as F–*lac*$^+$, continued to show the *lac*$^+$ phenotype even though the cells' capacity to transfer the F–*lac*$^+$ agent was reduced enormously. R-factors which had the ability to inhibit the function of F were termed *fi*$^+$ (for fertility inhibition) by Watanabe and his associates. R-factors that did not affect the functioning of F were termed *fi*$^-$. Egawa and Hirota, who studied this phenomenon, suggested in 1962 that the *fi*$^+$ R-factor might produce a cytoplasmic repressor which acted on F to switch off its conjugal, but not replicative, ability. Elinor Meynell and Naomi Datta presented an hypothesis in 1965 based on these facts. Their idea was that the inhibition of F fertility by R reflected a close relationship between F and *fi*$^+$ R-factors rather than a difference.

Meynell and Datta examined the relationship between F and the *fi*$^+$ R-factors by seeing if R$^+$F$^-$ cells were attacked by F-specific phage. It was already known that R$^+$F$^-$ cultures did not support enough F-specific phage multiplication to lead to visible lysis of the whole culture. However, the specificity of the F-specific phages lies in adsorption to the F-pilus, so that even if only a few cells of an R$^+$F$^-$ culture produced specific pili, some phage multiplication should be seen. In addition, since pilus production is correllated with the expression of conjugation, phage-

sensitive R^+ bacteria should be increased in HFT cultures. Their predictions turned out to be correct. When they infected *fi*$^+$ R-factor bacteria with F-specific phages, the phage multiplied in a small proportion of cells. This multiplication was detected by demonstrating a 20–1000-fold increase over the number of virus particles introduced. Moreover the proportion of F-specific phage-sensitive cells increased in HFT cultures in parallel with the proportion of cells which could conjugate and transfer drug resistance. Electron microscopy of HFT cultures showed a pilus resembling the F-pilus. One can see then that these studies show a close relationship between *fi*$^+$ R-factor and F, at least, in the genes determining sex pilus production and pilus specificity. A major difference between the functions of F-factor and R-factor is that the F-factor is normally de-repressed whereas the R-factors are normally repressed. In an F^+R^+ cell therefore, since the genes for both F- and R-pilus synthesis are present and both are repressed by the R-factor, it was thought that repression must be effected by an R-specific cytoplasmic repressor acting to negatively control pilus synthesis.

(b) *De-repressed (drd) mutants of* R-*factors.* If wild-type R^+ cultures conjugate only rarely because of pilus repression, mutant R-factors lacking the repressor should occur. A mutant of an *fi*$^+$ R-factor should give a culture similar to an F^+ culture in which virtually every bacterial cell has sex pili, can conjugate, and is sensitive to F-specific phage. This prediction of the Meynell–Datta hypothesis has been fulfilled. R-factor mutants were selected by Egawa and Hirota by introducing an R-factor into an Hfr strain and selecting for clones in which the function of F was not repressed. The R-factor mutant, termed R100-1, isolated in this way, was transferred by conjugation to an F^- strain. When R100-1 was carried in the F^- strain, a high proportion of the bacteria could transfer drug resistance and most cells could be seen in the electron microscope to be producing pili very similar to F-pili. Meynell and Datta also isolated de-repressed (*drd*) R-factor mutants by looking directly for variants which transferred drug resistance at high frequency rather than by seeking mutants which have lost the ability to repress F. All of these R-factor mutants show about a 300-fold increase in drug-resistance transfer frequency, are visibly lysed by sex-specific phages, and 50–90% of the bacteria are piliated. When these *drd* R-factors are transferred to Hfr, F^+, and F′ bacteria, they no longer repress F-function.

Yet the pili of F- and most R-factors are not totally identical. The pili differ to some extent antigenically and can be distinguished from one another by using antiserum made specific for one kind by adsorption of cross-reacting antibody. Nonetheless, when *drd* R-factors are present in an F^+ or Hfr or F′ cell, two specific types of pili are not present; rather, individual pili are demonstrably mixtures of the two antigens. Thus, while F- and R-factor retain their genetic autonomy, the pili proteins of the

fi$^+$ R-factor and of the F-factor are sufficiently similar to permit their copolymerization and assembly into a single 'hybrid' structure.

Although transfer inhibition of F by *fi*$^+$ R-factors has been explained in terms of a single repressor, from work by Finnegan and Willetts it is now clear that this phenomenon involves several distinct interacting components. One product, now called *fin* (for *f*ertility *in*hibition), specifies an inhibitor component of broad specificity and is functionally identical in most, if not all, *fi*$^+$ plasmids. The other component involved in transfer inhibition is a product, P, of a gene called *tra*P. The P product is synthesized slowly (or is slow acting) and, unlike the *fin* product, the P product(s) are rather plasmid-specific. Both the P product and *fin* product are required for transfer inhibition, and the absence of either one (*fin*$^-$ or *P*$^-$) leads to the de-repressed transfer state described above. Thus when an R-factor enters a cell its immediate retransfer is not inhibited until both the *fin* and P product are synthesized in sufficient quantity to bring about transfer inhibition; this explains the transient HFT phenomenon one observes in dealing with newly R-infected cells. The F-factor is ordinarily de-repressed and is considered to be *fin*$^-$. However, if an *fi*$^+$ R-factor enters an F$^+$ cell, the R-factor *fin* product can react not only with its own P product but also with the P product of the F-factor to form an F transfer inhibitor. This inhibitor acts to prevent synthesis or function of yet another F gene *J*, the absence of which in turn prevents the synthesis or function of a number of other genes concerned with pilus biosynthesis and other transfer functions. Thus transfer inhibition turns out to be a rather complex phenomenon which acts, in the more normal repressed R-factor state, to control the functioning of a whole cluster of genes required for conjugational DNA transfer, without interfering in any way with the genes required for plasmid replication, maintenance, or segregation.

(c) *fi*$^-$ R-*factors.* As noted before, not all R-factors have the ability to repress the function of F, and those that do not are called *fi*$^-$ R-factors. Initially one might be led to suspect that *fi*$^-$ R-factors are nothing more than *drd* mutants. If this were the case the transfer frequency of *fi*$^-$ R-factors would be high and the cultures should show sex pili. Neither of these expectations is borne out, however. The *fi*$^-$ R-factors are transferred by established cultures at no higher frequency than *fi*$^+$ or Col I factors. Similarly, established cultures of *fi*$^-$ R-factors are not visibly lysed by either F or I phages, nor visibly agglutinated by F or I antisera. The *fi*$^-$ R-factors do show the LFT–HFT phenomenon, and *drd* mutants of *fi*$^-$ R-factors can be isolated. The *drd* cultures of many *fi*$^-$ R-factors do, in fact, produce a sex pilus and are lysed by the I-specific phage and agglutinated by I antiserum! Thus the sex factor of many (but not all) *fi*$^-$ R-factors determines the production of pili similar to that determined by the Col I factor. Naturally occurring *fi*$^-$ R-factors are 'normally' repressed in transfer function quite like both the *fi*$^+$ and Col I factors.

Bacteria which harbour both an *fi*$^+$ and *fi*$^-$ de-repressed R-factor show no mixed pili, in contrast to the mixed pili produced by F and *fi*$^+$ R-factors. It may be that the pilus subunits determined by each are so grossly different from those determined by the other that they are incapable of copolymerization. Or it might be that the products of *fi*$^+$ and *fi*$^-$ R-factors may be synthesized and assembled in isolation from one another in the cell. It is still difficult to say which of these alternatives is more likely to be correct. The crux of the matter may well be the physical relationship of the genome of the sex factor to the sex pilus.

3.1.4 Other transmissible plasmids

Transferable factors which confer a variety of characteristics have been described in the Enterobacteriaceae. Many of these were initially recognized because of a property conferred upon the host which seemed unique, for example, lactose fermentation. The author and L. S. Baron in 1962 described a strain of *Salmonella typhi* that could ferment lactose. The *lac*$^+$ property was unstable, which suggested that it was not linked to the bacterial chromosome, and subsequent experiments showed that this *lac*$^+$ property was transmissible by conjugation. This transmissible element, resembling but distinct from F′-factors, was given the name F_o–*lac*$^+$. In a similar way the plasmid P–*lac*$^+$ was isolated from strains of *Proteus* causing urinary tract infections in the USA. More recently, two transmissible elements have been described which appear to be associated with bacterial virulence. These two, Hly and Ent, confer upon certain *E. coli* hosts the ability to synthesize an α-haemolysin and enterotoxin respectively. A common plasmid found along with Ent and Hly is one which carries a determinant controlling the biosynthesis of a surface antigen (K88). Many transmissible Col factors, in addition to Col I, have been described and include Col V-K-30, Col V-K-99, Col V-B-K-200, Col B-K-77, and others.

All of these observations imply the existence of a range of sex factors. One could describe the properties of each in detail and try to draw some conclusions. If, however, one ignores the particular characteristics carried by these plasmids and concentrates only on their sex-factor properties, a rather more satisfactory picture begins to emerge.

Because of the studies of the Meynells, and Datta, and Lawn, the relationship between the different sex factors can be assessed by looking at the specificity of the mechanisms controlling synthesis of the sex pilus, and at the properties of the sex pili themselves. Table 3.2 shows the results obtained for 66 strains carrying plasmids, all of independent origin, when tested for susceptibility to the F and I phages. It can be seen that there were four distinct classes, of which the first two fell unequivocally into typical F-like or I-like factors. The first, F-like, contained most *fi*$^+$ R-factors, no *fi*$^-$ R-factors, Col factors such as Col V-K-94 and Col B-K-77, as well as F, F_o–*lac*$^+$, Ent, Hly, and K88. The second class,

I-like, contained 20 *fi*⁻ R-factors and seven Col factors (most producing Col I). It contained one *fi*⁺ R-factor, namely R-62, which will be described in a subsequent chapter. The third class of cultures harbouring plasmids propagated both F and I phages. This class, we now know, is composed of bacteria carrying both an *fi*⁺ and an *fi*⁻ R-factor. The fourth class, composed of two *fi*⁺ and six *fi*⁻ R-factors, propagated neither the F nor the I phages. This fourth group very likely represents other classes of sex factors. For example, Hajra Khatoon, Rajul Iyer, and V. N. Iyer have isolated a sex-factor-specific phage, provisionally designated Ike, which is specific for some of the *fi*⁻ R-factors which failed to permit multiplication of the I- or F-specific phages. In a similar vein, Olsen and Shipley, as well as Brinton and his collaborators, have isolated phages specific for the pili produced by several different *fi*⁻ R-factors that are unrelated to F, I, or Ike pilus classes. In point of fact, as interest in plasmids and R-factors has grown more and more, investigators have examined enteric organisms other than *E. coli*, *Shigella*, and *Salmonella*, and have encountered a large variety of plasmids which do not neatly fit into the F or I groups. Datta, Hedges, and others have delineated the *fi*⁻ R-factors (and some *fi*⁺ R-factors) into a number of distinct groups based on their genetic properties. These groups will be considered in a later chapter (chapter 7). It has been convenient for the past several years to treat *fi*⁺ R-factors as F-like, and *fi*⁻ R-factors as I-like. Although this broad classification has served a useful purpose, the connotation that all R-factors (or all sex factors) belong to one or other of these two groups is an oversimplification. Many do, but others definitely do not. The 'others' in some cases may be genetic variants of typical F or I structures. F_o–*lac*⁺, for example, differs from other members of the F-related group, since it does not confer sensitivity to isometric F-specific phages but only to the filamentous F phage, M13. Finally sex factors which do not neatly fall into the F-like or I-like grouping are just what they seem to be: distinct entities (distinct plasmid 'species'?) which differ in both genetic and physicochemical organizations from the two prototypes. Indeed in subsequent chapters it will be shown that even sex factors determining the same sex-pilus type are by no means identical.

Table 3.2. Susceptibility of 66 naturally occurring plasmids to phages F and I. Data from Lawn *et al.* (1967) and Meynell *et al.* (1968).

Susceptibility to phage		R-factors		Col factors	Others	Total
F	I	*fi*⁺	*fi*⁻			
+	−	11	0	4	F_1 F_o-*lac*⁺, Hly, Ent, K88 and five others	25
−	+	1	20	7	0	28
+	+	5	0	0	0	5
−	−	2	6	0	0	8

It should be noted here that except for F, Col V (which seems to be a genetic hybrid between an F-like agent and a Col factor), and a rare natural isolate, the vast majority of transmissible plasmids are normally repressed with respect to transfer function and sex-pilus synthesis. It has been suggested that repressed sex factors are likely to be more abundant in Nature because the majority of cells produce no sex pili and are therefore resistant to male-specific phages. The sex-factor-specific phages are relatively common (in raw sewage samples, for example) and their continued presence would be ensured by the occasional cells which do make pili. Thus, in some ways, the use of F as the classical example of a sex factor is sometimes misleading. Most sex factors are not de-repressed. Most sex factors do not readily become integrated into the host chromosome. It should not be overlooked that the *E. coli* K-12 strain enjoyed a sojourn of many years as a laboratory strain before the discovery of its sexual prowess. Some properties of F may therefore now be more the selective product of a laboratory environment than be representative of the 'typical' sex factor encountered in Nature.

For the remainder of this book I generally plan simply to use the broad classification *fi*$^+$ and *fi*$^-$ when speaking of R-factors. If the terms F-like or I-like are employed, it is done with the strict meaning that the agent under discussion does indeed synthesize F- or I-pili, as the case may be. Even though R-factors and other sex factors do not constitute a homogeneous group, it nonetheless seems highly significant that roughly 70% of the transmissible plasmids of *E. coli*, *Salmonella* and *Shigella* studied thus far do belong to either one or the other of but two major classes.

3.2 Nontransmissible plasmids

3.2.1 Col factors

Both F- and I-like Col factors have already been described. There are also Col factors, of which type E is the best known, that are not self-transmissible and may have the property of inducibility: the amount of colicin released by some colicinogenic bacteria is enormously increased after (for example) ultraviolet irradiation. This is a lethal biosynthesis much like the induction of a lysogenic culture and, indeed, the number of copies of Col determinants increases during induction. I do not have space to do justice to studies on these Col factors here, and the reader is referred to full details about individual colicins and their genetic determinants in the reviews of Fredericq, Reeves, Nomura and others. Rather, the concept at this time is that a transfer agent can mediate the conjugal transfer of a second plasmid without the need of a physical attachment between the two.

If a Col E1, F$^-$ culture is mated with an F$^-$ organism, no transfer of Col E1 is detectable. On the other hand when the mating Col E1, F$^+$ × F$^-$, is performed, Col E1 transfer is accomplished with a rather high efficiency. The transfer of Col E1 is not associated with any other donor marker

except the sex factor. The F and Col E1 agents are not physically attached, however, since F is transferred at a higher frequency than the Col factor, and spontaneous segregants, which have lost F but retain Col E1, can be easily isolated. In this case one can say that the transmissible agent has *mobilized* the nontransmissible agent. Clearly, the classification of Col E1 as a nontransmissible plasmid is a misnomer. Perhaps a better term would be a nonself-transmissible plasmid. This is an awkward term, however, and in lieu of anything better the term nontransmissible plasmid will be continued to be employed throughout this book. The term nontransmissible plasmid is used in the connotation that it is deficient of, or lacks sufficient, genetic information to promote its direct genetic transfer. The concept of nontransmissible plasmids and plasmid mobilization applies most significantly in some R-factor systems that will be discussed in chapter 5.

3.2.2 Staphylococcal plasmids

A series of nontransmissible plasmids, associated with antibiotic resistance, have been identified in *Staphylococcus aureus*. It is doubtful whether there is any genetic or direct evolutionary relationship between the staphylococcal plasmids and the enteric R-factors, although resistance to many of the same drugs is involved in both. The staphylococcal plasmids fall into several groups, of which the best-studied are the penicillinase plasmids. These elements have a variety of markers, including determinants for penicillinase production, resistance to erythromycin, and resistance to a group of inorganic ions including mercury, cadmium, lead, and bismuth. Other plasmids carrying resistance to tetracycline, chloramphenicol and kanamycin are also described. Chapter 12 describes these elements more fully.

3.3 Temperate phages

So far phage λ has been employed as the representative temperate phage to describe some properties of a bacterial plasmid. The number of known temperate phages is quite large and many of them have properties somewhat different from those described for phage λ. Even the dividing line between virulent phages and temperate phages is not very sharp. It has already been pointed out that a single mutation in the *c*I gene of phage λ can lead to virulence. Other phages show a queer combination of virulent and temperate phage properties. For example, the male-specific filamentous single-stranded DNA phage, fd, under certain conditions do not lyse infected bacteria nor even kill them very frequently. On the contrary, infected cells continue to grow and divide while the filamentous progeny particles are extruded through the cell wall. If infected cells are maintained under conditions where reinfection is prevented, the ability to produce phage is gradually lost.

I will now briefly consider some specific examples of temperate phages which demonstrate something of the spectrum of genetic behaviour that may be encountered.

3.3.1 Phage P-22

In 1952, Zinder and Lederberg attempted to demonstrate genetic recombination between strains of *Salmonella typhimurium* LT-2. Recombinant species were found, but the production of these was traced to a prophage carried by one of the *Salmonella* strains. The temperate phage called P-22 has a chromosome of about 27×10^6 daltons (*E. coli*, $2{\cdot}5 \times 10^9$ daltons). When phage P-22 lysogenizes a host, it has a site of attachment at a preferred specific location on the chromosome, this site being near two genes determining the biosynthesis of proline. Thus phage P-22, like phage λ, has a unique attachment site. Also, as with phage λ, defective variants of P-22 which have incorporated the adjacent proline bacterial genes can be isolated, so that phage P-22 also can bring about specialized transduction. It was previously stipulated that if phage λ is grown lytically on a host, specialized transducing particles are not seen. This seems logical since one presumes that the prophage must interact with the adjacent chromosome in order to generate the transducing fragment. On the other hand, when phage P-22 is propagated on *Salmonella* strains and the progeny particles are examined to see if they contain any bacterial genes, the result is that transducing fragments are found not only for proline but also for all other bacterial genes. The amount of the bacterial chromosome that can be carried by a single transducing fragment appears limited by the amount of DNA which can fit inside the protein head of the virus. Therefore phage P-22 causes not only specialized transduction during prophage maturation but also generalized transduction during productive growth.

A simple way of visualizing generalized transduction is that, during the autonomous replication of the virus, fragments of the host genetic material are packaged by 'accident' into phage heads and become progeny particles. The frequency of this 'accident' for a given gene is about 10^{-6}; thus in a usual phage P-22 lysate, about 0·1% of all phage particles will contain some bacterial genes rather than viral DNA. On release from a lysed host cell, these particles can be adsorbed to a sensitive host and the injected nucleic acid is now available for recombination. It is important to see that the transducing agent itself is not identical with phage. A transducing fragment of this kind cannot lyse or lysogenize. The virus particle acts instead simply as a vector. To quote from Hayes book (see reference page 36): "It is surely one of the more bizarre manifestations of evolutionary adaptation that a potentially lethal virus should acquire the redeeming function of a gamete, rescuing some of its victim's genes for posterity!".

3.3.2 Phages P1 and P2

In 1951, G. Bertani found that a strain of *E. coli* (the Lisbonne–Carrère strain) was lysogenic for three distinct phages, namely P1, P2, and P3, which could form distinct plaques on *E. coli* as well as on strains of *Shigella dysenteriae*. Very little is known about phage P3 but phages P1 and P2 have been extensively studied. Since they both come from the same host it is instructive to see how much they differ from each other and from the phage λ prototype.

Phage P2, which is about the same size as phage λ, has received a good deal of attention because it cannot be induced by ultraviolet light or most other inducing agents. Nonetheless cells lysogenic for phage P2 are specifically immune, and virulent mutants of P2 can be isolated. Since Hfr strains lysogenic for phage P2 can be mated with F^- phage-sensitive cells without the occurrence of zygotic induction, it seems that phage P2 immunity is quite diverse from that described for phage λ. On the other hand this difference need not have such a complex basis since phage λ can, by mutation, become noninducible.

The λ prophage has a unique attachment site on the *E. coli* chromosome between genes *gal* and *bio*. Moreover, if *E. coli* is made polylysogenic for phage λ, additional λ prophages are all located at (or very near) the unique attachment site. Phage P2 has a preferred attachment site on the *E. coli* chromosome, this being near the genes determining histidine biosynthesis. Yet phage P2 can still establish itself as prophage in at least eight other chromosomal locations far removed from the original insertion site. The possession of more than a single chromosome attachment site is more reminiscent of F than phage λ. Yet, again, the difference between phages λ and P2 may not be so fundamentally complex. If the unique phage λ attachment site is deleted from the chromosome by mutation, phage λ can be found to insert at a new site far removed from the *gal–bio* region. Similar findings have been made when the P-22 attachment site is denied the phage. Thus phages λ and P-22 have the same potential to go to new chromosome sites, but P2 seems to have already evolved this characteristic to a high degree.

P1, the other phage released by the Lisbonne–Carrère strain, has been widely used because, like P-22, it mediates generalized transduction. Since the phage P1 chromosome is about 60×10^6 daltons in size, it can carry twice as much bacterial DNA per fragment as phage P-22 when the host DNA is mistakenly wrapped inside a phage head. The frequency of transduction for phage P1 is about the same order as for phage P-22; each bacterial gene has a probability of transduction of about one per 10^6 phage particles. Phage P1 is also inducible. The most remarkable difference between phage P1 and the other temperate phages we have considered so far is that P1 does *not* have a chromosomal site of attachment. In other words, phage P1 replicates autonomously in both its vegetative and prophage state. Clearly phage P1 has evolved a very effective method of

quiescent replication, which does not involve a specific recombinational system of insertion into the chromosome of the host. At first view this appears to be a most remarkable difference from phages λ, P2, and P-22.

3.3.3 Quiescent replication of phage λ as an extrachromosomal element

If one infects *Salmonella typhimurium* (or other *Salmonella* species) with wild-type phage λ, it can be shown that there is a complete absence of productive growth. In most cases the infection of *Salmonella* by phage λ is simply abortive. When cells are lysogenized, however, the λ genome is not inserted into the host chromosome. Rather the λ 'prophage' is maintained as about ten copies of an independent circular structure. Despite the multiple representation of the λ genome, the regulation of replication and the distribution of the λ copies at cell division is poor, as evidenced by the accumulation of λ^- daughter cells. Moreover the λ copies are not inducible and can be 'cured' from the bacterial host by the same agents (ethidium bromide, acridine orange) as eliminate the sex factor F. This phenomenon has been traced to a *Salmonella* gene whose product inhibits the effective functioning of the *N* gene product so that the phage may not follow either a normal productive or normal lysogenic cycle. Enough *O* and *P* gene products are produced, however, to permit some phage λ replication. In *Salmonella* therefore phage λ behaves phenotypically as if it were an N^- mutant. Indeed this phenomenon is mimicked in *E. coli* by infection of cells with phage λ N^- mutants.

In a somewhat similar vein a deletion mutant of λ phage, λ*dv*, has been isolated which also exists within an infected cell as a multicopy pool of independent circular elements. The λ*dv* plasmid is only about one-eighth the size of wild-type phage λ; it contains the λ immunity region and the *O* and *P* genes, but not *N* or *Q* or any genes outside the *c*I-*P* segment. In both the λ*dv* and the λN^- (genotypic and phenotypic) states the resulting quiescent replication of phage λ, as an extrachromosomal replicon, depends upon an escape from repression (because fully repressed DNA cannot replicate). This condition was met for λ*dv* by mutations in its immediate antecedent phage (it was a $c\mathrm{I}^-$ mutant). For a phage in which *N* function has been affected either by mutation or by a natural host product, the condition is met because the N protein indirectly regulates both *c*I and the late genes. Certainly it would be foolhardy to attempt to equate the quiescent replication of phage P1 with that of λ*dv* or phage λ in *Salmonella*. The λ quiescent plasmid state is characterized by multiple copies and a poor coordination with the division of the host bacterium. In contrast the P1 prophage is effectively distributed to daughter cells and is present as but one or two copies per host chromosome. Phage P1 has effectively coordinated its replication with that of the host bacterium.

It is important to see that all of the phages P1, P-22, P2, and λ, are distinctive biological entities that have solved their individual biological problems in an individual way. One can, of course, infer how a phage such

as P1 may have come about by the behaviour of the λN^- and λdv 'exceptions'. It seems to me, however, that the usefulness of these exceptional phage λ variants is to point out the high evolutionary potential within a plasmid population. There is often at least some vestige of potential in a plasmid for a form of genetic behaviour developed to a high degree in another (apparently unrelated) plasmid. Thus a plasmid, such as λ or an R-factor, may present a quite different constellation of genetic properties after but a single mutation or after introduction into a different bacterial host. Plasmids, by virtue of their infectivity or transmissibility, are potentially more polymorphic than bacterial species and phage λ reflects this most admirably.

3.4 Conclusions

I have tried to present a brief comparative genetic picture of bacterial plasmids. The plasmids have been classified into major categories, which seemed biologically meaningful. By now, however, it is evident that there is no clear-cut line of demarcation between transmissible plasmids, nontransmissible plasmids, and temperate bacteriophages. This is clear from table 3.3, where it is seen that the genetic properties of the plasmids that have been discussed form a spectrum rather than distinct categories. My presentation of plasmid properties has been frankly biassed in favour of the idea that the distinction between viruses and plasmids is not at all clear. This idea is certainly not new. As early as 1953, Hayes suggested that F might be some sort of defective phage. Similarly in 1963, Pierre Fredericq stated: "A phage is a particle that can establish effective contact with a cell and inject into it genetic material. What else is an F^+ cell?" In 1968, E. S. Anderson outlined the relationship between bacterial viruses and transfer factors, basing his argument on Lwoff's well-known definition of a virus as something with an organized infectious phase. The major proponent in recent years of the concept that transmissible bacterial plasmids and their associated pili are essentially viral in nature has been C. C. Brinton, Jr. Brinton considers transmissible plasmids and their pili as *epiviruses*, which are defined as those viruses which make no cell-free virion and must, therefore, infect by cell-to-cell contact. This is not to say that a sex factor is nothing more than a temperate phage that has evolved to the point where it carries its protein coat on the outside of the cell as a specialized structure we now call the sex pilus. Rather each of the scientists cited above simply recognizes the basic biological similarities between bacterial plasmids and the more classical concept of the virus. Indeed the animal virologist was quick to grasp the episome concept, and those working on bacterial plasmids avidly follow the literature of the virologist to gain new insights. Clearly it does not seem unreasonable to accept the idea of a viral origin for a plasmid (or vice versa). In this vein, Brinton considers that virion-forming DNA and RNA F-pilus phages have life cycles almost identical to the 'F epivirus'. In a more general vein,

Table 3.3. A simplified plasmid spectrum of *E. coli*.

Element	Direct transmissibility by conjugation	Free infective particles	Integration into host chromosome	Productive replication	No. of copies per host chromosome during normal host growth	Sex pili	Elimination by acridine orange	10^{-6} x Molecular weight	Guanine + cytosine content of DNA (%)
F	+	−	+	−	1-3	+ (F-like)	+	60	49
Col I	+	−	−	−	1-3	+ (I-like)	−	70	50
R-factor (R1)	+	−	−	−	1-3	+ (F-like)	+	75	52
F_o-*lac*$^+$	+	−	−	(+)[a]	1-8	+ (F-like)	−	65	52
Col E1	−	−	−	(+)[a]	10-30	−	−	6	50
λ	−	+	+	+	1-2	−	−	30	49
P1	−	+	−	+	1-2	−	−	60	46
λ*dv*	−	−	−	+	50-100	−	−	5	47
P2	−	+	+	+	1-2	−	−	22	50

[a] Under certain circumstances, e.g. ultraviolet irradiation.

Lawn, Meynell and Cooke (1971) point out that just as a bacteriophage primarily transfers its own genome but only infrequently transduces unlinked genetic material, so a bacterium converted into a genetic donor by the presence of an autonomous sex factor primarily transfers the sex factor at much higher frequency than unlinked genetic material. Moreover, when two sex factors are present, transfer of one is ordinarily independent of the other—there may even be, as with F and *fi*$^+$ R-factors, phenotypic mixing of the sex-factor products. The work on sex-factor pili therefore reinforces the view of a bacterial donor not as a bacterium endowed with the general property of gene donation, but rather as a cell containing one or more sex factors, each of which, like independent bacteriophages, is primarily responsible for the transfer of its own genome.

Many of the plasmids that have been described are the result of some sort of recombinational mechanism. One can trace the origin of F-*lac*$^+$ as a recombinational event between F and an *E. coli* K-12 host, but one can only infer the origin of the 'naturally occurring' F_o-*lac*$^+$ isolated from the blood stream of a patient with typhoid. It need not be assumed that the acquired *lac*$^+$ property is in any way essential to the plasmid, although it could provide some selective advantage for survival under certain environmental conditions. The fact that a *lac*$^+$ (*Salmonella*) is so difficult to identify in the laboratory is a trivial advantage. More likely it is simply because we are so aware of the *lac*$^+$ property of enteric species that F_o-*lac*$^+$ and P-*lac*$^+$ were isolated and studied. Genetic elements such as F_o-*lac*$^+$ are likely to be a reflection of a large pool of plasmid-linked genes in bacteria.

It is also clear that plasmids recombine with other plasmids. Certainly an agent such as Col V is most easily understood as a recombinational assemblage between an F-like plasmid and a nontransmissible plasmid determining colicin synthesis. To what extent existing plasmids are really recombinational assemblages of more than one replicon remains to be seen, but it will be shown that this is an important concept when dealing with R-factors.

Enough basic information on plasmids in general has been presented to begin a detailed discussion of R-factors. This does not mean that temperate phages, and F and Col factors are left behind. It will be necessary to draw continually on the concepts that have been established with different plasmids to understand the biological and clinical manifestations of transmissible drug resistance.

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3.1 Transmissible plasmids

3.1.2 Col I plasmid

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(See references chapter 12)

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4

The prevalence and ecology of R-factors

It is the intent of this chapter to serve as a prelude to the discussion of R-factor genetics and molecular biology. The genetics of R-factors have a rather limited and isolated meaning unless considered in terms of their prevalence and ecology. To paraphrase John Tyndall, the brightest flashes in the world of genetic theory are incomplete until they have been proved to have counterparts in the world of fact.

4.1 The changing pattern of infection by enteropathogenic species

Because of the clinical and public health implications of R-factors, most data on their prevalence and ecology are available in relation to disease states caused by Gram-negative organisms. The pattern of enteric disease in technologically advanced societies had begun to change well before the introduction of antimicrobials. Widespread epidemics caused by both *Salmonella* and *Shigella* have become relatively less common in coincidence with improvements in sanitation and water-quality control. In most of the western world, shigellosis causes sporadic outbreaks among poorer people and in institutions.

Shigella infection remains as one of the handmaidens of war, and can still cause extensive epidemic disease in those parts of the world that are not blessed with a significant gross national product. A most recent striking example of the latter situation was an extensive epidemic of bacillary dysentery due to the Shiga bacillus (*Shigella dysenteriae* type 1) which occurred during 1969 and 1970 throughout Central America and Mexico. The epidemic was remarkable both because of its extent and its severity. Indeed almost one-fifth of the population of El Salvador was affected and, in line with the central theme, the Shiga bacillus, initially drug-sensitive, acquired an R-factor, R-Su-Sm-Tc-Cm, as the epidemic progressed. Typhoid and other enteric fevers have been largely brought under control in most parts of the world. Of course, other forms of salmonellosis, notably gastroenteritis, still remain as one of the most important problems in communicable disease. There are an estimated 2000000 human cases annually in the USA alone, at an economic burden of some $300000000. But the salmonellosis seen is usually relatively mild and self-limiting, and probably it often goes undetected. In short, extensive epidemic disease by the 'feared' pathogens of the enteric group has become increasingly uncommon and, especially in those areas of the world where the epidemic problem still exists, it is recognized that the answer to disease control rests more with the sanitary engineer than with medicine. The modern problems of shigellosis and salmonellosis are therefore predominantly characteristic of an urban endemic disease, which spreads sporadically in small local outbreaks but is also commonly seen as an isolated case here and there. Both bacillary dysentery and

Salmonella gastroenteritis display well-marked seasonal variations and, at least for shigellosis, there is no prolonged immunity after infection.

Table 4.1. Increasing incidence of drug resistance among *Salmonella* and *Shigella* strains.

Year and location	Organisms	No. of strains examined	Patterns and prevalence of drug resistance (%)			
1958 Japan[a]	*Shigella* sp.	6563	3·8 (Su-Tc-Sm-Cm)			
1966		351	56·0 (Su-Tc-Sm-Cm)			
			Tc	Sm	Km	Tc, Sm
1956–1958 Great Britain[b, c]	*Shigella sonnei*	1888	3·1	0·4	-	-
1959–1960		2942	8·5	5·6	-	0·4
1961–1962		1328	10·2	6·2	-	1·6
1963–1965		2504	22	16·0	-	7·9
1967		533	12	60	0·2	9·0 most R^+
1968		491	21	73	2·5	15·0 most R^+
1969		611	42	70	1·5	34·0 most R^+
Oct.–Dec. 1968 USA[d]	*Shigella sonnei*	47	42 Sm			
April–June 1969		19	63 Sm			
July–Sept. 1969		34	79 Sm			
Oct.–Dec. 1969		68	82 Sm (24·9% R^+)			
			Tc	Cm	Ap	
1958–1959 Netherlands[e]	*Salmonella typhimurium*	-	2	0·7	0	
1963		10836	16·9	3	0	
1964		11292	19·2	1·6	0	
1965		9628	30	1	11·6	
1966		10467	22	1	15 (most also Tc^r)	
			Tc			
Before 1948 USA[f]	*Salmonella typhimurium*	100	<1			
1956–1957		100	5			
1958–1960		158	14			
1962		213	38 (many R^+)			
1963–1964 Great Britain[g]	*Salmonella typhimurium*	712	21 (Su-Sm), R^+			
1964–1965		450	61 (Su-Sm-Tc) R^+			
1955–1970 France[h]	*Salmonella vienna*	17	1 strain (Ap-Su-Sm-Cm-Tc-Km)			
1972		649	99 (at least 3 antibiotics)			
			77 (transmissible Ap-Su-Sm-Cm-Tc-Km)			

[a] Mitsuhashi (1969)
[b] Farrant and Tomlinson (1966)
[c] Davies *et al.* (1968, 1970)
[d] Farrar and Eidson (1971)
[e] Manten *et al.* (1971)
[f] Center for Disease Control, USA
[g] Anderson (1968)
[h] LeMinor (1972)

It was pointed out in chapter 1 that the increased incidence of multiple antibiotic resistance in Japan led to the discovery of R-factors. The inevitable question is whether this experience has been duplicated throughout the world. Has there, indeed, been an increasing incidence of multiple antibiotic resistance in well-characterized enteric pathogens and, if so, does the situation resemble that experienced in Japan?

Table 4.1 shows several well-documented increases in resistance for *Salmonella* and *Shigella* infection in Northwestern Europe and the USA. In every instance large-scale epidemiological surveys have demonstrated a steady increase in resistance to antibiotics commonly employed for treatment of each disease. The large numbers of cultures surveyed often precluded an extensive examination for the transmissibility of the antibiotic resistance, although every investigator performed at least enough experiments to suggest that a significant proportion of the antibiotic resistance(s) under study was indeed transmissible and often of the multiple type. This implication is further reinforced by the data presented in table 4.2, taken from isolated observations of the incidence of R-factors in *Salmonella* and *Shigella* throughout the world.

The data shown in table 4.2 are by no means intended to be comprehensive; they represent but a cross-section of numerous published reports. Furthermore, it is doubtful whether the studies can be compared directly with one another, since the methodology used to detect resistant strains and to detect R-factor transfer varied a good deal. Nonetheless the data seem clear enough. Although the incidence of R-factors in the total *Salmonella* and *Shigella* populations varies throughout the world, it is obvious that the vast majority of drug-resistant isolates owe their resistance to the possession of an R-factor. It seems a reasonable conclusion that, at present, R-factors are the major source of drug-resistance genes in *Salmonella* and *Shigella*. An examination of tables 4.1 and 4.2 reveals that the predominant R-factor type in these various investigations often differs not only from species to species, but also from geographical location to geographical location. The two best-studied situations are Japan and Great Britain.

In Japan the first R-factors isolated from *Shigella* sp. in 1958 carried resistance to tetracycline, streptomycin, sulphonamides, and chloramphenicol, but whereas this pattern of R-factor-mediated resistance in *Shigella* sp. has been predominant to the present time, it is apparently uncommon elsewhere in the world. Resistance to kanamycin and ampicillin is still relatively uncommon in Japan, but is found, particularly to ampicillin, in quite a significant (and increasing) proportion of *Shigella* isolates in both the USA and Europe. This does not mean that these drugs have not been employed in Japan to treat disease, but apparently only that extrachromosomal resistance determinants for these drugs are not as commonly available in the local enteric gene pool.

A survey of the records of incidence of drug resistance in cultures of *Salmonella typhimurium* isolated in Great Britain shows a rather different history. If we start with resistance to sulphonamides and streptomycin in 1963, the predominant R-factor type showed successively resistance to tetracycline, ampicillin and kanamycin, followed by chloramphenicol. So among *Shigella* in Japan there appears to be a more stable population of

Table 4.2. Prevalence of R-factors in *Salmonella* and *Shigella*. Resistance is denoted by the phenotype abbreviations Ap (ampicillin), Cm (chloramphenicol), Km (kanamycin), Tc (tetracycline), Sm (streptomycin), Su (sulphonamides).

Year and location	Organism	Strains	Incidence of R-factors in bacterial population (%)		Predominant resistance pattern conferred by R-factors
			total	drug-resistant	
1952 Japan[a]	*Shigella* sp.	3550	0·03	-	Tc-Sm-Su
1966		351	56	92	Tc-Sm-Su-Cm
1964 Hungary[b]	*Shigella* sp.	103	61	-	Tc-Sm-Su-Cm Sm-Cm Tc
1967 Greece[c]	*Shigella* sp.	31	71		Su-Sm Su-Cm Tc-Su-Sm-Cm-Ap
1967 USA[d]	*Shigella flexneri* and *Shigella sonnei*	539	13	85	Tc-Sm Tc-Sm-Su Tc-Sm-Su-Ap Sm-Ap
1965 USA[e]	*Salmonella* sp.	254	9·8	71	Tc-Sm Tc-Sm-Su Tc-Sm-Su-Ap
1966 USA[f]	*Salmonella* sp.	32	18·7	75	Su-Sm
1964 Great Britain[g]	*Salmonella typhimurium*	450	24	61	Tc-Su-Sm
1967 Greece[h]	*Salmonella* sp. including:	43	49	99	
	Salm. typhi	(21)	(19)	(100)	Tc-Sm-Cm
	Salm. paratyphi	(9)	(67)	(100)	Tc-Sm-Su-Cm-Ap (Km)
1969 Great Britain[i]	*Shigella sonnei*	611	51	-	Su-Sm-Ap Su-Sm-Tc-Ap
1970 Switzerland[j]	*Salmonella*	348		21	Su-Sm-Tc Km-Sm
	Shigella	37		87	Tc-Ap

[a] Mitsuhashi (1969)
[b] Kétyi and Verténvi (1965)
[c] Kontomichalou (1967)
[d] Farrar and Dekle (1967)
[e] Gill and Hook (1966)
[f] Smith (1966)
[g] Anderson (1968)
[h] Kontomichalou (1967)
[i] Davies *et al.* (1970)
[j] Lebek (1972)

R-factor drug-resistance determinants, whereas in *Salm. typhimurium* strains in Great Britain there seems to be a more dynamic situation with the progressive accretion of drug-resistance determinants.

Other than *Salmonella* and *Shigella*, the best-recognized enteropathogenic species are the various specific serotypes of *E. coli* associated with epidemic diarrhoea of the newborn. Indeed, acute diarrhoeal disease is a leading cause of infant morbidity and mortality in all parts of the world. Neomycin has generally been regarded as the treatment of choice for such infections, and a recent inquiry to the Center of Disease Control in the USA revealed that fully 25% of the strains collected from epidemic outbreaks in various areas of the US are now neomycin–kanamycin-resistant, probably by virtue of R-factors.

A most striking illustration of the appearance of R-factors in pathogenic *E. coli* during the course of an outbreak has been described by Sidney Ross and Waheed Kahn. This outbreak, caused by *E. coli* serotype 055:B5, occurred among ten infants, none of whom had received prior antibiotic therapy. Initially the infecting strain was resistant only to sulphonamides. After a course of oral neomycin therapy, however, many of the infants began to shed *E. coli* 055:B5 cells in their stools, and these organisms were resistant to ampicillin–kanamycin–streptomycin or to ampicillin–kanamycin–streptomycin–tetracycline. These resistance genes were uniformly transmissible and, indeed, by the end of treatment, many of the 'normal' *E. coli* bowel residents of these infants were multiply resistant as well. The changes in antibiotic resistance observed in this outbreak, one supposes, reflect on a small scale what has been observed over the years for the *Salmonella* and *Shigella*. The change from sensitive to resistant can be regarded as a result of the interaction and transfer of R-factor plasmids between pathogenic and nonpathogenic species inhabiting the intestine.

Despite the general tendency for enteropathogenic bacteria to become increasingly resistant to antibiotics, it would be a mistake simply to assume that this has not been an uninterrupted process. The data of W. N. Farrant and A. J. H. Tomlinson determined for *Shigella sonnei* clearly demonstrate that the rise and fall of restricted local outbreaks of disease are often associated with a change in the colicin type and, as well, in the antibiotic-resistance pattern of the causative organism.

One striking example of this kind has occurred in Atlanta, Georgia (W. E. Farrar, personal communication). Sporadic outbreaks and endemic foci of *Shig. sonnei* in Atlanta had been predominantly due to only one or two colicin types, of which about 20% carried R-factors conferring resistance to streptomycin–tetracycline–sulphonamides. After a steady state lasting roughly three years, the 'local' *Shig. sonnei* has abruptly been supplemented by a new colicin type of *Shig. sonnei*, which is rather uniformly (thus far) drug-sensitive. The means by which an epidemic strain suddenly dies out and is subsequently replaced by an apparently

different type is not absolutely clear. It may be that the epidemic or endemic strain fails to infect new individuals at the rate at which convalescent carriers ceased excreting the organism or, as Farrant and Tomlinson speculate, that new epidemic strains possess 'special' inherent characteristics which give them selective advantage over the majority of other strains of the same species. Might such 'special' abilities also be plasmid-borne?

It is significant in the latter context to note that fully 80% of the cases of diarrhoeal disease seen by physicians cannot be ascribed to any known bacterial, viral, or parasitic aetiological agent. Even making allowances for diarrhoeal disease caused by functional, pyschological, or chemical effects, it is clear that a significant proportion of these cases are infections caused by 'normal' bowel residents. An increasing body of data suggests that many episodes of diarrhoeal disease (variously described by such colourful terms as 'Montezuma's Revenge', 'Guppy-Tummy', 'Delhi Belly' or, more delicately, as 'Traveller's Diarrhoea'), are indeed caused by 'normal' *E. coli* serotypes or other enteric species which possess a plasmid specifying the synthesis of an enterotoxin. These plasmids are analogous to the Ent plasmid associated with scours in calves and toxigenic disease in swine.

It seems additionally significant that for the initial isolates of the Shiga bacillus from the recent Central American outbreak, which were drug-sensitive, the oral (50% infective) dose was some 10000 cells, whereas isolates made at virtually the same time which had acquired an R-factor possessed an oral (50% infective) dose of some 1000 organisms. I hasten to add that the R-factor itself is not the culprit for the apparent increased infectivity of this strain, and many possibilities exist to explain these findings. But it does seem worthwhile to consider the idea that factors, such as Ent, contributing to the pathogenicity of organisms, may become more widely disseminated among bacterial species because of the selection of a large reservoir of R-(sex) factors within enteric pathogens. The contribution of plasmids to bacterial pathogenicity is more thoroughly covered in chapter 11.

4.2 Infection by nonenteropathogenic Gram-negative bacilli in hospitalized patients

Most surveys performed on the incidence of R-factors in enteric species have been performed on institutionalized patients. It has been recognized for the past several decades that infections with the common enteric species *E. coli, Klebsiella, Proteus, Serratia,* and *Pseudomonas* have increased appreciably in hospital practice. As an example of the ascendance of Gram-negative infections, one can cite the experience at the Boston City Hospital in the USA. In 1935, only some 12% of the episodes of bacteraemia and 9% of the bacteraemic deaths were caused by enterobacteria, but by 1957, 34·5% of the bacteraemic deaths were attributed to Gram-negative species. The increased prevalence of these

serious infections is related to a number of diverse factors. For example, previously devastating infections caused by the pyogenic Gram-positive cocci, the tubercle bacilli, and other infectious agents are now often primarily treated on an out-patient basis with antimicrobial therapy. The hospital population has therefore changed significantly, and the patients at risk are far more likely to be debilitated individuals at the age extremes and with chronic disabling disorders. Many surgical procedures today require long periods of hospitalization, coupled with the use of cytotoxic and immunosuppressive drug therapy. Not surprisingly then, there is a direct temporal relationship between the change in incidence and severity of Gram-negative infections seen in hospitals, and the increased use of antimicrobial drugs. Resistant enteric organisms of relatively low pathogenicity may emerge in large numbers under the selection of antibiotics. These organisms are more likely to invade the tissues when the host's resistance has been diminished.

Additionally an individual admitted to a hospital without an overt infection has a significant risk of acquiring one. Such hospital-acquired (nosocomial) infections are increasingly associated with Gram-negative species. Table 4.3 shows, for example, the incidence of bacteria isolated from skin infections acquired during hospitalization compared with infections of the same sort acquired in the community. Whereas well over one-half of the infections acquired in the community were caused by Gram-positive pyogenic cocci, the reverse was true in the hospital-acquired infections, in which Gram-negative species predominated. Indeed, in many surveys, Gram-negative bacilli other than *E. coli* account for 36% of the bacterial isolates from all forms of hospital-acquired infection, in contrast to only 18% of the isolates in all community-acquired infections.

Table 4.3. Incidence of bacteria in nosocomial and community-acquired infections of the skin and subcutaneous tissues. [Data from Barrett *et al.* (1968).]

Organism	Community-acquired infection[a] (%)	Hospital-acquired infection[b] (%)
Staphylococcus aureus	44	21
Group A *Streptococcus*	21	0
Enterococcus	0	14
Escherichia coli	9	18
Klebsiella-Enterobacter	9	14
Proteus	6	11
Pseudomonas aeruginosa	3	11
Others	8	11
	100	100

[a] Community-acquired infection was defined as clinically active infection that was present or that could be assumed to be incubating at the time of hospital admission.

[b] Patients were considered to have a hospital-acquired infection if they had clinical evidence of active infection that was not present or possibly incubating at the time of admission but was clearly manifest at the time of survey.

The national average in the USA is roughly five patients with nosocomial infections per 100 patients discharged, with more than 51% of all forms of nosocomial infection caused by *E. coli, Proteus, Klebsiella, Enterobacter,* and *Pseudomonas.*

It is against this background that the data of table 4.4 are presented on the prevalence of R-factors in nonenteropathogenic enteric species. These studies deal almost exclusively with clinical isolates. It is obvious that a significant proportion of the organisms examined were multiply resistant because they harboured R-factors. This is not to say that drug-resistant Gram-negative bacteria originate primarily in hospitalized patients. As will be discussed shortly, enteric bacteria harbouring R-factors are not uncommon in 'healthy' individuals who have no recent history of hospitalization or exposure to antimicrobials. It should be recognized, nonetheless, that not only is one dealing with a special population at risk, but also that environmental selection in many hospitals provides ample opportunity for colonization of patients, hospital personnel, and hospital equipment with antibiotic-resistant strains, which may serve as a reservoir for nosocomial infections.

How intense is this selective force in hospitals? Well, Gardner and Smith report that during January–February, 1968, almost three-quarters of the patients admitted to a surgical ward received, on average, three antimicrobial agents for fifteen days! Prophylactic antibiotics were administered to 48% of these patients. Isenberg and Berkman likewise give a fascinating insight into the selective pressures in our institutions. They point out that, in an average hospital, fully one-third of the patients receive antibiotics, which are administered intramuscularly, intravenously, or directly into an infusion set. No matter what the route of parenteral injection, no physician or nurse will carry out the procedure without first clearing the syringe needle by the time-honoured process of expelling a small portion of the syringe contents. The resultant aerosol, says Isenberg and Berkman, rarely exceeds 0·1 ml. Nevertheless, they estimate that, in the average three-hundred bed hospital, this procedure alone adds 15–30 litres of high-potency antimicrobial agents to the patients' environment yearly. Under such massive selection, the hospital emerges as an environment which caters to resistant microbial forms.

This intense selective pressure changes from time to time as new drugs are introduced and older drugs become less useful. Figure 4.1 shows, for example, the changing incidence of drug use in an urban university hospital in the southern USA. Ampicillin (and cephalothin) use has increased enormously since 1965, whereas the use of tetracycline, chloramphenicol, and sulphonamides decreased substantially. The use of streptomycin and kanamycin (not shown) remained relatively constant. In this same hospital the incidence of ampicillin-resistant *E. coli* isolates has increased progressively and, at present, fully 47% of the strains owe their ampicillin resistance to the presence of an R-factor. As was the case

Table 4.4. Prevalence of R-factors in clinical isolates. See table 4.2 for identification of Ap, Cm, Km, Tc, Sm, and Su.

Year and location	Organism	Source	Strains	Incidence of R-factors in bacterial population (%)		Predominant resistance pattern conferred by R-factors
				total	drug-resistant	
1966 USA (Boston)[a]	*Escherichia coli*	Various	10		70	Tc-Su-Sm-Cm-Km
	Proteus		8			Tc-Su-Sm-Cm
	'Klebsiella aerogenes'		21		25	Su-Sm
						Su
	Serratia		7		33	Tc-Su-Sm-Cm
					43	Tc-Su-Sm-Cm-Km
1966 USA (Boston)[b]	Various, include:	Urinary tract	100	53	69	Tc-Su-Sm
	(*Escherichia coli*)		(65)	(49)	(76)	
	(*Proteus*)		(18)	(55)	(54)	
	(*Klebsiella*)		(10)	(60)	(60)	
	(*Pseudomonas*)		(7)	(71)	(75)	
1967 Germany[c]	*Escherichia coli*	Various	439	90	99	Tc-Su-Sm-Cm
						Su-Sm-Cm
						Tc-Su-Sm
1967 USA (Washington, DC)[d]	*Escherichia coli*	Various	63	28	50	Tc-Su-Sm-Km-Ap
						Tc-Su-Sm-Cm-Km-Ap
1968 USA (Birmingham, Alabama)[e]	*Escherichia coli*	Various	398	18·6 (University Hospital)	53	Tc-Sm-Ap
				38·9 (Veterans Hospital)	62	Tc-Sm
				15·7 (Childrens Hospital)	60	
1966 USA (New York)[f]	*Escherichia coli*	Lesions	25		68	Various
	Klebsiella–Enterobacter	Lesions	28		68	Tc-Sm-Cm-Km
						Tc-Sm-Cm
	'Coliforms'	Intestinal carriers	18		66	Sm

Year and location	Organism	Source	Strains	Incidence of R-factors in bacterial population (%)		Predominant resistance pattern conferred by R-factors
				total	drug-resistant	
1967 Greece[g]	*E. coli* and *Klebsiella pneumoniae*	Patients before antibiotic treatment	25	32	50	Tc
		Patients after antibiotic treatment	35	83	83	Tc-Su-Cm Tc-Su-Sm-Ap Tc-Su-Sm-Km-Ap
1968 USA (Virginia)[h]	*Klebsiella pneumoniae*	Various	81		79	Sm-Cm-Km Tc-Sm-Ap Tc-Sm-Cm
	Enterobacter aerogenes	Various	90		14	Tc-Sm-Ap
1969 USA[i]	*Klebsiella pneumoniae*	Various	358	58	91	Tc-Sm-Cm-Km
1970 Switzerland[j]	*Escherichia coli*	Various	840	-	63	Tc; Ap; Su-Tc; Su-Sm-Tc-Ap-Cm
	Klebsiella		349		41·5	Su-Sm-Tc-Ap-Cm
	Proteus		330		26·4	Su-Ap Su-Tc-Ap
1972 (January–June) Great Britain[k]	Enterobacteria (*E. coli*, *Klebsiella*, *Proteus*, etc.)	Burns	339	-	54	Tc-Km-Cm-Ap
	Pseudomonas aeruginosa		196	-	11·2	Tc-Km-Cm-Ap

[a] Egawa *et al.* (1969)
[b] Smith and Armour (1966)
[c] Lebek (1969)
[d] Falkow (unpublished work)
[e] Gunter and Feary (1968)
[f] Salzman and Klemm (1966)
[g] Kontomichalou (1967)
[h] Hinshaw *et al.* (1969)
[i] Gardner and Smith (1969)
[j] Lebek (1972)
[k] Lowbury *et al.* (1972)

for enteropathogenic enteric species, table 4.3 indicates that when a Gram-negative clinical isolate displays multiple drug resistance, that resistance is not often due to R-factors.

Tables 4.1, 4.2, and 4.4 show that R-factors are not restricted merely to pathogenic species or just to closely related bacteria such as *E. coli* and *Shigella* sp. R-factors are found in all enteric species. Since R-factors are acquired by genetic transfer, this observation may appear to be a bit odd—ordinarily one does not expect mating between diverse organisms. The answer to this apparent promiscuity of genetic transfer lies in the inherent autonomy of R-factors, which will be explored in some detail in later chapters. There are also some striking differences in the incidence of R-factors among particular enteric species. These differences may reflect variability in recipient ability for R-factors or may simply reaffirm the difference in the distribution of microorganisms in the community as compared with a hospital—both of these alternatives could apply. The case of *Klebsiella pneumoniae* and *Enterobacter aerogenes* may serve as an instructive example. On a molecular basis these two organisms show a marked divergence in their genetic fine structure despite their close phenotypic similarity (T. Hemmaplardh and S. Falkow, unpublished work). Yet these organisms are often lumped together; in fact it is a common practice for hospital laboratories to report them as a single group. Perhaps this obscures important epidemiological differences. One study cited in table 4.3 showed that the incidence of R-factors in *Klebsiella* was about six times more frequent than in *Enterobacter*. Moreover the prevalent R-factor type in each was dramatically different. My own

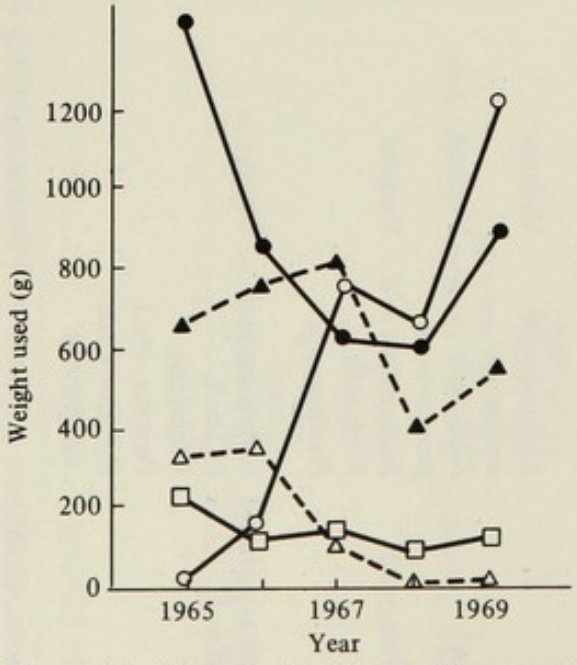

Figure 4.1. Changing incidence of drug use in a single hospital (data from W. Edmund Farrar, personal communication, 1970). ○, Ampicillin; △, chloramphenicol; ●, tetracycline; □, streptomycin; ▲, sulphonamides.

experience has been that *Klebsiella* is a far more common culprit in serious nosocomial epidemic disease than is *Enterobacter*.

These observations are mentioned here only to point out that the genetic properties of the host are equally as important as the genetic properties of the R-factor, and must always be taken into account. Moreover the genetic plasticity of some hosts, coupled with the intensive drug selection in the environment, may spawn a Gram-negative flora in the hospital environment which is quite different from that in the community at large. Nosocomial infection will again be considered in some detail in chapter 10.

4.3 The prevalence of R-factors in normal intestinal flora

The normal human intestine contains large numbers of enteric species, which obviously could constitute an enormous potential host population for R-factors. If R-factors are so prevalent in enteropathogenic species and in clinical isolates of nonenteropathogenic enteric organisms, what then is the incidence of R-factors in the intestinal tract of healthy people in the community at large?

One immediately recognizes, of course, that it is difficult to find individuals in a modern society who have not experienced some form of antimicrobial therapy. Consequently several investigators have gone to remote regions, where antibiotics have not been employed, to sample the flora of the inhabitants and their domestic animals. These data, listed in table 4.5, from 'pre-antibiotic' societies show that R-factors are indeed sometimes present. The significance of finding R-factors in the absence of any form of concentrated antibiotic selection will be discussed at some length later (chapter 9) when the possible origins of the drug-resistance genes carried by R-factors are considered.

The reported incidence of R-factors in the normal intestinal bacteria of healthy urban adults and children of the Netherlands, Great Britain, and USA is shown in table 4.6. Grouped together with these studies are several surveys of the incidence of R-factors in raw sewage and polluted water, which may also reflect the incidence of infectious drug resistance in normal intestinal bacteria. The total incidence of drug-resistant bacteria shows rather wide variation from study to study, reflecting, no doubt, both differences in local ecology and differences in the methodology employed. The study cited in tables 4.5 and 4.6 of normal human intestinal carriage of resistance in the Netherlands, for example, shows the different range and results obtained with differing methods of selection for drug-resistant strains. Notwithstanding methodological and local differences, however, it appears quite clear that a significant number of presumably healthy people carry antibiotic-resistant enteric species in their intestinal tract. And, in every instance, a large proportion of these resistant bacteria harbour a transmissible R-factor. In the main, published data over the years show that the incidence of resistance in normal *E. coli* and coliforms has risen like that of the enteropathogenic enteric species, but at a slower rate.

Table 4.5. Prevalence of R-factors in 'pre-antibiotic' societies. See table 4.2 for identification of Ap, Cm, Km, Tc, Sm, and Su.

Year and location	Organism	Source	Number investigated	Incidence of drug-resistant bacteria (%)	Incidence of R-factors in drug-resistant bacterial population (%)	Predominant resistance patterns conferred by R-factor
1968 E. Malaysia (N. Borneo)[a]	Various	Stool	1017 strains	40 (resistant to two or more drugs)	12	Su-Sm-Tc-Ap Su-Sm-Tc-Cm
1968 Rhodesia[b]	Various	Stool	47 faecal samples	10 (contained drug-resistant bacteria)	0	
1968 Solomon Is.[c]	*Escherichia coli* *Alcaligenes* sp.	Stool Soil			"Two strains from 40 specimens"	Su-Sm
1969 Australia[d]	*Escherichia coli*	Stool	247 strains	16	<1	

[a] Davis and Anandan (1970)
[b] Mare (1968)
[c] Gardner *et al.* (1969)
[d] F. J. Skerman and S. Falkow (unpublished work)

If normal bowel bacteria have been acquiring more drug resistance because of R-factors in recent years, has there been any reflection of this in less serious human infections, which are most often successfully treated in the physician's office rather than in a hospital? Naomi Datta has examined this question by studying infection of the urinary tract as seen by general practitioners (Datta, 1969). Urinary tract infections are particularly suitable for such a study for a number of reasons. They occur in adults at a frequency second only to respiratory infections. Over 65% of the infections are caused by *E. coli* and, in general, the patient's own faeces are the immediate reservoir of infection[4]. Among the *E. coli* infecting 37 patients studied by Datta, seven strains (19%) were resistant to one or more of the drugs tested. This resistance was plasmid-associated in six of seven strains. Simultaneous stool cultures indicated that carriage of predominantly resistant intestinal *E. coli* correlated with resistant urinary infection.

A particularly striking feature of Datta's work was that she followed the effect of the ordinary therapeutic courses of sulphonamide, ampicillin or tetracycline on the resident intestinal flora. The most significant effect was observed in patients undergoing tetracycline therapy. Before therapy some 22% of the patients studied were found to harbour tetracycline-resistant organisms in their bowel. After one week of therapy, the *E. coli* excreted showed uniform tetracycline-resistance coupled with an increase in many strains of resistance to ampicillin, streptomycin, chloramphenicol and sulphonamides as well. The observation, to me, is quite interesting since it has been my impression from published studies such as those reported in table 4.6, as well as our own clinical experience, that R-factors isolated from healthy individuals or existing in patients before antibiotic therapy commonly show resistance to only one or two drugs, particularly tetracycline and sulphonamides–streptomycin. Yet it seems that R-factors isolated from epidemics, from hospitalized patients, and other individuals after antibiotic therapy, more often exhibit resistance patterns involving three or more 'resistances'. In the main, drug resistance mediated by R-factors is not yet a critical clinical problem in or out of the hospital, simply because there is generally a sufficiently large choice of suitable therapeutic agents which can be employed against most common resistant strains. Datta warns us, however, to watch out for further spread of resistance and, particularly, for further accumulation of multiple linked-resistance genes, all of which can be favoured by the use of only one drug (Datta, 1971).

The surveys cited in this chapter generate a number of questions. Among the more obvious are: What were the selective pressures which led to the accumulation of R-factors in members of 'pre-antibiotic'

[4] A further significant finding is that, of the 140 or so possible *E. coli* O serogroups, usually only relatively few O types are responsible for the major fraction of infections of the urinary tract.

Table 4.6. Prevalence of R-factors in the intestinal tract of healthy people.

Year and location	Organism	Source	Number investigated	Incidence of drug-resistant bacteria (%)	Incidence of R-factors (%)	Incidence of R-factors in drug-resistant bacterial population (%)	Predominant resistance patterns conferred by R-factors
1968–1969 Netherlands[a]	*Escherichia coli*	Stool	400 military kitchen personnel	38–45	18–21	–	
			86 office employees	23–38	8–16	62% of all multiresistant *E. coli* transferred one or more resistances	No predominant pattern reported: Tc was most common determinant transferred, followed respectively by Sm, Cm and Km
			77 vegetarians	36–52	9–16		
			87 babies (<6 months, living at home)	32–49			
1969 Republic of Ireland[b]	Predominantly *Escherichia coli*	Stool	100 faecal samples urban infants	71	–	89	Tc
			100 faecal samples rural infants	54		76	Sm-Ap
1969 Great Britain[c]	*Escherichia coli*	Stools of patients before admission for elective surgery	139 strains	81		65	Tc; Tc-Sm-Su
	Klebsiella and *Enterobacter*		50 strains	34		7	Su-Cm-Km
	'Coliforms'	Stool	37 strains	23		3	Various

Year and location	Organism	Source	Number investigated	Incidence of drug-resistant bacteria (%)	Incidence of R-factors (%)	Incidence of R-factors in drug-resistant bacterial population (%)	Predominant resistance patterns conferred by R-factors
1970 USA[d]	*Escherichia coli*	Stool	36 normal subjects	50	12	50% of multiresistant strains	Tc; Sm-Ap
1970 Great Britain[e]	'Coliforms'	River water		16·6		70·2% of Cm transmissible	
1969 USA[f]	'Coliforms'	Raw sewage	20 samples	~1		50	Tc-Sm-Cm Sm-Tc Sm-Tc-Ap
1970 USA (Washington, DC)[g]	*Escherichia coli*	River water	5 samples	8		50	Su-Sm-Ap Sm-Ap Sm-Tc-Ap

[a] Guinee *et al.* (1970)
[b] Moorhouse (1969)
[c] Datta (1969)
[d] S. Falkow (unpublished work)
[e] Smith (1970)
[f] Sturtevant and Feary (1969)
[g] S. Falkow (unpublished work)

societies? What are the origins of the resistance genes carried by R-factors? Are there distinct R-factor populations in hospitals and the general community or is the degree of antibiotic selection the only difference? Do certain bacterial hosts favour certain R-factor types or vice versa? What may be the effect of R-factors on the subsequent evolution of enteric bacteria—and of enteric disease? These questions and surely others in the mind of the reader are not just questions of medical importance; in a large sense they could also be applied, in modified form, to other plasmid classes. In the succeeding chapters of this book, especially chapters 9, 10, and 11, many of these questions will be raised again and examined in light of the genetic and molecular organization of R-factors.

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The genetic properties of R-factors

People who study R-factors have a relatively unique opportunity. They can monitor both the extent of change and the genetic basis of change in natural bacterial populations which have been subjected to a deliberate massive alteration in environment. Consequently thousands of bacterial strains have been isolated from hospital patients and the community at large, and their R-factors studied to some degree. It is, however, difficult enough to describe the genetics of the classical F-factor, which has been retained in a single strain of *E. coli* since the twenties; it is considerably more difficult to describe genetic properties of a large group of genetic elements which share the common property of carrying drug-resistance genes. Thus a detailed discussion of the genetic structure and behaviour of R-factors necessarily revolves around only a relatively few examples which, for historical, technical, or even accidental reasons have been selected for laboratory testing.

I have avoided wherever possible the use of specific epithets for R-factors. For example, probably the best studied R-factor is one isolated in 1958 by Rintaro Nakaya, which confers resistance to sulphonamides, streptomycin, chloramphenicol and tetracycline (see reference p.7). Nakaya termed this R-factor NR-1. Other workers designate the same R-factor as 222, others as R100. As this R-factor has been disseminated from laboratory to laboratory and subjected to largely uncontrolled differences in selective conditions and genetic manipulation, subtle (and not so subtle) differences have emerged. For example, David Hoar (personal communication) has found the linkage of Su, Sm determinants to be different in R100 to that in 222. Similarly, Robert Rownd thinks that the replication of NR-1 may be quite different from that reported for 222. Although I do not wish to underestimate the importance of such differences in understanding R-factor genetics, it seems important not to emphasize individual differences but to concentrate on what appear to be unifying general principles.

5.1 Transmissibility and autonomous replication of R-factors

The frequency of transfer of R-factors from natural isolates to suitable recipient bacteria is generally quite low. At the most, the frequency is about 10^{-4} per R^+ cell in one hour of mixed incubation. In practical terms this means that if equal volumes of an R-factor strain and a drug-sensitive recipient strain are mixed, no more than 0·01% of the recipient cells receive the R-factor within 90 minutes. After overnight incubation, from 1%–100% of the recipient cells may be converted. We now recognize that this relatively low transfer frequency is due to the normally repressed state of R-factor sex pili. The transmissibility and autonomy of R-factors

can be explored more thoroughly at the cellular level, therefore, if one studies R-factor-mediated conjugation with a de-repressed mutant. Figure 5.1 demonstrates the kinetics of transfer of a de-repressed F-like R-factor (Su-Sm-Cm-Km-Ap) from one *E. coli* to another.

The two bacterial populations are mixed and at timed intervals samples are withdrawn, agitated violently to separate mating pairs, and plated on an appropriate medium to score for R-factor transfer. Initially there is a lag before transfer commences, which represents the time required for contact and effective pair formation. Transfer begins, however, within 5 minutes after mixing the two cell populations and is completed by 15 minutes. In this experiment 87% of the recipient cells had been infected with the R-factor by 15 minutes. One may also ask how soon after a cell receives an R-factor can it, in turn, be a donor? In figure 5.1 it can be seen that the second round of transfer can commence within 15 minutes after receipt. In other words the recipient cell can replicate the incoming R-factor DNA, synthesize the R-pilus (and presumably other essential proteins), and be able to transfer the R-factor very quickly

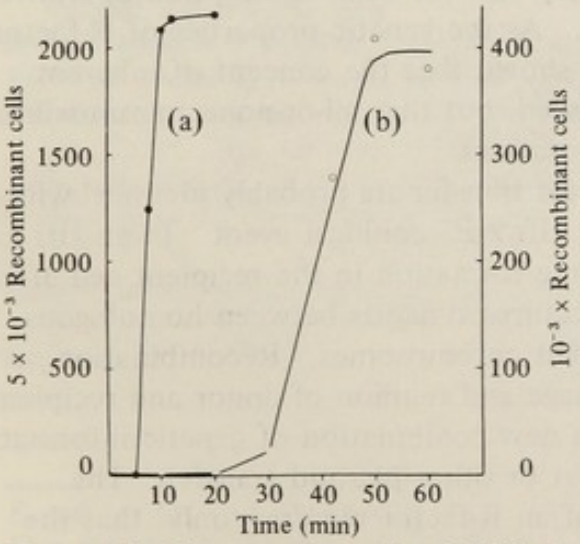

Figure 5.1. Kinetics of transfer of F-like R-factor in *E. coli*. (a) *E. coli* K-12 cells containing the R-factor R1 (R-Su-Sm-Cm-Km-Ap) were mixed at time 0 with a tenfold excess of drug-sensitive recipient organisms at 37°C. At timed intervals samples were removed and agitated violently in a mixer. Samples were then diluted 5000-fold and plated onto a medium selective for recipient cells which had received the R-factor. (b) *E. coli* cells containing R1 were mixed as in (a), but at timed intervals samples were removed immediately into fresh medium containing 10^{12} plaque-forming units of phage T_6/ml. The R1 donor cells were immediately lysed by the large number of phage T_6 ('lysis from without'), but a recipient cell was chosen which was resistant to this viral action. After 10 min incubation a third strain was introduced, which is also resistant to phage T_6 and additionally carries a chromosomal mutation conferring resistance to nalidixic acid. After incubation for 20 min the cell suspension was diluted 1000-fold and plated onto a medium selective for nalidixic acid-resistant cells which have received the R1 R-factor. This experiment demonstrates the time it takes for a recipient to receive R1 and, in turn, pass it on to another suitable recipient.

indeed. Since under the conditions of the experiment the replication time of the bacterial host is nominally 65 minutes, one is justified in concluding that the R-factor is transferred independently of chromosome replication and has a much faster replication time.

The transfer of resistance can be interrupted by vigorous agitation or by lysis of the donor with a bacteriophage, since transfer depends upon direct cell contact. It is important to note that no matter how often or how soon the interruption takes place in this experiment, all of the resistance determinants are transferred together and do not segregate from one another. The R-factor behaves as a single unit of replication and transmission or, in other words, under these experimental conditions R-factor transfer is an all-or-none phenomenon. The combined results of this one transfer experiment seem clear. R-factors are transmissible by direct cell contact and replicate autonomously from the host chromosome as a single genetic element. These conclusions simply confirm the initial impression of the Japanese investigators who first studied R-factors. These concepts were the basic cornerstone of all early genetic experiments with R-factors and are probably still the basic assumption operative in most epidemiological surveys. As the genetic properties of R-factors are examined in detail, it will be shown that the concept of inherent autonomony is firmly established, but that all-or-none transmissibility is subject to some interesting variations.

The specific stages of R-factor transfer are probably identical with those described earlier for an F^+ or Hfr $\times$ F^- conjugal event. In an Hfr $\times$ F^- cross, recombinant chromosome formation in the recipient cell after transfer of genetic material requires synapsis between homologous segments of donor and recipient chromosomes. Recombination *per se* very likely results from breakage and reunion of donor and recipient chromosome pieces to yield a new combination of genetic information. This is *not* the case in R-factor or other plasmid transfer. The achievement of the transfer of an R-factor requires 'only' that the incoming autonomous replicon be immune to the nucleases of the cell, and that its functions be properly transcribed and translated by the host bacterium. Thus the autonomous replication of the R-factor dispenses with the necessity of recombination with the chromosome to achieve survival and removes the obstacle presented by DNA nonhomology as a barrier to the success of gene transfer. Consequently, whereas the transfer of chromosomal material between enteric species is largely restricted to *E. coli*, *Salmonella* sp. and *Shigella* sp., this restriction in host range, in general, does not apply to R-factors and other transmissible plasmids.

5.2 Host range of R-factors

R-factors are promiscuously transferred among the Enterobacteriaceae and to other Gram-negative bacteria outside the family, as summarized in table 5.1. In some instances, for example in *Vibrio cholerae* and *Yersinia*

pestis, transfer has been demonstrated experimentally only in the laboratory. In most cases, however, transfer has been demonstrated both in the laboratory and by isolation from clinical material.

The frequencies of R-factor transfer differ considerably among species and transfer also depends upon whether an organism is employed as a donor or a recipient. One can only generalize broadly, but 'good' R-factor donors are, in order of their quality: *E. coli*, *Shigella* sp., *Citrobacter*, and *Klebsiella–Enterobacter* sp. 'Good' recipients are *Klebsiella–Enterobacter* sp., *E. coli*, *Shigella* sp., *Citrobacter* sp., and *Pseudomonas aeruginosa.* Of course, there are wide strain differences. Many laboratories employ the *E. coli* K-12 strain as a standard donor and recipient of R-factors, and it is excellent in both respects. Among natural *E. coli* isolates, however, one may find that as many as 50% of the strains show little if any recipient

Table 5.1. Host range of R-factors.

Escherichia coli—virtually all serotypes; *Shigella flexneri, Shig. boydii, Shig. sonnei, Shig. dysentereae,* and *Alkalescens–Dispar* group.
Salmonella typhimurium, Salm. typhi, Salm. panama, etc.; *Arizona* group and *Citrobacter freundii, Edwardsiella tarda*
Proteus mirabilis, P. vulgaris, P. rettgeri, P. morganii, and *P. providence*
Klebsiella pneumoniae, Enterobacter aerogenes, E. cloacae, Serratia marcescens
Pseudomonas aeruginosa; Aeromonas hydrophila, Alcaligenes faecalis, Aeromonas liquifaciens, Argrobacterium tunifaciens, Rhizobium meliloti
Vibrio cholerae, El Tor vibrio, 'NAG' vibrios
Yersinia pestis, Y. pseudotuberculosis etc.

Table 5.2. Frequencies of transfer of R-factors to various enteric species.

Donor strain[a]	R-factor	R-factor type	Frequency of transfer/Hr to organism		
			E. coli	*Shig. flexneri*	*Salm. typhimurium*
E. coli	Su-Cm-Tc-Sm	fi^-	$2{\cdot}4 \times 10^{-3}$	1×10^{-4}	5×10^{-7}
Shig. flexneri	Su-Sm-Tc	fi^-	5×10^{-3}	1×10^{-4}	$<10^{-8}$
K. pneumoniae	Su-Sm-Tc-Cm	fi^+	1×10^{-3}	5×10^{-4}	5×10^{-4}
			Salm. typhi	*K. pneumoniae*	*P. mirabilis*
E. coli	Su-Cm-Tc-Sm	fi^-	5×10^{-7}	5×10^{-3}	2×10^{-5}
Shig. flexneri	Su-Sm-Tc	fi^-	2×10^{-4}	5×10^{-4}	1×10^{-7}
K. pneumoniae	Su-Sm-Tc-Cm	fi^+	2×10^{-2}	2×10^{-2}	3×10^{-6}

[a] Each of the donor strains was a recent clinical isolate harbouring the indicated transmissible drug resistance. Matings were performed with each of the indicated recipients, which were resistant to nalidixic acid (100 μg/ml). Approximately 5×10^7 donor cells were mixed with 1×10^8 recipient cells in minimal-salts medium containing glucose. After 1 h samples were removed and plated on minimal-salts–agar containing 100 μg of nalidixic acid/ml, and either 25 μg of tetracycline/ml or 50 μg of chloramphenicol/ml (S. Falkow, unpublished work, 1966).

ability. All workers pretty much agree that species of *Salmonella*, *Proteus*, and *Serratia marcescens* are generally not avid donors or recipients of R-factors in the laboratory. Table 5.2 records some representative frequencies of transfer between a variety of enteric species.

5.3 Genetic determinants carried by R-factors

The R-factors discovered in Japan conferred resistance to one or more of the drugs sulphonamides, streptomycin, chloramphenicol, and tetracycline. Resistance to kanamycin, neomycin, and to the penicillins and cephalosporins were subsequently described. It has been found that R-factors may, in fact, carry genetic determinants for quite a variety of chemical and physical agents. All of the properties shown in table 5.3 have been reported to be transmissible. It must be admitted that there has not been any systematic testing of R-factors for resistance to a large spectrum of drugs or chemicals. David Smith and his associates began a search for previously undefined resistance genes in 1967, when it was noted that less than 5% of the R-factors isolated in their laboratory mediated the Su-Sm-Cm-Tc pattern encountered in Japan. Drugs tested included the aminoglycoside antibiotics, bluensomycin, spectinomycin, and gentamicin; additionally resistance to a variety of metal ions was investigated. Resistance to these antibiotics was indeed found on some

Table 5.3. Resistance determinants carried by R-factors.

Antibiotics	Abbreviation used in text for determinant
Sulphonamides	Su
Chloramphenicol	Cm
Streptomycin (including spectinomycin, bluensomycin and actinamine)	Sm
Penicillins and cephalosporins	Ap
Kanamycin–neomycin (including framycetin and paromomycin)	Km
Tetracycline	Tc
Gentamicin–viomycin	Gm
Furans	Fn
Nalidixic acid	Nx
Trimethoprim	Tp
Fusidic acid	Fs
Lividomycin	Lv
Other determinants	
Heavy metals	
Hg^{2+}	Hg
Ni^{2+}	Ni
Co^{2+}	Co
Colicin resistance (including I, A, H, K, V, B and E)	Col
Ultraviolet light	Uv

R-factors, as was resistance to mercuric chloride, nickel sulphate, and cobalt chloride. This extension of the antibiotic resistance that is possible to be found on R-factors is partially understood in terms of cross-resistance. Thus a single genetic locus apparently specifies resistance to streptomycin, spectinomycin, and bluensomycin. Similarly a gene determining resistance to neomycin–kanamycin may in some cases also specify resistance to paromomycin and gentamicin A. Resistance to kanamycin, the entire gentamicin complex, and to nebramycin may be mediated by an entirely separate gene(s). These resistance patterns are now understood in terms of specific modifying enzymes with distinct substrate specificities, and will be considered in some detail in chapter 9. Since 1967 there have also been reports of R-factor genes specifying resistance to trimethoprim, fusidic acid, and lividomycin and even to the furans and to nalidixic acid. R-mediated resistance to these latter two determinants is questionable and certainly not at all common.

Several investigators have investigated resistance to colicins mediated by R-factors. They theorized that since enterobacteria are so often exposed to colicins in their natural environment, resistance to colicins might be carried as part of an R-factor. One must be careful when talking about colicin resistance, however, to distinguish between insensitivity or indifference to colicin protein and immunity because the gene determining colicinogeny is present. It seems fitting and proper that as many as 30%–40% of all I-like R-factors, in fact, produce colicin I and are consequently resistant to its action. This observation could suggest that these R-factors are formed by linkage of drug-resistance determinants with Col I. In other cases resistance to colicins I, A, H, K, B, V, and E has been found but no colicinogeny could be demonstrated. The basis for these resistances has not yet been determined—it seems unlikely to be due to an alteration in surface receptors. Whatever the mechanism, the resistance to the colicins is determined by genes that are an integral part of the R-factor. It has been suggested that colicin-resistant bacteria have a higher probability of causing extraintestinal disease in humans than colicin-sensitive strains. If this is the case the association of colicin resistance with R-factor bears careful scrutiny.

R-factors have been described which alter the sensitivity of the host towards ultraviolet light. Sometimes the host is more sensitive, sometimes more resistant, and sometimes the host sensitivity remains unchanged. This observation is not particularly surprising in view of the observations that F, Col agents, and phage also may alter the response of their hosts to ultraviolet light.

The degree of resistance to antibiotics conferred upon a host when an R-factor is transferred from one bacterial species to another may often change dramatically. In my experience this is especially true of Sm and Ap. As a general rule, for example, Sm levels are lower in *E. coli* than in *Salmonella*, *Shigella* or *Klebsiella–Enterobacter* harbouring the same

R-factor. It has also been reported that tetracycline resistance tends to be low in *Salmonella* (10–25 μg/ml) but higher (between 100 μg and 250 μg/ml) in *Shigella* and *E. coli*. Table 5.4 shows some levels of resistance conferred on host bacteria by an F-like R-factor. The important point is that although resistance may change from host to host, the levels in all hosts are sufficiently high to preclude use of the drug clinically.

Table 5.4. Degree of drug resistance in different enteric species infected with a single R-factor.

Strain carrying F-like R-factor (Su-Sm-Tc-Km-Ap)	Resistance (μg/ml)[a]					
	Su	Sm	Tc	Cm	Km	Ap
E. coli K-12	>1000	20	100	150	200	250
Shig. sonnei	>1000	200	100	200	200	250
Salm. typhimurium	>1000	125	25	150	200	100
P. mirabilis	>1000	100	200	100	250	25
K. pneumoniae	>1000	150	100	100	200	250
Resistance range before R-factor infection	1–50	0·3–10	1·6–7·0	1–12	0·3–5	3·1–7·5

[a] The inoculum used was approximately 10^3 bacteria/ml into media containing the antibiotics.

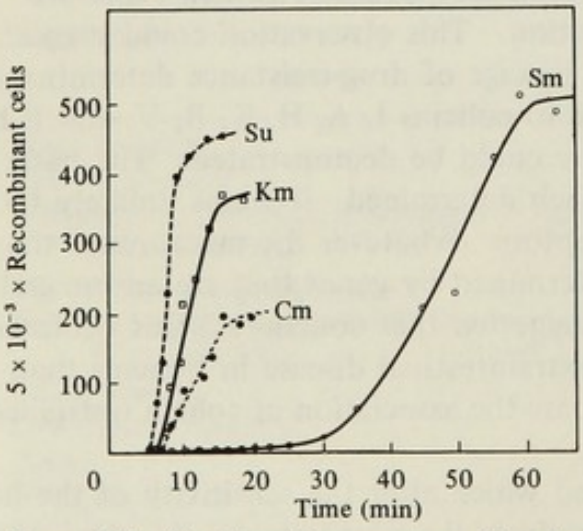

Figure 5.2. Kinetics of expression. *E. coli* cells containing the R-factor (R-Su-Sm-Cm-Km-Ap) were mixed at time 0 with a tenfold excess of drug-sensitive recipient organisms at 37°C. At timed intervals, samples were removed, and agitated violently in a mixer. Samples were diluted 5000-fold and immediately plated onto a medium containing the indicated antibiotic. The differences indicate the time it takes for a recipient cell to express resistance to the antibiotic. This experiment differs from figure 5.1 in that the mating mixture is plated immediately on drug media. In figure 5.1, cells were plated immediately, but drugs were not added for 70 min. Thus figure 5.1 shows the kinetics of transfer and figure 5.2 the kinetics of phenotypic expression of drug resistance. (Data from R. P. Silver and S. Falkow, unpublished work, 1968.)

The expression of drug resistance by a cell that has received an R-factor is quite rapid. Figure 5.2 shows the kinetics of drug-resistance expression after transfer of an F-like R-factor into a sensitive *E. coli* recipient cell. This experiment was performed in a manner similar to that shown in figure 5.1 except that cells were immediately plated on media containing antibiotics. Ordinarily when a kinetic experiment is performed it is best to wait at least an hour before adding drugs to experimental plates. The reason is clearly shown. It may take up to thirty minutes before recipient cells express their full resistance potential. The data from a number of experiments indicate that the order of expression to resistance is Su, Km, Tc, Cm and, finally, Sm. I would emphasize that the curves shown in figure 5.2 do not necessarily reflect the order of entry of the resistance determinants. For example, if the colonies selected on sulphonamide plates are tested it is found that all of the other resistance genes are also present. The different numbers of colonies selected on each drug reflects only the time it takes for cells which have received an R-factor to become resistant. Taken together, figures 5.1 and 5.2 demonstrate the rather dramatic changes that can befall a cell within about fifteen minutes after receiving an R-factor. One need only imagine the potential clinical implications of even a single similar event in the human gut.

5.4 Transduction of R-factors

R-factors can be transduced. It is not clear how significant phage-mediated transfer may be in Nature but it is certainly an important phenomenon to the microbial geneticist. Initial studies demonstrated that multiple resistance could be jointly transduced from a quadruply resistant F-like donor (Su-Sm-Tc-Cm) to sensitive *E. coli* strains by phage P1, and to *Salm. typhimurium* by phage P-22. The two systems, however, give quite different results. In *E. coli*, P1 commonly transduced all of the resistance traits and the transduced R-factor was transmissible by conjugation. In contrast the transduction of the R-factor to *Salm. typhimurium* by phage P-22 resulted in segregation of resistance determinants. Transductants received either Su-Sm-Cm or only Tc (table 5.5). Not only were the resistance determinants consistently segregated but also the majority of these phage P-22 transductants were unable to transfer their resistance by conjugation. Group E *Salmonella* sp. transduced by different phages, $\epsilon 15$ and $\epsilon 34$, show the same pattern of transducing only fragments of the R-factor. This 'defect' in *Salmonella* is not due to the host. The same *Salmonella* strains, which receive the identical R-factor by conjugation, inherit the entire R-factor gene complement and show normal transfer.

A large number of R-factors have now been studied by transduction. When phage P1 has been employed, there has been very little segregation of drug resistance and the vast majority of transductants display conjugal ability. The pattern of R-factor transduction by phage P-22 shows

considerable variation, however. Indeed the pattern is not necessarily uniform for R-factors with the same combination of drug-resistance markers and similar sex pili type (table 5.6). Some *fi*$^-$R-factors and a rare *fi*$^+$R-factor are transduced *in toto* by phage P-22. More commonly, transduced *fi*$^-$ factors show no segregation of drug resistance even though they may be nontransmissible. In 1968, Drabble and Stocker showed that about 7% of such transductants may reacquire transmissibility of resistance

Table 5.5. Differences in transduction of R-factors by phages P1 and P-22. Phage P1 was propagated on an *E. coli* strain carrying an F-like R-factor, R-Su, Sm, Tc, Cm. The purified phage particles were then mixed with a drug-sensitive *E. coli* strain and, after a suitable period of incubation, the cells were plated on a medium that was selective for the indicated resistance. Cells appearing on the drug plates were tested for the inheritance of additional resistance genes and also tested to determine whether the drug resistance could be transmitted by conjugation. Phage P-22 was propagated on a strain of *Salm. typhimurium* harbouring the same R-factor. The purified phage P-22 particles were mixed with a drug-sensitive *Salm. typhimurium* strain, and resistant transductants were isolated and analysed as described for the phage P1 experiment.

Phage	Recipient	Selection for resistance to:	Pattern of resistance (%)			Transmissibility by conjugation
			Sm, Cm, Tc Su	Su, Sm, Tc	Su, Sm, Cm, Tc	
P1	*E. coli*	Chloramphenicol	0	0	All	All
P1	*E. coli*	Tetracycline	10	2	88	All
P-22	*Salm. typhimurium*	Chloramphenicol	All	0	0	None
P-22	*Salm. typhimurium*	Tetracycline	0	All	0	2%

Table 5.6. Pattern of phage P-22 transduction of various R-factors with the same resistance phenotype. (Modified from Watanabe *et al.*, 1968.)

R-factor	Type	Selection	Drug resistance of transductants	Frequency of transduction[a]	Transfer[b]
N-1 Su, Sm	*fi*$^-$	Tetracycline	Tc	$2{\cdot}7 \times 10^{-9}$	1/109
		Sulphonamides	Su-Sm	$9{\cdot}5 \times 10^{-7}$	0/180
N-3 Su, Sm, Tc	*fi*$^-$	Tetracycline	Tc-Su-Sm	$1{\cdot}9 \times 10^{-9}$	72/159
		Sulphonamides	Tc	$2{\cdot}0 \times 10^{-9}$	5/176
			Su-Sm-Tc	$2{\cdot}7 \times 10^{-10}$	16/21
			Su-Sm	8×10^{-9}	5/6
K-R_3 Su, Sm, Cm	*fi*$^+$	Chloramphenicol	Su-Sm-Cm	1×10^{-6}	0/180
S-a Su, Sm, Cm	*fi*$^-$	Chloramphenicol	Su-Sm-Cm	$1{\cdot}6 \times 10^{-5}$	60/60

[a] The frequency of transduction is the number of drug-resistant cells per adsorbed virus particle.
[b] Ability to transfer transduced drug resistance by conjugation.

after an F-merogenote is introduced into the transductant. The majority of *fi*$^+$R-factors transduced by phage P-22 show the segregation pattern discussed previously. Tc is not transduced with other resistance genes and few transductants are transmissible even after infection with an F-merogenote. The Km resistance determinant, when present on an R-factor, always segregates with the Su, Sm, Cm resistance genes. In contrast the Ap gene as part of an F-like R-factor is transduced at a very low rate and rarely cotransduced with any other resistance. *fi*$^-$ R-factors carrying an Ap gene do not behave in this way; rather the transductants selected as ampicillin resistant acquire some or all of the other resistances of the factor concerned. In the main most phage P-22-mediated transductants are nontransmissible and, of course, not all R-factors carry the Tc gene. As a general rule, however, there is a rather high degree of correlation in phage P-22 transductants between conjugal transmissibility and resistance to tetracycline.

The different patterns of phage P1- and P-22-mediated transduction of R-factors probably reflects in large part the size of the DNA molecule that can be incorporated into the phage particle. As pointed out previously, the size of a phage P1 transducing fragment equals more or less the molecular size of the phage P1 chromosome (about 60×10^6 daltons). A similar situation is likely for phage P-22, but here the molecular size is reduced by about half (27×10^6 daltons). Thus one reasonable inference is that phage P1 is simply big enough to accommodate the entire structure of practically all R-factors whereas P-22 ordinarily cannot carry the whole unit but only R-factor 'fragments'. This would seem to explain why various drugs-resistance markers and the ability for conjugal transfer are so often segregated in transduction with phage P-22. Those R-factors that are transduced *in toto* by phage P-22 may reflect a smaller size of the R-factor DNA.

There are several fundamental conclusions that can be discerned from the transduction studies. The drug-resistance determinants are segregated from one another, clearly indicating that these genes are indeed different and that multiple drug resistance is not some pleiotropic effect of a single gene. The most important conclusion is that the genes determining drug resistance and those determining conjugal ability are distinctly separable. In 1961 Watanabe and Fukasawa designated the genetic region of an R-factor that controlled plasmid functions as the resistance-transfer factor, RTF, to distinguish it from drug-resistance genes. These authors also constructed a preliminary genetic map of an R-factor based on the segregation which was observed in both phage P1 and phage P-22 transductants.

The data of table 5.5, for example, are consistent with the following genetic model:

Su-Sm-Cm-Tc-RTF[5] .

[5] The data are really not good enough to distinguish this model from Su-Sm-Cm-RTF-Tc, which is an alternative.

If one examines the data it can be seen how this follows. The segments carried by phage P1 are:

Tc-RTF
Sm-Cm-Tc-RTF
Su-Sm-Cm-Tc-RTF .

The segments carried by phage P-22 are:

Tc-RTF
-Tc
Su-Sm-
Su-Sm-Cm- .

Such genetic maps are useful but the data do not tell anything about the relative size or distance between the genes. The data of table 5.6 clearly show that these parameters are not necessarily the same for various R-factors. It has now been determined that the Km gene maps between Cm and Sm and, in at least one R-factor, Ap has been found rather closely linked to RTF. Figure 5.3 shows two alternative circular models of R-factor genetic structure formulated on the basis of transduction studies.

The transduction experiments suggest that an R-factor is a single unit of linked genes since both RTF and the drug-resistance genes are cotransduced by phage P1. On the other hand, what about the phage P-22 transductants? If the R-factor is fragmented by transduction, how do the drug-resistance genes replicate in the absence of RTF? The two basic alternatives seem to be (a) that the drug-resistance genes are, in fact, replicons in their own right and (b) that they no longer replicate autonomously but instead become integrated into the host chromosome. The results of experiments by Dubnau and Stocker seem to support the latter hypothesis. They

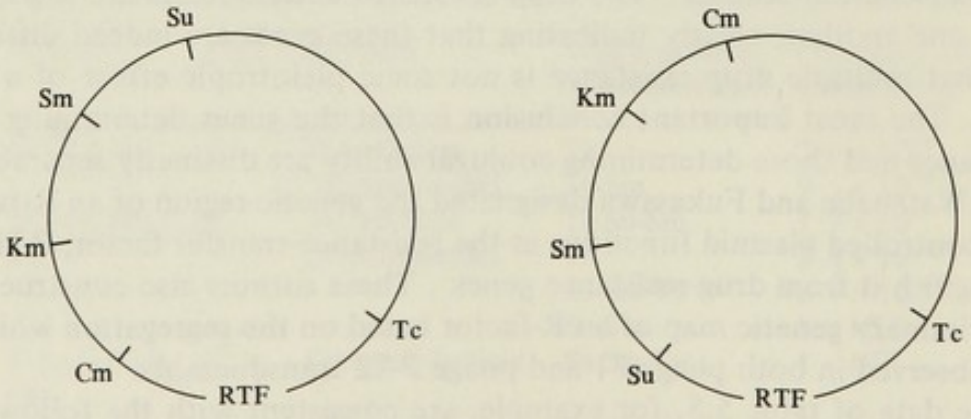

Figure 5.3. Two models of the genetic structure of an R-factor. These two alternative models have been presented on the basis of transduction studies in both *E. coli* and *Salm. typhimurium*. The distances between genes is purely arbitrary. The models are circular to reflect the other known physical (chapter 6) and genetic (chapters 7, 8) properties of R-factors.

found that the R-factor fragment transduced by phage P-22 becomes integrated at the P-22 prophage attachment site, which, you may recall (chapter 2), is adjacent to the host's proline (*pro*$^+$) genes. Thus the nontransmissibility of R-factor pieces seems to be the result of their integration along with a defective phage genome. Watanabe and Ogata have further studied this point. They employed a *Salm. typhimurium* recipient that had physically lost a segment of its chromosome extending through the *pro* genes and phage P-22 attachment site. The reasoning was that if transduced defective R-factors are integrated exclusively at the phage P-22 attachment site near *pro*$^+$, then no drug-resistant transductants will be found when the deletion strain is employed as a recipient. The results showed that indeed the frequency of Tc transduction was markedly depressed as compared with that for a 'normal recipient'. Nonetheless the frequencies of transduction of Su, Sm, and Cm are not similarly affected. They also found therefore that the majority of Tc transductants are probably integrated at the P-22 prophage attachment site, but the sulphonamide-, streptomycin- and chloramphenicol-resistant transductants are either integrated at some other, as yet unknown, site(s) or still retain some ability for autonomous replication.

5.5 Reversible dissociation of R-factors

So far we have dealt only with the R-factors that are transferred as a single unit at conjugation and are transduced by phage P1 as a single linkage group—but this genetic picture is not always the case. In 1965, E. S. Anderson and M. J. Lewis made some important observations which enlarged the views of many investigators about the epidemiology, ecology, and possible origins of R-factors. They established that the transfer (RTF) segment of an R-factor and the genes determining resistance may sometimes behave as essentially independent elements. The initial studies were performed with a strain of *Salm. typhimurium*, designated as RT, which belongs to phage type 29. This strain carried resistance to sulphonamides, streptomycin, ampicillin, tetracycline and furans. These resistances, except for furans, all proved to be transmissible. The basic difference in the behavior of the R-factor RT, from those already discussed, was that the resistance determinants of RT were almost always segregated during conjugation to *E. coli* K-12 recipients and other salmonellae. Anderson designated the RTF of strain RT as Δ, and, because he thought that Δ could carry many bacterial genes in addition to (or instead of) drug-resistance determinants, Δ was called simply a transfer factor. Genetically the Δ transfer factor of strain RT appeared to be *fi*$^-$.

The frequency of transfer of drug resistance from strain RT to an *E. coli* K-12 F$^-$ strain is recorded in table 5.7. In contrast with previous examples, the drug-resistance genes did not form a single linkage group. Yet each of the individual resistances maintained an independent relationship with Δ. Anderson considered that, effectively, the RT strain

carried three R-factors, ΔAp, ΔSu-Sm (the Su and Sm genes, while distinct, were always found together) and ΔTc. Let us take this one step further and examine the outcome of a mating between a converted strain carrying ΔSu-Sm and a drug-sensitive recipient (table 5.8). If the progeny from this cross are plated on a medium containing the drugs, it is observed that ΔSu-Sm has been transferred at a frequency of about 10^{-2} and, in addition, a smaller fraction (10^{-4}) of the cells have received Su-Sm alone, that is, without Δ. If the progeny of the cross are plated on a drug-free medium, it is found that 50% or more of the drug-sensitive recipients, which have simply been grown in contact with the donor, have received Δ alone[6]. Thus Δ and the Su-Sm determinants behaved as independent genetic entities.

Anderson conceived that the phenomenon observed for strain RT was an example of a reversible dissociable state. His view was that in a majority of donor cells Δ and Su-Sm were dissociated independent replicons and the cells for the most part transfer only Δ. In a minority of cells Δ and Su-Sm are 'associated' and are transferred as the unit ΔSu-Sm.

Table 5.7. Segregation of Δ and drug-resistance genes during transfer from *Salmonella typhimurium* RT_1. *Salm. typhimurium* strain RT was grown in mixed culture with a drug-sensitive *E. coli* K-12 F^-. After mixed cultivation for 16-18 h the mixture was plated onto a medium on which only *E. coli* cells could grow. The *E. coli* progeny were subsequently assayed for the inheritance of Δ and drug resistance.

Parental strains	Progeny	Frequency
Salm. typhimurium RT	K-12 Δ	5×10^{-1}
(ΔSu, Sm, Ap, Tc)	K-12 ΔAp	2×10^{-2}
×	K-12 ΔSu-Sm	1×10^{-2}
E. coli K-12 F^-	K-12 ΔSu-Sm, Ap	1×10^{-3}
	K-12 ΔTc	$<10^{-6}$

Table 5.8. Transfer of Δ and Su-Sm from a converted *E. coli* K-12 strain. A strain of *E. coli* K-12 that had received ΔSu-Sm was mated with a drug-sensitive *E. coli* F^- strain. After 16-18 h at 37°C, the mixture was plated onto selective media and the number of cells receiving Δ and Su-Sm were enumerated (frequency).

Parental strain	Progeny	Frequency
E. coli K-12 ΔSu-Sm	K-12 Δ	5×10^{-1}
×	K-12 ΔSu-Sm	1×10^{-2}
E. coli	K-12 Su-Sm	1×10^{-4}

[6] The reader may wonder how it is possible to detect Δ alone. One method (reported by Anderson in 1966) depends upon a change in phage sensitivity when a *Salmonella* receives Δ. For example *Salm. typhimurium* phage type 36 shows the pattern of phage type 6 after receiving Δ. This is a consequence of a phenomenon called restriction-modification, which will be discussed in chapter 7.

This association was seen as unstable, so that once in the recipient the Δ and Su–Sm again dissociated into separate replicons. Rarely Su–Sm was found in recipients without Δ, which underlined the fact that Δ and the drug-resistance determinants were independent. Recipients which received Su–Sm alone could not transmit this resistance any further by conjugation unless the cell line was reinfected with Δ or some other suitable transfer factor. This mobilization of Su–Sm is really quite reminiscent of the mobilization of the nontransmissible plasmid Col E1 by F that was discussed previously (chapter 2).

The association and disassociation of Δ and Su–Sm is clearly a very dynamic situation; the ΔAp complex behaves in very much the same way. The ΔTc factor, however, is quite stable. In table 5.7 one sees that the initial transfer of ΔTc from strain RT, was a rare event (10^{-6} or less). Yet once the transfer has taken place ΔTc is subsequently transferred at a frequency of 5×10^{-1} or higher—really much like Δ alone. This finding indicates that although Δ and the drug-resistance determinants are separate entities, occasional recombination may take place to yield a single unit of transmission and replication.

The discovery of the inherent independence of the Δ transfer factor and resistance determinants suggests one mechanism by which R-factors, such as the one in strain RT, could be formed. The prediction is that natural isolates will be found which carry transfer factors such as Δ, as well as others which carry multiple drug-resistance determinants that are nontransmissible but which replicate autonomously from the chromosome and can be mobilized. To establish such a hypothesis, however, one requires a method of identifying these 'raw' building blocks of R-factors in cultures isolated from natural sources.

It had already been shown that if a cell line carrying only Δ is mixed with a cell line carrying only Su–Sm (or some other nontransmissible drug determinant), Δ is transferred into the Su–Sm cells to re-form the Su–Sm 'complex'. In this way Su–Sm can once more be transferred in a subsequent conjugal act. This same basis was employed by Anderson in 1965 to develop a resistance mobilization test which could be used to detect the presence of transfer factors in wild-type strains. This test is summarized in table 5.9. Drug-sensitive clinical isolates, which serve as potential donors, are mixed in broth with an intermediate strain that carries a known nontransmissible drug-resistance determinant such as Su–Sm. After a period of incubation a third strain, the final recipient, is introduced into the mating mixture. The final recipient has a selective marker, such as a chromosomal mutation to nalidixic acid resistance, which can be employed to distinguish it from the donor and intermediate strain. Neither the donor strain nor the intermediate strain can alone transfer Su–Sm resistance to the final recipient. However, if the potential donor strain does in fact carry a transfer factor it will in all probability be

transferred to the intermediate strain during the incubation period and it will 'associate' with the Su–Sm genes. In turn, the transfer factor and Su–Sm determinant (or just the Su–Sm determinant) will pass into the final recipient when it is added to the incubation mixture. The transfer is detected by plating this 'triple cross' on a medium containing both nalidixic acid and streptomycin. The appearance of colonies indicates that a transfer factor must have been present in the potential donor strain. Of course, the drug resistance of the intermediate strain can be any nontransmissible resistance that can be mobilized, and the selective agent for the final recipient may be varied to suit the experimental conditions.

Using this test Anderson showed that 57 out of 90 drug-sensitive isolates of *Salm. typhimurium* harboured an *fi*$^-$ transfer factor capable of mobilizing nontransmissible drug-resistance genes. Other investigators have employed essentially the same methods to show that roughly 40% of drug-sensitive Gram-negative bacteria carry a transfer factor (table 5.10). The distribution of organisms carrying transfer factors include *E. coli*, *Shigella* sp., *Klebsiella–Enterobacter*, *Proteus* sp. and, of course, *Salmonella* sp. The nature of the transfer factors identified by Anderson and others in clinical isolates of drug-sensitive bacteria is not clear. Some are clearly Col factors, but most do not carry any known determinants and must be classified simply as sex factors. It has only been determined that a large reservoir of these agents exists in Nature. Neither is the significance of the widespread distribution of sex factors at all clear. The obvious suspicion is that some selective advantage is operative, but, as pointed out by Hayes on page 807 of his book (see reference p.36), all the features of sexuality conferred on a host cell by a transmissible plasmid can be explained as benefitting the plasmid as much as the bacterium. Hence, one is not sure who is doing what to whom.

Table 5.9. Resistance mobilization test for detection of transfer factors in drug-sensitive strains of enterobacteria.

The components

1 Donor: *E. coli* (drug-sensitive) presumed to carry transfer factor
2 Intermediate: *E. coli* carrying known nontransmissible drug-resistance determinants
3 Final recipient: *E. coli* F$^-$, carries chromosomal resistance to nalidixic acid (Nx)

The cross

1 Donor and intermediate incubated together for 6 h at 37°C. This permits any sex factor to be transmitted to intermediate
2 Add final recipient. Sex factor (if present) 'mobilizes' nontransmissible drug-resistance determinants into final recipient. Incubate for 18 h at 37°C.

Detection

1 Plated onto medium containing nalidixic acid + drugs specified by nontransmissible determinants
2 Appearance of colonies indicates transfer factor in donor. No colonies means no transfer *or* an inability of the sex factor to mobilize nontransmissible determinants

A significant proportion of the drug-resistant enterobacteria isolated from clinical material do not show demonstrable transfer. An examination of table 5.9 shows that the triple cross could be easily modified to determine whether any of these clinical isolates possess resistance genes that can be mobilized. One substitutes a known donor containing a transfer factor, and the drug-resistant clinical isolate becomes the intermediate strain. The data concerning the incidence of nontransmissible, but mobilizable, drug resistance in enteric bacteria are not yet very extensive. Nonetheless, Smith, Humphreys and Anderson have recently recorded examples of mobilizable nontransmissible plasmids carrying Ap; Tc, Su–Sm; Ap–Su and Ap–Su–Sm. A nontransmissible plasmid specifying Km has also been found. The most common nontransmissible determinants susceptible to mobilization appear to be Su–Sm and Tc. You can appreciate that at the practical level it is often difficult by means of a simple genetic cross alone to distinguish between a cell line carrying an R-factor which is a single unit of transmission and one which carries an independent transfer factor plus a nontransmissible R-plasmid. Both kinds of strains will show resistance transfer to a suitable recipient. Only a careful study of the segregation of sex-factor activity from drug resistance among the recipient cells will reveal their fundamental independence. The segregation of the resistance pattern in recipients may only denote that two distinct single-unit R-factors were present in the donor cell. Retransfer of drug resistance is not a sure sign of a single unit since a cell which has received a mobilized drug-resistance plasmid will most likely have received the transfer factor as well. A rather extensive genetic analysis is required and even then one is often required to show, by transduction or molecular studies, that one is dealing with an independent transfer factor and a nontransmissible R-plasmid rather than a single unit.

Table 5.10. Incidence of transfer factors in wild-type *E. coli* determined by the resistance-mobilization test. This survey was undertaken with strains isolated from an isolated rural area north of Brisbane, Australia: F. J. Skerman and S. Falkow (unpublished work).

Source	Total number of strains	Number carrying sex factors	Pilus type[a]		Untypable[b]
			F	I	
Human	43	12	3	3	6
Bovine	134	58	34	4	20
Equine	75	25	15	2	8
Totals	252	95 (38%)	52 (55%)	8 (9·3%)	34 (35·7%)

[a] Determined from sensitivity to the male phages MS-2 and If1.
[b] Predominantly fi^- types.

5.6 R-factors: single units or dissociated?

When Anderson and his associates published their findings, workers in the field were suddenly faced with two sets of quite divergent evidence concerning the genetic structure of R-factors. One set of data strongly supported the concept that R-factors were single units of transfer and replication. Anderson's data suggested that R-factors were often dissociated independent elements. During the period 1965 to 1970 there was a spirited controversy about this in an attempt to find some unity in the data. There were assertions that the nontransmissible drug-resistance determinants seen by Anderson were defective single units, much like the phage P-22-mediated drug-resistant transductants already seen in *Salmonella*, and were simply complemented for their missing functions by a suitable transfer factor. This was a reasonable argument but it did not adequately explain the high frequency of segregation between drug-resistance determinants seen by Anderson and others. The use of the terms 'associated' transfer and plasmid 'complexes' by Anderson caused difficulty in the minds of some investigators. Anderson and his associates did not specify the nature of the reversible association between Δ and the drug plasmids. Some investigators found it difficult to conceive of any mechanism of associated transfer of plasmid complexes which did not involve covalent linkage or separate and independent transfer but not a 'nebulous' association. Anderson's rebuttal was that it was ingenuous to think that all possible types of association between transfer factors and other plasmids were known.

Over the past few years the controversy has been resolved. Anderson and Natkin, as well as later experiments from my own laboratory, showed that 'complexes' such as ΔSu–Sm and ΔAp were not transduced by phage P1 as a single unit but were, in fact, two distinct replicons. This view was further confirmed by molecular studies by Christine Milliken and Roy Clowes, as well as by others, which showed that Δ and the Su–Sm and Ap determinants resided on easily distinguishable separate circular DNA molecules and mobilization of drug resistance by Δ did not appear to require covalent linkage. Moreover it was clear that the nontransmissible drug-resistance plasmid was capable of existing in a cell without any transfer factor being present. All of the nontransmissible drug-resistance plasmids discovered by Anderson (Ap; Su–Sm) were found to be small, about 6×10^6 daltons, and present in cells as multicopies (see chapter 6).

From the standpoint of plasmid evolution, it is also interesting to examine the behaviour of 'known' plasmids such as F, Col I, Hly, K88, and Ent when they are examined in a 'triple cross' similar to that devised by Anderson. All of the cited plasmids can mobilize a nontransmissible Su–Sm plasmid (table 5.11). The *fi*$^-$ factors mobilize this particular determinant at a much higher frequency than do *fi*$^+$ factors; but all do mobilize the Su–Sm determinant at a significant level. Thus, by definition, all of these plasmid combinations are behaving as R-factors. These results

show that one does not necessarily require a transfer factor with a 'special' propensity for drug resistance but that, in a sense, plasmids are as promiscuous in terms of their interrelationships as they are in terms of their bacterial host range. Of course, we still do not understand the mechanisms of plasmid mobilization. However, it does not appear to be simply a passive phenomenon in which the noninfectious DNA accidentally enters a conjugation tube.

It is now believed that what had been observed in Japan and Great Britain showed the two extremes of the potential genetic behaviour of R-factors. As a result of antibiotic usage, the genetic nature of the existing plasmid reservoir, and other unknown selective pressures, one can assume that there was a selection for those R-plasmids best suited to survive in a particular ecological situation. Let me remind the reader that the ecological situations that were sampled in Japan and Great Britain were rather different. The Japanese experience from the outset and until quite recently principally revolved around isolates of *Shigella* sp. and *E. coli*. By far the most common R-factor studied in Japan was an *fi*$^+$ Su-Sm-Cm-Tc plasmid which behaved genetically as a single unit of transmission and replication. Anderson's work in Great Britain primarily revolved around strains of *Salm. typhimurium* phage type 29. Drug resistance in these strains appeared successively. The overwhelming majority of the R-factors were *fi*$^-$ and behaved genetically as independent transfer and drug-resistance determinants. The reasonable conclusion is therefore that both kinds of genetic behaviour can occur and that resistance-*transfer* systems can be broadly classified into two groups, defined by Anderson and Natkin as Class 1 (called a plasmid cointegrate by Clowes) and Class 2 (called a plasmid aggregate by Clowes). In Class 1 the resistance determinant(s) and the transfer factor form a covalently bonded complex which is maintained and transferred as a single linkage group. The R-factors discovered in Japan are the classical examples. In Class 2 transfer systems the resistance determinant(s) and the transfer factor are discrete plasmids, independent of each other in the host cell,

Table 5.11. Mobilization of a nontransmissible streptomycin determinant (Su-Sm) by various transfer factors. (Guerry *et al.*, 1974.)

Plasmid	Sex pilus type	Relative frequency of Su-Sm mobilization
F-factor	F	1·0[a]
Hly	F	0·142
K88	F	1·03
Ent P307	F	0·88
Col I	I	539
ΔAus	I	201

[a] $1{\cdot}38 \times 10^{-5}$ per donor cell.

and are transferred together occasionally by an unknown mechanism that probably does not include covalent linkage. The typical examples of this second class are the ΔAp and ΔSu–Sm plasmids, although the F–Col E1 system described in chapter 3 is clearly an analogous system.

It would be a mistake to think that these two classes of genetic behaviour are completely distinct or mutually exclusive. For example, Guerry, van Embden and I prepared a strain carrying a typical Class 1 R-Cm factor as well as a separate nontransmissible R-plasmid encoding for Tc. The phenotype R-Cm–Tc was actually the result of two distinct plasmid species and was capable of both Class 1 and Class 2 resistance transfer. Phage P1-mediated transduction of this strain showed that the R-Cm and Tc were ordinarily transduced independently of each other. Yet when selection was made for both Tc^R + Cm^R, we could isolate (at low frequency) a single transmissible R-factor which transmitted both Cm and Tc as a single unit. By the same token some R-factors like ΔTc were originally distinct but associate (recombine) to form a stable transmissible single unit. Such findings give an interesting insight into how R-factors can acquire new resistance determinants as selective pressures change and how a Class 2 transfer system may evolve into a Class 1 transfer system. The reverse is also possible. An R-factor isolated in Japan, which behaves as a typical Class 1 representative by conjugation and phage P1 transduction in *E. coli* and *Shigella*, can, nonetheless, dissociate in part after it has been transferred to *Proteus mirabilis*. One can then transfer both a complete R-factor unit and a drug-sensitive transmissible plasmid from this species. This latter plasmid has all the properties one would attribute to a transfer factor be it called Δ or RTF. The molecular basis of this observation will be considered in the next chapter. The data can be anticipated, however, by stating that at least some single-unit R-factors can dissociate into more than one replicon which can reversibly associate and dissociate. Similar phenomena for the dissociation of certain single-unit R-factors into component replicons have been observed following transfer into *E. coli* and *Salmonella*. Assuming that R-factors are often assemblages of more than one replicon, one can substitute the word recombination for association and probably come a good deal closer to understanding the genetic structure of R-factors. One can picture a transfer factor and a nontransmissible R-plasmid forming a single unit, following a site-specific cross-over mechanism analogous to the Campbell model for insertion of phage λ into the *E. coli* chromosome. If this insertion is unstable, reversal of the process will again yield two distinct replicons. In a recombination assemblage of more than one replicon, one must assume that one replicon is subordinate (repressed) to the other. Perhaps the stability of the repression determines the stability of the associative recombination. Thus an R-factor which can stably replicate as a single unit in *E. coli* may, in *Proteus*, lose or relax replication control so that

each replicon is individually expressed. Of course, not all R-factors need be assemblages of replicons nor, if so, need the recombination be reversible.

It is probably fair to say that currently the majority of R-factors isolated from enteric bacteria, including *Salmonella* sp. and *Proteus*, ordinarily behave as typical Class 1 examples that are maintained and transferred as a single linkage group under most circumstances. I suppose that one could make the case that Anderson and his associates were dealing with a more 'primitive' kind of R-determinant and, given time, drug selection would ultimately lead to the more efficient single-unit Class 1 transfer system. At the moment it seems best to simply accept the data at face value. Namely, that in addition to the class of R-factors consisting of a single linkage group possessing the genetic determinants of replication, transfer, and antibiotic resistance, there are autonomously replicating, non-selftransmissible R-plasmids of smaller size and whose transfer can be promoted by virtually any transfer factor which may be present in the same cell. The major point is that R-factors often seem to be a product of dynamic plasmid–plasmid interactions. To pursue this further, it is necessary to go on to consider the molecular nature of R-factors and other plasmids.

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6

The molecular nature and replication of R-factors and other plasmids

6.1 R-factors are circular molecules of DNA

R-factors are DNA structures which are accessory to the chromosome of the bacterial cell. This much has been explicit in the discussion thus far. Since the R-factor DNA structure is physically distinct from the host chromosome it can be isolated by a variety of techniques. Most recent isolation methods are primarily based on the fact that R-factor DNA within the host cell appears to be present as twisted covalently-closed circular molecules. R-factor DNA is certainly not unique in this respect since it is established that many bacterial, viral, mitochondrial, and other prokaryotic and eukaryotic chromosomes are, much of the time, circular structures as well. The full significance of the circularity of chromosomes is not known. The covalently closed ring chromosome is generally considered as the 'resting' state between rounds of replication. It is believed that closed ring molecules are more resistant to attack by exonucleases. Although this may be true, it seems more likely that circularity is more strongly related to having the replication origin and terminus adjacent to each other so that replication control and the distribution of DNA replicas are 'simplified'.

Circular DNA molecules, in the general sense, have special physical and chemical properties. It should be understood, however, that the same DNA species may exhibit several alternative circular forms. As mentioned earlier, in chapter 2, linear duplex DNA of phage λ can be circularized *in vitro* by annealing the complementary or 'sticky ends' of the molecule. These ring molecules formed *in vitro* are held together solely by hydrogen bonds and there is a 'gap' in each strand between the 3′-hydroxyl and 5′-phosphoryl termini. Such hydrogen-bonded rings can be covalently closed *in vitro* by the enzyme polynucleotide ligase (found normally in most bacterial cells), which covalently joins the 3′- and 5′-termini. Circular molecules of phage λ held together only by hydrogen bonding are not normally found in natural systems. It has not been unequivocally demonstrated that R-factors and other transfer factors have 'sticky' ends like phage λ, yet, as detailed in a later section, R-factor and other plasmids cyclize very quickly *in vivo*, which suggests that special mechanisms facilitate a reaction similar to that seen in the model studies *in vitro* with phage λ.

Bacterial cells, shortly after infection with R-factors and other plasmids, show two types of covalently joined ring structures (cf. plate 1). One type is covalently closed in both strands. This type has a supercoiled configuration and will be referred to as a twisted covalently-closed form. In the literature this form has been synonomously called a supercoil, closed circle, RF1 and species 1. The other ring form found *in vivo* has

one strand which remains unsealed and has the other strand covalently joined. This ring form will be called a 'nicked' form; in the literature it has been called an open circle, relaxed circle, RF2 or species 2.

All twisted covalently-closed molecules, be they phage λ, R-factor or polyoma virus DNA, have certain common features. The compact supercoiled configuration provides the molecule with a decreased intrinsic viscosity and decreased sensitivity to shear. Consequently a twisted covalently-closed ring sediments faster than a corresponding 'nicked' ring. The ratio of the two sedimentation velocities varies with the physicochemical environment. As an example, in about 1·0 M-sodium chloride, the twisted covalently closed ring sediments in a linear neutral sucrose gradient at about 1·5 times the rate for a corresponding 'nicked' ring form. Under the same conditions the 'nicked' ring will sediment about 1·14 times the rate of a linear duplex (figure 6.1).

Twisted covalently-closed molecules have other special properties. These include: (a) a high sedimentation rate in solvents (such as alkaline sucrose, pH 12·5) which ordinarily will completely separate the strands of linear DNA; (b) a markedly increased resistance to elevated temperatures; and (c) a smaller change in buoyant density than that which is seen with linear or 'nicked' ring structures after interaction with an intercalative dye such as ethidium bromide. These 'special' properties of twisted covalently-sealed ring forms can be simply understood in terms of the inability of the covalently-sealed strands to unwind in a topological sense and to separate physically from one another. There is no such restraint to strand separation in a linear double-stranded duplex, nor in a 'nicked' ring, because of one or more free (unsealed) ends. The twisting of the covalently-closed ring is a spontaneous formation of

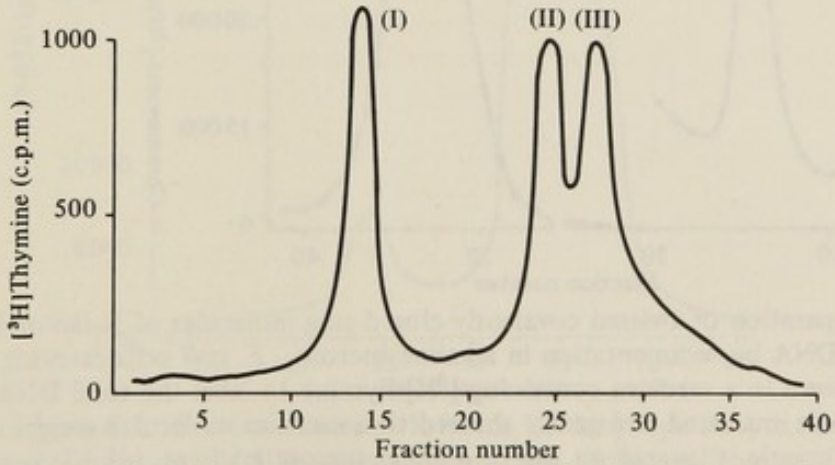

Figure 6.1. Sedimentation of twisted covalently-closed ring form (I), 'nicked' (open) ring form (II) and a linear duplex form (III) of R-factor DNA in a linear neutral sucrose gradient. [^{3}H]Thymine-labelled R-factor DNA was layered over a 5–20% neutral sucrose gradient made up in 1·0 M-NaCl solution. Centrifugation was carried out at 100000*g* for 2 h. Fractions were collected through a hole punctured in the bottom of the tube and were assayed for acid-precipitable radioactivity.

tertiary (superhelical) turns, reflecting the topological restriction that any change in the average pitch (Watson–Crick turns) of a double helix must result in a change in the tertiary structure. The number of superhelical turns is about 4–6 per 1×10^6 daltons of DNA. A pH range of 11·5–12·0 is sufficient to separate the strands of linear and 'nicked' circular DNA completely, but the strands of a covalently-closed ring are physically constrained from separating. If the pH is raised to about 12·5 the covalently sealed ring 'collapses' into a tightly compact structure. This feature can be exploited in a practical vein. For example, if the DNA of an *E. coli* cell harbouring an R-factor is extracted at a pH of about 12·5 and centrifuged in a linear gradient of sucrose at the same pH, one finds, as shown in figure 6.2, that the covalently-sealed R-factor molecule sediments about 3·8 times faster than the host DNA. If the conditions of pH are returned to normal, the denatured collapsed covalently-closed DNA 'snaps back' into a twisted covalently-closed ring form. The reduced binding of the intercalative dye ethidium bromide can also be exploited in a practical way. When ethidium bromide binds to DNA it causes a change in the average pitch of the base pairs of the molecule. For a covalently-closed circular DNA this results in a corresponding increase in the superhelix density of the molecule (actually the superhelix density is initially negative, and in the presence of the dye becomes zero and then

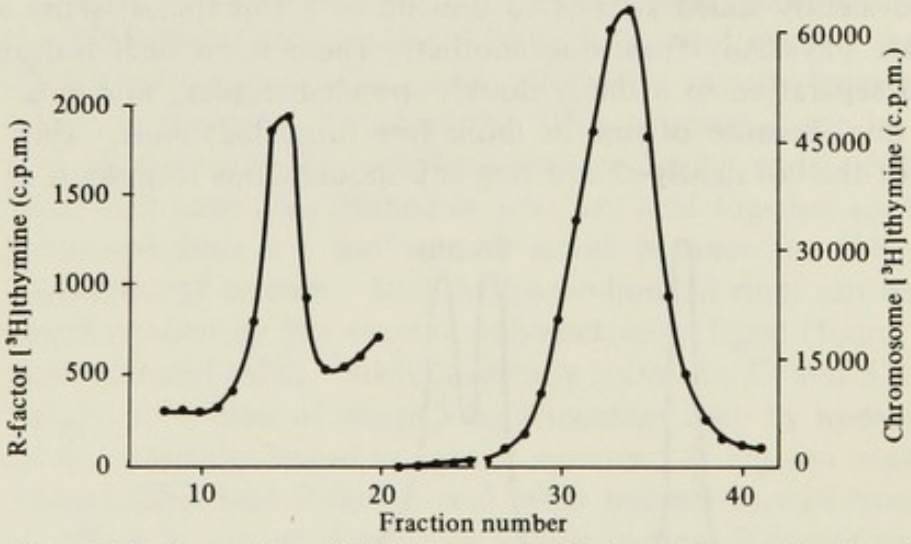

Figure 6.2. Separation of twisted covalently-closed ring molecules of R-factor DNA from the host DNA by sedimentation in alkaline sucrose. *E. coli* cells carrying an R-factor are grown in a medium containing [^{3}H]thymine to label the total DNA of the cell. The DNA is extracted and gently sheared to a uniform molecular weight of about 100×10^6. A sample is layered on top of a linear sucrose gradient, pH 12·5, and centrifuged for 30 min at 115000*g*. Fractions are collected through a hole punctured in the bottom of the tube and the DNA in each fraction is counted for radioactivity. The R-factor DNA, comprising about 1·5% of the total DNA of the cell, is seen to sediment as a discrete peak about 3·8 times faster than the large mass of linear host DNA. Note that it is necessary to plot the data with two scales in order to detect the R-factor.

positive). This conformational change is thermodynamically unfavourable and results in a restriction of the dye binding. The net result is that the twisted covalently-closed molecules bind less dye under saturating conditions than do 'nicked' rings or linear duplexes. The binding of dye reduces the buoyant density of all DNA, so that in buoyant solutions of caesium chloride the density of twisted covalently-closed molecules is greater (because less dye is found) than that of corresponding 'nicked' rings and linear DNA. As shown in figure 6.3, the twisted covalently-closed ring molecules of an R-factor can be cleanly separated from the bulk of the host material by equilibrium centrifugation of a whole-cell DNA extract in caesium chloride in a saturating concentration (about 200 μg/ml) of ethidium bromide. It is important to see that the introduction of but a single-strand scission, such as a single 'nick' by a nuclease, has a profound effect on the behaviour of a twisted covalently-closed molecule (figure 6.4). The topological restraints are relieved and the molecule relaxes to a 'nicked' ring form, which displays a 'normal' degree of dye-binding and a 'normal' response to denaturation by heat and alkali.

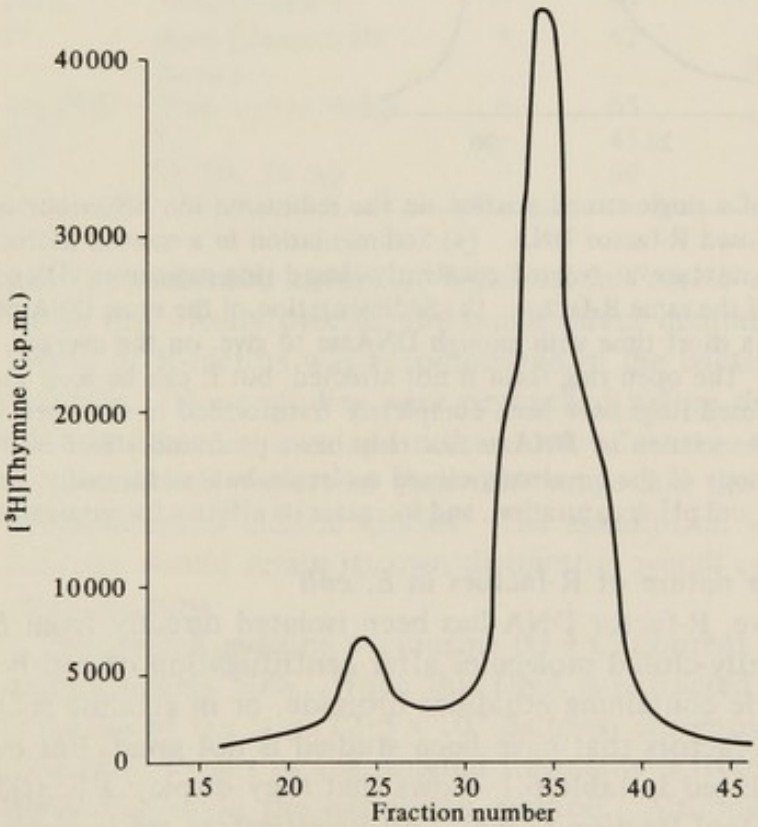

Figure 6.3. Demonstration of R-factor DNA by dye buoyant-density equilibrium centrifugation. [^{3}H]Thymine-labelled DNA extracted from an *E. coli* R^+ cell is centrifuged to equilibrium in caesium chloride solution containing 200 μg of ethidium bromide/ml. After centrifugation the bottom of the tube is punctured with a needle and fractions are collected and assayed for radioactive DNA. A discrete peak of twisted covalently-closed ring molecules is clearly separated from the huge mass of chromosomal material. 'Nicked' and linear molecules of the R-factor would band in about the same region as the chromosomal material.

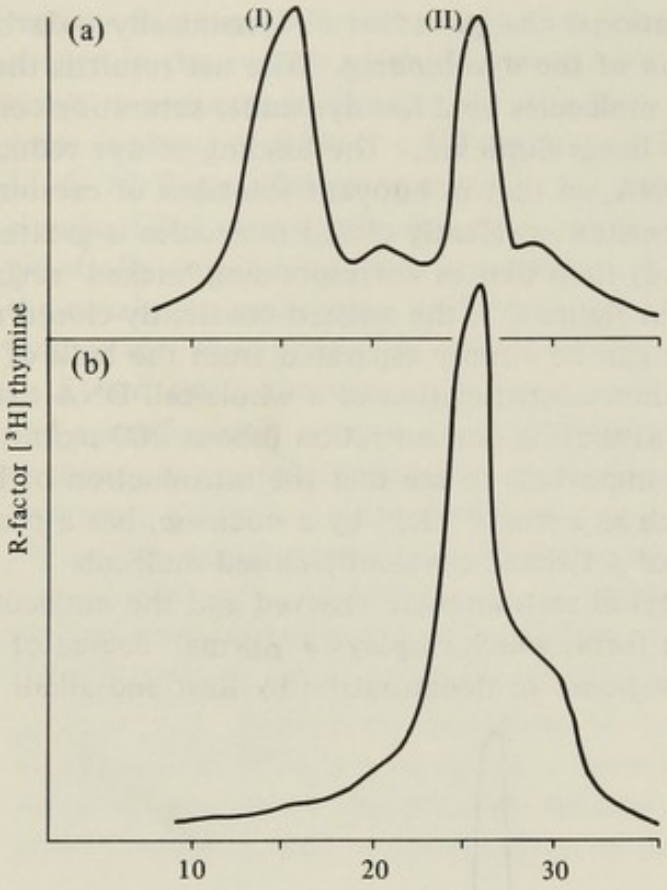

Figure 6.4. Effect of a single-strand scission on the sedimentation behaviour of twisted covalently-closed R-factor DNA. (a) Sedimentation in a neutral sucrose gradient of an equal mixture of twisted covalently-closed ring molecules (I) and open ring molecules (II) of the same R-factor. (b) Sedimentation of the same DNA mixture after incubation for a short time with enough DNAase to give, on the average, one 'nick' per molecule. The open ring form is not affected, but it can be seen that the twisted covalently-closed rings have been completely transformed to the open ('nicked') ring form. The single scission by DNAase not only has a profound effect on the sedimentation behaviour of the covalently-closed molecule but, additionally, removes its resistance to heat and pH denaturation, and increases its affinity for intercalative dyes.

6.2 The molecular nature of R-factors in *E. coli*

As described above, R-factor DNA has been isolated directly from *E. coli* as twisted covalently-closed molecules after centrifugation of cell lysates in caesium chloride containing ethidium bromide, or in alkaline sucrose. The number of R-factors that have been studied is not great, but even the limited selection listed in table 6.1 shows that they display a considerable range of size and mol fraction G + C. All investigators are in agreement that for the R-factors listed in this table there are a limited number of R-factor copies per *E. coli* host chromosome (this is not always the case as will be noted later). When the cellular DNA is very carefully extracted it can be shown that more than 75% of the total complement of R-factor molecules can be isolated as twisted covalently-closed molecules. Since these circular structures presumably represent a resting stage between

rounds of replication, this is not an unexpected finding because, at any given instant, few R-factors would actually be in the act of replication. Genetic transfer experiments, coupled with physicochemical studies, are consistent with the conclusion that both resistance and transfer functions of the R-factors presented in table 6.1 are associated with the single circular-molecular species within *E. coli* strains.

Table 6.1. Molecular nature of several R-factors and other transfer factors in *E. coli.*

R-factor	Resistance determinants	*fi* type	$10^6 \times$ Molecular weight	Guanine + cytosine content (%)	Number of copies per cell
R1*drd* 19	Su, Sm, Tc, Cm, Km, Ap	+	65	51·5	1-3
R6	Su, Sm, Tc, Cm, Km	+	65	51·5	1-3
222	Su, Sm, Tc, Cm	+	70	51·5	1-3
R-15	Su, Sm	−	46	49	1-3
RP4	Tc, Km, Ap	−	40	60	1-3
S-a	Su, Sm, Cm, Km	−	25	62	3-5
Col I	None (colicin I)	−	63	50	1-3
F	None (classical sex factor)	+	62	48·5	1-3
Ent P307	None (enterotoxin)	+	65	50	1-3
RTS-1	Km	−	45	46	1-3
r^{16}	Su, Sm, Tc, Ap	−	69	50	1-3

6.3 The molecular nature of R-factors in *Proteus mirabilis*

To be historically precise, the initial direct demonstration of the molecular nature of R-factors was accomplished in the bacterial host *Proteus mirabilis*. These studies were performed before the circularity of R-factor DNA had been shown and rather took advantage of the promiscuity of R-factors with respect to their host range and the diversity in the DNA composition of enteric species. The assumption was that the DNA of an R-factor would retain its own distinctive overall composition no matter what the host.

The overall guanine + cytosine (G + C) content of *E. coli* DNA is approximately 50%. When this DNA is extracted from the cell, there is breakage of the large ($2{\cdot}5 \times 10^9$ daltons) chromosome and, ordinarily, the molecular weight of the isolated fragments is from 10×10^6 to 200×10^6 depending upon the extraction method. Although the average content of G + C is 50% the heterogeneity of composition for the isolated fragments is essentially Gaussian about this mean, and individual fragments may be found approximately ±4% from the mean value. (It can be seen that if two bacterial species differ in average overall G + C composition by about 6%, it is fair to conclude that they have virtually no molecules of similar composition in common.) The distribution of G + C about the

mean can be visualized by equilibrium centrifugation of the DNA in caesium chloride, since the buoyant density of DNA fragments in a caesium chloride density gradient is directly related to G + C content. In most cases when the DNA of R-factor-containing *E. coli* is compared with DNA isolated from an isogenic R^- strain, the banding of the DNA species is grossly identical, indicating that the DNA of the R-factor has a G + C content within the range of the cellular DNA.

As pointed out above, R-factors can be transferred between practically all species of enteric bacteria. While most enteric species possess G + C contents of 50% or higher, *Proteus mirabilis* possesses an average G + C content of 39%. One would suspect that, by virtue of its independence of the bacterial chromosome, the R-factor DNA residing in a *Proteus* cell would be clearly separated in a caesium chloride gradient distinct from the host genetic material. This assumption proved to be correct, as illustrated in figure 6.5. The R-factor DNA can be seen as a smaller DNA fraction clearly separable from the bulk chromosomal material.

Figure 6.5 shows an important additional feature. The R-factor DNA is present as two quite distinct fractions which, on the basis of their density, are calculated to have G + C contents of 50% and 56%. It was not immediately clear if this finding meant that the breakage of R-factor DNA during isolation resulted in these two distinct G + C fractions, or if this meant that there were two distinct replicons. Other studies of F-merogenotes and colicin factors in *Proteus* had heretofore revealed only a single satellite fraction of 50% G + C. The situation was clarified in

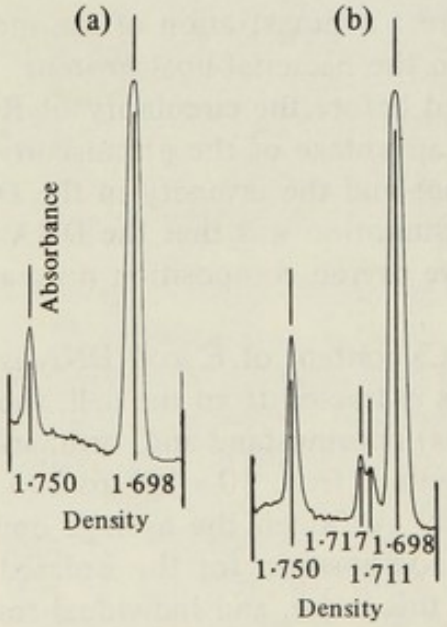

Figure 6.5. Caesium chloride centrifugation profile of DNA isolated from *Proteus mirabilis* before (a) and after (b) the acquisition of an *fi*$^+$R-factor (R-Su-Sm-Tc-Cm). The band of 1·750 g cm^{-3} in each represents an internal density standard. The band of 1·698 g cm^{-3} is the DNA of *P. mirabilis* (39% G + C). The satellite R-factor bands have buoyant densities of 1·711 g cm^{-3} (50–52% G + C) and 1·717 g cm^{-3} (56–58% G + C).

large measure when Robert H. Rownd and his associates discovered that the total amount of the satellite DNA fraction and the proportion of the 50% and 56% G + C subfractions exhibited enormous variations over the growth cycle of the *Proteus* host cells. This phenomenon is illustrated in figure 6.6, which shows the banding pattern of the DNA isolated from samples of the same culture of cells in the early logarithmic phase of growth and in the early stationary phase of growth. Not only is there a fantastic increase in the quantity of R-factor DNA during growth but, in addition, it can be seen that the relative proportion of the 50% and 56% G + C fractions can be varied by changing the antibiotic content of the growth medium. The mechanism whereby differing antibiotic selection affects the proportion of R-factor subunits will be discussed later on. For the time being, however, the basic observation itself is compatible with the conclusion that the separate DNA peaks represent autonomous units which are replicating at different rates.

The latter conclusion was confirmed more precisely by experiments reported during 1969–1970 from the laboratories of Royston C. Clowes and Stanley N. Cohen, as well as from the author's own. These workers fractionated the R-factor DNA from *Proteus* under very careful isolation conditions. These studies revealed that for R-factors 222, R1, and R6 (see table 6.1) the 50% G + C subfraction could actually be resolved into two distinct components corresponding to 49% and 51·5% G + C.

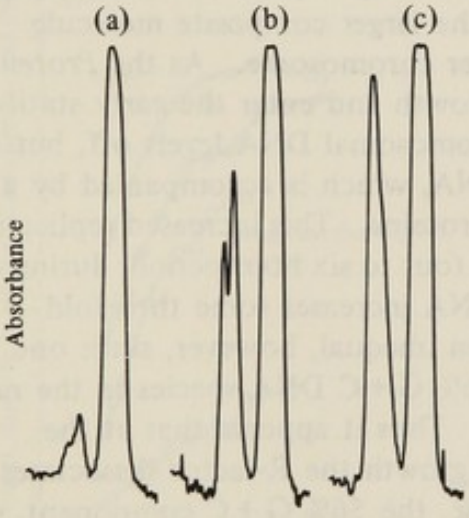

Figure 6.6. Analytical caesium chloride density-equilibrium centrifugation of DNA isolated from *P. mirabilis* carrying an *fi*$^+$ R-factor (R-Su, Sm, Tc, Cm). (a) The two satellite bands of 50% and 56% G + C as they appear in cells grown to the logarithmic phase of growth in a medium containing streptomycin, tetracycline, and chloramphenicol. (b) The DNA isolated from a parallel culture grown to the stationary phase of growth in the presence of streptomycin. (c) The DNA isolated from a parallel culture grown to the stationary phase of growth in a medium containing chloramphenicol. Each gradient contains about 8 μg of DNA. The large band to the right in each case is *P. mirabilis* chromosomal DNA with a buoyant density of 1·698 g cm^{-3} (39% G + C). (Redrawn from Falkow *et al.*, 1969.)

The 56% DNA fraction was, however, homogeneous. Examination of the three fractions in the electron microscope revealed that each contained tightly twisted and open circular DNA molecules. The measured molecular size of the molecules had interesting implications. Table 6.2 summarizes the results for the R-factor, R1, although these data are representative of many F-like R-factors.

Table 6.2 shows that the buoyant density, G + C content, and molecular weight for the largest of the three circular species of R1 (subfraction III) have a most pleasing agreement with the calculated values which would result from the associative joining of the two smaller units (subfractions I and II). The idea that the largest molecular species represented a complete R-factor genome was confirmed by examining the molecular nature of the identical R-factor after it was retransferred to *E. coli*. In this host, only a single circular molecular species, the 51·5% G + C, 65 x 10^6 daltons moiety, was found. This single molecular class was present, on the average, as a single copy per bacterial chromosome. The questions raised by these observations were manifold. What was the key to the dissociation of the R-factor molecule in *Proteus*? Why was it so stable in *E. coli*? What did the two smaller subunits represent?

A careful examination of the behaviour of the R-factor dissociation in *Proteus* led to a more sharply defined phenomenon than was originally thought. R-factors do not dissociate immediately after beginning their sojourn in *Proteus*. Rather, during the early exponential phase of growth, the predominant R-factor species is the larger composite molecule present as about five to ten copies per chromosome. As the *Proteus* cells leave the late logarithmic phase of growth and enter the early stationary phase of growth the synthesis of chromosomal DNA levels off, but there is a burst of synthesis of R-factor DNA, which is accompanied by a marked increase in R-factor-specific proteins. This increased replicative and biosynthetic activity continues over a four to six hour period, during which time the total amount of R-factor DNA increases some threefold (figure 6.7). The replication has been unequal, however, since one finds the 48% G + C, 51·5% G + C, and 56% G + C DNA species in the ratio of approximately 5 : 5 : 50 respectively. Thus it appears that at the beginning of the stationary phase of growth the R-factor dissociates into its component parts and, one of these, the 56% G + C component, shows

Table 6.2. Properties of R1*drd* R-factor DNA isolated from *Proteus mirabilis*.

DNA subfraction	G + C content (%)	Buoyant density in CsCl (g cm^{-3})	Contour length of circular molecules (μm)	10^{-6} x Estimated molecular weight
I	49	1·709	28 ± 1	53
II	56	1·716	5 ± 0·5	12
III	51·5	1·711	33 ± 0·8	65

a remarkably increased replication rate. This increased rate of replication is not without lethal consequencies to the host *Proteus* cell, since there is lysis of up to 30% of the bacterial population.

More recently it has also been possible to identify more precisely the nature of the 48% and 56% G + C R-factor subunits which appear in *Proteus*. This was accomplished in a direct manner by Stanley Cohen and Christine Miller by a clever combination of genetical and physical studies. R-factor containing *Proteus* cells were mixed with drug-sensitive *E. coli* and permitted to mate over many generations. The cells were plated and those *E. coli* cells that were still drug-sensitive were examined for the presence of any circular DNA species. Sure enough, some of these drug-sensitive cells contained a 53×10^6 daltons, 48% G + C, molecule. This element was transmissible from cell to cell and, in an independent study by Daniel K. Haapala and myself, it was demonstrated that this sex factor could reinfect *Proteus*, where it appeared as a 48% G + C, 53×10^6 daltons, species present as about five copies per chromosome over the entire growth cycle. The identity of this molecule with the dissociated subunit seen in *Proteus* was confirmed by DNA–DNA hybridization studies. Thus the intermediate-sized molecular species seen in *Proteus* is identified as the transfer portion of the R-factor and is the molecular equivalent of the hypothetical RTF of Watanabe and the Δ transfer factor of Anderson.

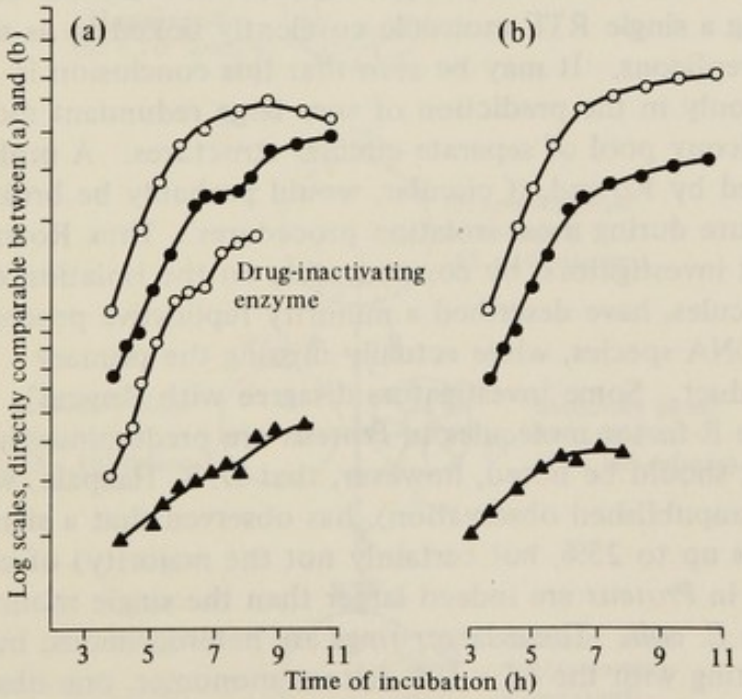

Figure 6.7. Change in biosynthetic activity over the growth cycle of a *Proteus* R^+ culture. (a) A culture of *P. mirabilis* carrying an R-factor (R-Su-Sm-Tc-Cm-Ap) was diluted into a medium containing 100 μg of chloramphenicol/ml. Samples were removed periodically and assayed for cell number, DNA content, total cellular protein, and a specific R-factor drug-inactivating enzyme. (b) A *P. mirabilis* R^- strain carrying a chromosomal mutation to chloramphenicol treated identically. ○, Cell number; ●, DNA content; ▲, total cellular protein.

The smallest molecular species seen in *Proteus* (56% G + C, 12×10^6 daltons) is not independently transmissible from *Proteus*. By DNA hybridization it can be identified as part of the 65×10^6 daltons molecule in *E. coli* and, furthermore, it is found to be primarily associated with the expression of drug resistance. It appears therefore that this replicon carries the majority of the drug-resistance genes. Indeed the only drug-resistance gene not identified on this 12×10^6 daltons molecule is that for tetracycline, which appears to be part of RTF. The reader may recall that the Tc gene was found to be closely linked to RTF by genetic analysis (chapter 5). The studies summarized above have been achieved primarily with five or six F-like R-factors of independent origin. In each case it is clear that there is dissociation into a transfer factor and resistance determinants, which replicate independently under different replication control systems.

This replication scheme is not universally accepted. Rownd and his associates have presented the alternative proposal that the R-factor subunits are not ordinarily independent, but rather that most of the time the 56% G + C replicons are covalently added in tandem to a single RTF molecule. This mechanism, Rownd thinks, explains the shift in average buoyant density of R-factor DNA from 1·710 g cm^{-3} to about 1·716 g cm^{-3} over the growth of the culture. In short, Rownd envisages the R-factor in *Proteus* as an enormous (theoretically about $6{\cdot}5 \times 10^8$ daltons) structure containing a single RTF molecule covalently linked to as many as fifty resistance replicons. It may be seen that this conclusion is basically different only in the prediction of very large redundant molecules rather than a multicopy pool of separate circular structures. A molecule of the size suggested by Rownd, if circular, would probably be broken into a linear structure during most isolation procedures. Thus Rownd contends that most investigators, by concentrating on the isolation of circular DNA molecules, have described a minority replicative process involving circular DNA species, while actually missing the primary replicative end product. Some investigators disagree with Rownd's contention that the R-factor molecules in *Proteus* are predominantly such huge structures. It should be noted, however, that D. K. Haapala, working in my laboratory (unpublished observation), has observed that a significant proportion (perhaps up to 25%, but certainly not the majority) of circular R-factor molecules in *Proteus* are indeed larger than the single monomeric ring usually seen in *E. coli*. These larger rings are heterogeneous, but not randomly so. Starting with the 65×10^6 daltons monomer, one observes twisted covalently-closed rings of 77×10^6, 92×10^6, and, rarely, larger molecular species. That is, each is larger by about 12×10^6 daltons—the size of the drug-resistance replicon. These data support Rownds's idea that there is some generation of oligomers consisting of one RTF replicon and two or more tandemly linked drug-resistance replicons. In the same

vein it may be noted that the production of circular oligomers, through errors in replication, was proposed by Bazaral and Helinski to account for the presence of significant amounts of Col E1 DNA in the form of dimers and trimers in *P. mirabilis*. A diagrammatic representation of the replication features of *E. coli* and *P. mirabilis*, representing all points of view, is presented in figure 6.8. Presumably no one mode of replication in *P. mirabilis* is exclusively used and there may well be differences from R-factor to R-factor and from *Proteus* host to *Proteus* host. For example, Rownd has employed a *P. mirabilis* host different from that of other laboratories and has studied the R-factor NR-1 exclusively. The observations from my own laboratory largely deal with the R-factor R1*drd* 19 in a standard laboratory strain of *P. mirabilis* called Pm-1. It should be emphasized, however, that all investigators unanimously agree that the R-factor can dissociate in *Proteus* hosts into separate units of replication, that the smaller 56% G + C unit contains the bulk of the drug-resistance genes and is represented as a multicopy pool, and that the transfer factor, RTF, is present as but a few copies per cell and is devoid of drug-resistance determinants except Tc.

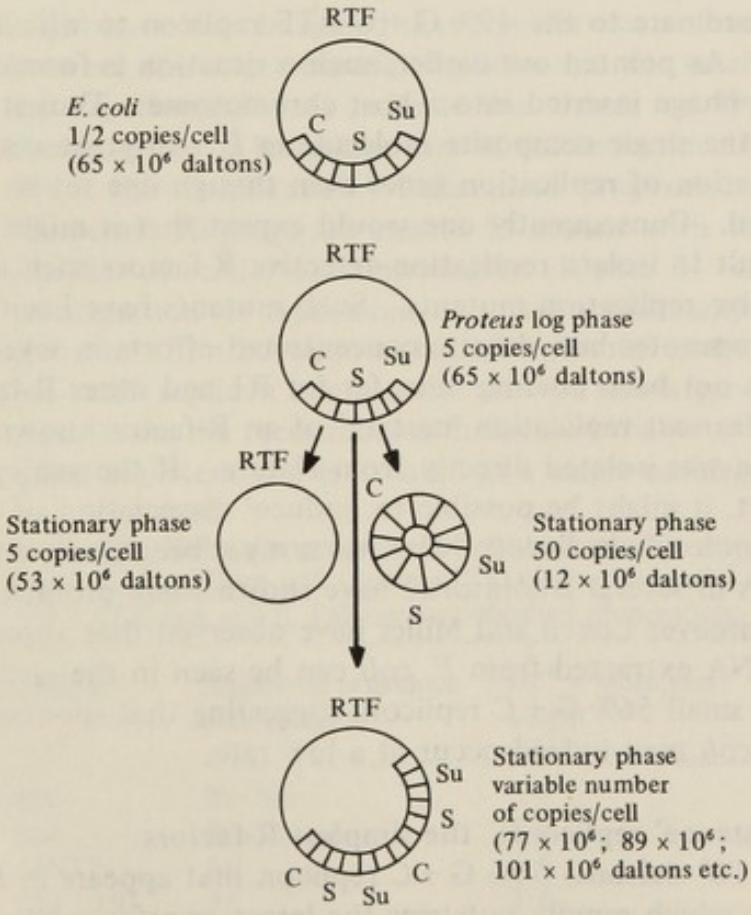

Figure 6.8. A diagrammatic representation of the molecular nature of R-factors in *E. coli* and in *P. mirabilis*.

One may be tempted to generalize about the molecular nature of R-factors from the observations of *fi*$^+$ R-factor behaviour in *Proteus*. It should be quickly noted, however, that only a few *fi*$^-$ R-factors have been cursorily examined in this species. Several R-factors have been reported to display only a single 49–51% G + C molecule in *Proteus*, showing neither relaxed replication or dissociation into subunit replicons. Moreover it should also be noted that several R-factors of independent origin have been discovered which show relaxed replication but do not dissociate into discernible subunits in *Proteus*. The point is that although the F-like R-factors studied thus far seem to behave pretty much in the same way in *Proteus*, there is little information about other R-factor classes.

Nonetheless the studies on the molecular nature of R-factors thus far do provide a rather more reassuring picture to accompany the genetic observations discussed in the previous chapter. It seems clear that an R-factor can represent an assemblage of two (or more?) replicons, each of which possesses distinct determinants that control replication. In the case of the R-factor, R1, in *E. coli*, the 56% G + C replicon, containing the bulk of the resistance genes, replicates as part of a composite molecule and appears to be subordinate to the 49% G + C RTF replicon to which it is covalently joined. As pointed out earlier, such a situation is formally analogous to a prophage inserted into a host chromosome. Thus it would be predicted that the single composite molecule in *E. coli* possesses a multiple representation of replication genes even though one set is ordinarily repressed. Consequently one would expect that it might be exceedingly difficult to isolate replication-defective R-factors such as temperature-sensitive replication mutants. Such mutants have been readily isolated for F-merogenotes but, despite concentrated efforts in several laboratories, it has not been possible thus far for R1 and other R-factors. Indeed the only clear-cut replication 'mutant' of an R-factor known at the time of writing was isolated directly from Nature. If the analogy with prophage is correct, it might be possible to 'induce' dissociation of a stable composite molecule in *E. coli*. This has not yet been done, although preliminary studies in several laboratories have shown some promise in this direction. Moreover Cohen and Miller have observed that about 1% of the total R1 DNA extracted from *E. coli* can be seen in the electron microscope as the small 56% G + C replicon, suggesting that spontaneous dissociation in *E. coli* may indeed occur at a low rate.

6.4 The 'drug resistance' replicons: the simplest R-factors

The smaller (12×10^6 daltons) 56% G + C replicon that appears in *Proteus* replicates at a rate which rapidly outstrips the larger transfer replicon. It would be interesting to know what would occur if this drug-resistance replicon were present alone in *E. coli*. Would it be a multicopy pool or would the number of copies be limited? Unfortunately no one has been

able to experimentally manipulate the 12×10^6 daltons replicon from *Proteus* into another host. Nonetheless, as discussed in chapter 5, nontransmissible drug-resistance plasmids do occur naturally in *E. coli*, *Salm. typhimurium*, and other enteric species, and a number of examples have now been studied at the molecular level in some detail. The molecular properties of several of these plasmids are presented in table 6.3. In every case, they are isolated as small (5×10^6 daltons to $9{\cdot}4 \times 10^6$ daltons) covalently-closed circular molecules which are present as multiple-copies (9–30) within the cell over the entire growth cycle. When a bacterial cell harbouring one of these plasmids is infected with a transfer factor such as F, Col I, or even a single-unit R-factor, the transfer factor and the small resistance plasmids remain as distinct circular molecules with the transfer plasmid present as but one or two copies per cell, while the small resistance plasmid still appears as a multicopy pool (figure 6.9). At the time of conjugation the transfer plasmid can mobilize the smaller resistance plasmid at a frequency of about 10^{-2}–10^{-6} per conjugal event, depending upon the transfer factor. Recipients of drug resistance, except in rare instances, also receive the transfer agent. Yet both genetic entities are found within the recipient as distinct circular molecules. Thus, while it might have been imagined that transfer was accomplished by recombination between the larger transfer replicon and the small drug-resistance replicon, the experimental data indicate that this is not the case; transfer factors do not seem to form any sort of stable recombinational union to bring about the mobilization of the small drug-resistance plasmids. These laboratory findings are clearly in accord with the Class 2 transfer systems of Anderson described in chapter 5. Moreover, and again in confirmation of Anderson, one can occasionally detect associative recombination between a small Tc plasmid and a transfer agent in which the small replicon has become part of the larger transfer replicon. In these cases the recombinant molecule behaves as a Class 1 single-unit transfer system and is isolated as a single covalently-closed circular

Table 6.3. Molecular properties of nontransmissible drug-resistance plasmids. [Data taken from Milliken and Clowes (1973); Guerry *et al.* (1974); Smith *et al.* (1974); and J. H. Crosa and S. Falkow, unpublished observations (1974).]

Plasmid	Antibiotic resistance determinants	10^{-6} × Molecular weight	G + C (%)	Number of copies per chromosome
SSu	Su-Sm	5·7	61	6–8
SSu	Su-Sm	5·0	56	30–50
A(NTP1)	Ap	5·6	46	17–18
RSF1030	Ap	5·6	48	20–40
T	Tc	6·5	-	4–7
NTP4	Ap-Su-Sm	8·8	-	5–8
ASu	Ap-Su	8·1	56	6–9

molecule present as only one or two copies per cell. In most cases, such recombinant forms are not the expected product of a simple addition of one replicon to another. Rather the recombinant molecules are somewhat smaller than the sum of the sizes of the two individual replicons, indicating that the recombinant replicons were not formed by a Campbell-type model of direct insertion. Thus one can 'create' a composite R-factor *in vitro* which mimics the behaviour of some naturally occurring R-factors. To my knowledge no one has yet prepared a recombinant molecule between a nontransmissible drug-resistance plasmid and the F-factor, or any F-like transfer factor, to determine whether it might show dissociative replication in *Proteus*.

The molecular studies of the small nontransmissible R-plasmids, the simplest R-factors, provide a firm base to re-enforce the genetic view given in chapter 5 that R-factors cannot be defined as any single kind of plasmid type. It is perhaps most intriguing to consider the small nontransmissible plasmid class as the basic or raw material of drug-resistance genes in bacterial populations, although, I suppose, one could alternatively suggest

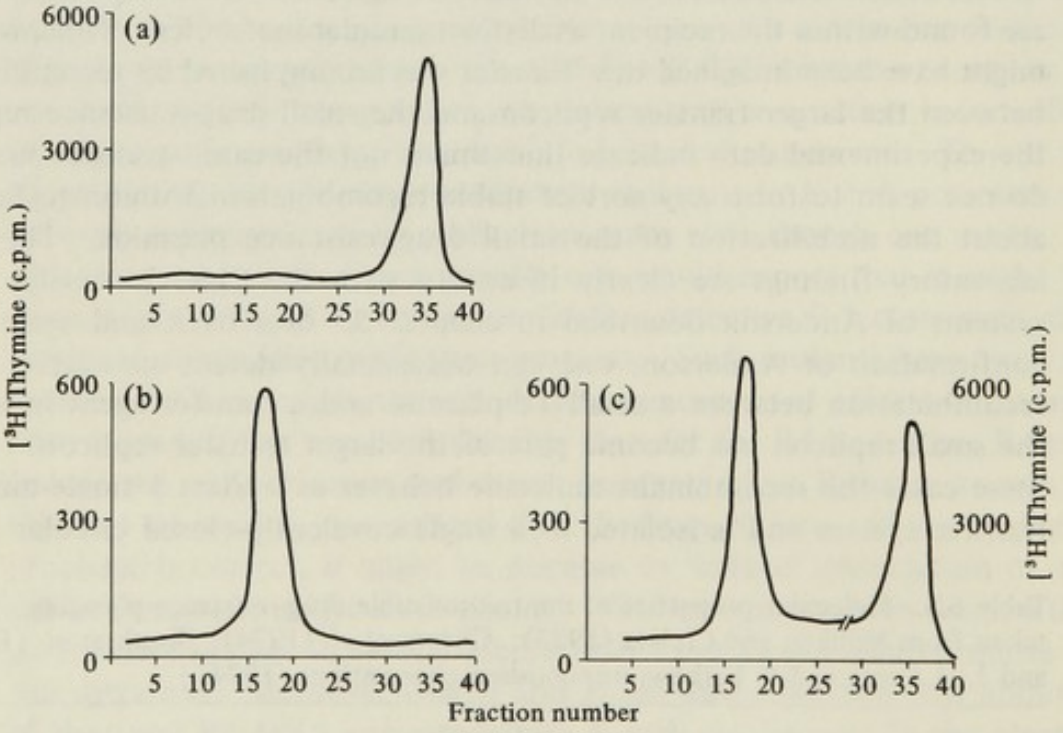

Figure 6.9. Independence of transfer factors and nontransmissible drug-resistance replicons. (a) Sedimentation of twisted, covalently closed rings of Su-Sm (5×10^6 daltons) isolated from *E. coli*. (b) Sedimentation of twisted covalently-closed rings of Col I (63×10^6 daltons) isolated from *E. coli*. (c) Sedimentation of the plasmid DNA isolated from an *E. coli* strain mixedly infected with Col I and Su-Sm. [A profile identical to that shown in (c) is found when recipients which have received both the Col I and Su-Sm plasmids by conjugation, are examined.] Note that there is a tenfold difference in scale, which reflects the larger number of copies per cell of Su-Sm (30-50) relative to Col I (1-2). (P. Guerry, J. van Embden and S. Falkow, unpublished work.)

that the smaller plasmids were derived from the dissociation of a larger genetic element. At the present time there appear to be at least three distinct kinds of genetic element called R-factors that can be separated from clinical isolates. One type is a nondissociable single unit of replication and transmission. The second type, so far confined to fi^+ plasmids, is generally a single unit of replication and transmission but contains component replicons into which the R-factor may dissociate under certain physiological conditions. The third is the type discovered by Anderson in which the transfer factor and drug-resistance genes are represented by distinct replicons within the cell under virtually all conditions of growth. This latter type, by recombination, could presumably form either of the two unitary types of R-factor.

Not all nontransmissible plasmids isolated from enteric bacteria are of small size and present in multicopy gene pools. For example, Anderson, Mayhew and Grindley have isolated a type of nontransferring Km plasmid that is about 36×10^6 daltons in size and present as but one or two copies per cell. This plasmid, as well as similar nontransferring plasmids of large size ($>20 \times 10^6$ daltons) we have observed carrying as many as five distinct resistance genes, form Class 2 transfer systems with F and other transmissible plasmids. In such cases it is not clear whether these nontransmissible plasmids are simply defective transfer factors, whose missing functions are complemented by a transfer factor, or whether they are being mobilized by the transfer factor in much the same way as the small multicopy replicons. The nontransferring Km plasmid is fi^+, suggesting that it may be a defective type. At any rate, these nontransmissible forms are not common and are fundamentally different from the resistance replicons such as Su–Sm and Ap.

The basic molecular features of plasmids such as Su–Sm and Ap are not unique. They bear at least a superficial similarity to those of the Col E1 plasmid (see chapter 3) in that they are noninfectious, about 5×10^6 daltons in size, and are present as a multicopy pool throughout the growth cycle of the host cell. You probably have noted that plasmids seem to be divisible into two distinct classes on the basis of whether their replication is under stringent or relaxed control. Roy Clowes, and others have made the distinction that larger plasmids such as F, Col Ib, and many single-unit R-factors replicate under stringent control and are present as but a few copies per cell. In contrast, Col E1, Su–Sm, Ap, and other small plasmids seem to be relaxed with respect to replication and are present as multicopy pools. These smaller relaxed plasmid classes are almost like a controlled phage infection such as seen with λdv or certain λN^- mutants described earlier (chapter 3). Clowes suggested that perhaps the control of plasmid replication was size related and that only smaller plasmids were 'relaxed'. The trouble with such distinctions is how small is small? For example, relaxed replication is not totally in the province of nontransmissible plasmids; the transmissible R-factor R6K (Ap–Sm), which is 25×10^6

daltons in size and 45% G + C, shows relaxed replication in both *E. coli* and *Proteus.* Polyxeni Kontomichalou, Mitani and Clowes have shown that in *E. coli* R6K ranges from 13 copies per cell in the logarithmic phase of growth to 38 copies per cell at the stationary phase of growth. This plasmid behaves similarly when in *Proteus* and, what is more, does not dissociate into subunits in either host. On the other hand, we have seen naturally occurring R-factors as small as 20×10^6 daltons that replicate under stringent control. Moreover an fi^+ plasmid which replicates under stringent control (one to two copies per cell) can be selected on a medium containing very high concentrations of antibiotic to a mutant form which normally contains four to eight copies per cell. Thus the distinction between stringent and relaxed replication cannot be absolutely distinguished solely on the basis of molecular size or whether the plasmid is transissible or nontransmissible. Nevertheless the analysis of the plasmid complement of a number of clinical isolates has shown that the small multicopy class of bacterial plasmids is quite common indeed.

6.5 The replication of R-factors

6.5.1 General models for the replication of circular molecules

Before describing the specific experimental information concerned with R-factor replication, it is useful to consider some general features of the replication of circular DNA molecules. If we assume that the starting point is a twisted covalently-closed ring form, it is apparent that the process of DNA replication of R-factors and other circular models must be divisible into several sequential processes, which include initiation, chain growth (elongation), and the separation of daughter molecules. Superimposed on these processes is the problem of how the covalently-closed DNA unwinds during replication, since topology alone forbids such a molecule to replicate semiconservatively without it untwisting. The experimental approaches to the study of replication have been greatly influenced by two general models: the *Cairns model*, and the *rolling circle model* proposed by Gilbert and Dressler.

The Cairns model (plate 2) was based on the observation that a replicating circular *E. coli* chromosome exhibited a Y-shaped region interpreted as a growing fork. It was envisioned that replication began at a specific initiation site (origin) without ring opening and that the ring doubled symmetrically as the growing point moved around the circle until it reached a terminus, at which point two equivalent daughter chromosomes were formed. In this model both strands have equal roles. Cairns recognized that this model demanded some sort of 'swivel' to unwind the strands but did not specify its precise nature. A different and asymmetrical type of replication of cyclic molecules, the rolling circle, was proposed by Gilbert and Dressler—based, in part, on an attempt to resolve the swivel problem as well as to account for the generation of multiple copies of a genome, as occurs in phage replication (plate 2). This model

envisioned that one strand (−strand) of a circular duplex remains closed during replication and serves as a template. The other strand (+strand) is cut by an endonuclease at a specific site and is elongated by the covalent addition of new bases to the free 3′-hydroxyl end of the strand. As the bases are added according to the dictates of the closed template, the 5′-phosphate free end of the strand is peeled out as a single-stranded 'tail' which is thought to attach to a cellular site, probably the cell membrane. Thus the 'old' +strand is peeled off, as a 'new' +strand is synthesized. This replication mode can be continuous and is called a rolling circle since, as the free end is peeled off, the closed template rotates as the growing point continues around the circle. Single- or double-stranded progeny can be generated by the rolling circle, dependent upon whether the 'tail' is duplicated as it is being displaced. The major impetus of this model was that replication could be continuous to generate multiple copies and that the two strands of the parental duplex were processed differently at initiation. As might be expected, both of these basic models have been modified and extended as new experimental data have been accumulated. Their value has not been that they accurately describe the general process of replication, because no one really expected that they could. Rather these models stimulated many laboratories to design accurate quantitative experiments to determine whether DNA replication was symmetrical (Cairns-like) or asymmetrical (rolling circle-like) and whether the initiation of replication was different on the strands of parental DNA molecules. As a result, it is now considered likely that the overall replication of most DNA species fits a symmetrical model best, whereas the asymmetrical model is best fitted to those instances involving the conversion of cyclic into linear molecules, as seen in the replication of the phage ϕX174, late stages of phage λ replication, and the conjugal transfer of DNA.

Both the Cairns and the rolling circle model conceded that DNA replication was initiated at a fixed point on the DNA molecule—the origin. There is considerable evidence to sustain the view that, in fact, the origin of plasmid replication occurs at a unique site on the molecule. Although replication proceeds unidirectionally from a fixed origin in the case of the phage P2 and the plasmid Col El (M. Lovett, C. Katz and D. R. Helinski, private communication), in other systems, such as *E. coli*, phage λ, and the animal virus SV40, replication proceeds bidirectionally. Bidirectional replication has, thus far, not been unequivocally demonstrated in any R-plasmid or transfer factor, although it is likely to be common. Some organisms display either unidirectional or bidirectional replication, depending on the growth conditions. The preliminary indication from several systems is that the two replication forks travel at about the same rate early in the replication cycle, but that one fork reaches a genetically determined terminus before the other. These data would suggest that the terminus of replication is not immediately adjacent to the origin, as was initially

assumed in the Cairns and rolling circle models, but may be near-by or, if the rates of travel of the two forks are equal, directly opposite the origin.

Given the fact that there is a unique origin for replication, how does initiation of new DNA synthesis, and elongation of the newly synthesized plasmid DNA, take place? The requirement for protein for the initiation of new rounds of bacterial chromosome replication is well documented, although the initiator proteins have yet to be isolated and characterized. Under conditions where protein synthesis is inhibited by amino acid starvation or chloramphenicol, the rounds of chromosomal replication that have already begun are completed but no new rounds of DNA replication are initiated. It should be noted that the distinction is made here between the protein(s) required for the initiation step and those required for chain elongation. That is, already initiated chains continue to grow normally even under conditions where protein synthesis has been inhibited. F, F-*lac*$^+$, R-factors and other large plasmids also require protein synthesis for their replication. They too complete rounds of replication already in progress, but do not reinitiate new synthesis. As will be pointed out more fully in subsequent sections of this and the next chapter, although our knowledge of the initiation of plasmid DNA synthesis is scanty, a reasonable model for its control must involve specific host-mediated cytoplasmic factors working in concert with plasmid-specific initiation and/or repressor substances. Not all plasmids cease their replication in the presence of a cellular inhibitor of protein synthesis, however. Noteworthy among these are Col El and the R-plasmid RSF1030 (Ap), which continue to replicate in the presence of chloramphenicol for many hours accumulating as many as 3000 copies of covalently-closed supercoiled DNA per cell. Obviously the protein requirement for the initiation of replication for these plasmids is under different control from that for most others. Another small plasmid Su-Sm also replicates in the presence of chloramphenicol but to a much more limited extent. The phenomenon is not restricted just to small nontransmissible plasmids; the transmissible R-factor R6K also replicates for at least three doublings after the host chromosome has ceased to replicate in the presence of chloramphenicol (M. Ortiz, L. H. Crosa and S. Falkow, unpublished observations). All of these 'exceptional' plasmids, Col E1, RSF1030, Su-Sm, and R6K do share the common property of being present as multicopy pools within their host cells. One may not generalize too much about these common features at present, other than that they suggest major differences in the control of replication for various classes of plasmids.

Very likely the major advances in our understanding of the initiation of plasmid DNA synthesis will be derived from a better understanding of the early biochemical events in the elongation of DNA chains. The three known DNA polymerases of *E. coli*, polymerases I, II, and III, all require a free 3′-hydroxyl end to start the synthesis of a DNA strand, and can synthesize DNA only in a 5′ to 3′ direction. However, in a double-

stranded molecule the two strands are antiparallel and therefore basically nonequivalent. Thus, in principle, one strand could be synthesized continuously in the 'normal' direction of replication, while the other strand must be growing biochemically away from this direction by some other mechanism. Over the past seven years or so, several lines of evidence show that this alternative is almost certainly a discontinuous mechanism involving the synthesis and joining of short DNA segments. The main contribution was Okazaki's finding that virtually all of the radioactive label incorporated into replicating *E. coli* DNA during a brief pulse can be recovered as short single-stranded DNA fragments (about 1000–2000 nucleotides long). These fragments are all synthesized in the 5′ to 3′ direction and, when followed over a period of time, are subsequently found in high-molecular-weight DNA through a mechanism which involves polynucleotide ligase. Experiments which permit the accumulation of 'Okazaki fragments' (for example in a ligase-deficient host) show that in some systems these fragments hybridize almost equally well with *both* strands of DNA, while in other systems, for example phage P2, the fragments hybridize with just one of the DNA chains. These data suggest that discontinuous synthesis can occur either on just one strand or on both strands. It is conceivable that a bidirectional movement of the replication fork may require a mechanism of two-strand discontinuous replication, whereas those DNA species, such as phage P2, which replicate unidirectionally may use only a one-strand discontinuous synthesis, the other being synthesized continuously in the 5′ to 3′ direction. Although most of the findings of discontinuous synthesis were carried out with bacterial DNA and the DNA of virulent bacteriophages, recent studies by Leonard Katz, Peter Williams, Ron Leavitt and Donald Helinksi (unpublished observations) have shown that at least one strand of the nascent DNA in a replicating Col E1 molecule is synthesized in short discontinuous fragments. It is not unreasonable to expect, therefore, that most plasmids replicate, at least in part, by a discontinuous mechanism. Once one accepts that DNA replication involves discontinuous synthesis, the question arises how this discontinuity of chain growth is controlled—that is, what is the mechanism of initiation and termination of synthesis of the DNA fragment? A good deal of experimental evidence now implicates RNA in the initiation of DNA replication at the origin of replication, as well as in the initiation of synthesis of the DNA fragment at specific sites on the template strands. The evidence for the role of RNA has, in large part, come from the observation that the antibiotic rifampicin, an inhibitor of RNA polymerase, inhibits the replication of Col El, F, R6K, RSF1030, phage λ, and many other species of DNA. The possible role of RNA in the initiation of Col El, RSF1030 (Ap), and R6K DNA has been further supported on the basis of finding RNA-containing supercoiled DNA in *E. coli* cells synthesizing DNA in the presence of chloramphenicol. The generation of these molecules, which undergo a

transition from the covalently-closed form to a 'nicked' form after treatment with ribonuclease, depends upon plasmid DNA synthesis and is prevented by rifampicin and actinomycin D. The properties of these RNAase-sensitive plasmid DNA species suggest that at least one strand of the growing DNA is synthesized from an RNA primer at the origin of replication. Furthermore, RNA has been found associated with short Okazaki fragments, indicating that the short DNA fragments are formed by extension of even shorter RNA chains that are synthesized on the parental DNA strands. Thus a hypothetical scenario for discontinuous replication would envisage that, as the parental strands unwind, there is synthesis of short RNA chains along one or both strands of the DNA template by an RNA polymerase. This RNA polymerase would first bind at some specific site, presumably a specific nucleotide sequence that signals chain initiation. The RNA polymerase would then initiate synthesis of a short RNA chain (estimated to be 50–100 nucleotides in length) which terminates at a specific 'stop' point on the chromosome. Presumably such 'start' and 'stop' points may recur at frequent intervals along one or both of the DNA strands. A DNA polymerase would then extend the RNA primer in the 5′–3′ direction after forming a phosphodiester bond between the ribo- and deoxyribonucleotides. The elongation of the chains by the DNA polymerase is followed by removal of the RNA segments with RNAase H (which hydrolyses RNA in a DNA–RNA hybrid). The gaps thus created between adjacent DNA fragments are filled in by the action of a DNA polymerase, so that finally the completed DNA fragments can be linked to each other by the action of DNA ligase to form a continuous stretch of newly replicated material.

As noted earlier, one feature that is superimposed on the problems of DNA initiation and elongation is the problem of the molecular swivel which acts to unwind the strands as replication proceeds. It is easy to see that the simplest swivel is the one postulated by the rolling circle model, that is, a single-strand scission in the double helix. Yet, in certain systems, replicative intermediates can be isolated in which the parental strands remain covalently closed. Specifically there are two distinct regions in such replicative intermediates—one part superhelical and unreplicated and the other containing two open circular branches of equal size which contain the nascent DNA. Such molecules of Col E1 are shown in plate 3. This plate also shows that as replication proceeds to completion there is a continuous reduction of the superhelical content until two equivalent daughter molecules are formed. The question, of course, is how the DNA can unwind during replication and still retain its covalently-closed conformation. Clearly such a swivel would have to be transient and movable. A likely candidate for such a transient swivel is the unwinding or ω-protein isolated from *E. coli* by James Wang. This 110000 dalton protein appears, at least *in vitro*, to act as a 'swivelase' or 'untwistase'. It introduces a swivel reversibly into DNA, and its departure

leaves no mark on the DNA with the exception that a number of superhelical turns are lost during its transient presence. The postulated role for ω-protein *in vivo* requires that the enzyme effectively brings about local unwinding through a transient nicking and resealing process. This would be sufficient to permit chain elongation to take place. Apparently as the growing replication fork progresses, new swivel points are created ahead. It is interesting to note here that an analogous protein, the so-called untwistase mentioned above, has been described in mammalian cells by Champoux and Dulbecco.

It seems clear that at some point in the replication process a break in at least one of the two polynucleotide strands must occur, if not to initiate replication then in order to release the two daughter molecules. The best candidate for a nicking enzyme that plays a role in the vegetative duplication and, possibly, the transfer of R-factors and other plasmids is the *relaxation complex* described by Don R. Helinski and his coworkers, notably Don Clewell, Don Blair, Bruce Kline and Yankel Kupersztoch. Under mild conditions of cellular lysis covalently-closed supercoiled molecules of Col El, F, Col I, R6K, and other plasmids can be isolated complexed to about 240000 daltons of protein. This is designated the relaxation complex because, when it is exposed to agents which affect protein structure, the twisted covalently-closed DNA molecule undergoes relaxation to a 'nicked' open circular form. The nicking event in the DNA of Col E1 has been found to take place at a single site, in a specific strand, at (or near) the site in the DNA of the origin/terminus. The end result is the formation of an open circular molecule containing a nick or gap and a free 3′-hydroxyl group. Initially it was proposed that replication was triggered in the cell after the relaxation event had occurred, and proceeded by a rolling circle mechanism by elongation of the free 3′-hydroxyl parent strand as a primer, or by the Cairns mechanism whereby the nick acted as a swivel. The more recent observation that replicating Col E1 remains covalently closed during replication has given rise to the alternative speculation that the protein components of the relaxation complex play a regulatory role in the replication process, or that they function *in vivo* through the transient nick–reseal process. A particularly attractive alternative is that, since the site of the nick is adjacent to the origin/terminus of the Col E1 molecule, the role of the relaxation proteins may be involved in the termination of replication to permit separation of the daughter molecules (L. Katz, P. Williams, R. Leavitt and D. Helinski, private communication).

In this section I have attempted to give a brief overview of some of the basic features of replication deduced from studies of the bacterial chromosome, certain bacteriophages, animal viruses, as well as plasmids, particularly Col E1. The various models presented for several phases of replication have been designed to be compatible with the known genetic and molecular properties of circular genomes, together with the fact that

the DNA polymerase can only synthesize DNA in the 5′ to 3′ direction while the parental strands of DNA are antiparallel. The dilemma that one faces is perhaps best typified by Col E1. Replicating molecules of Col E1 (or at least a presumed replicating structure) have been observed in several laboratories, which are partially covalently closed, circular molecules with two fork points (θ, Cairns structures), or circular molecules with linear double-stranded 'tails' (σ, rolling circle structures). A critical evaluation of all these possibilities still remains to be done and a similar assessment of these potential replicative structures in the synthesis of R-factors has yet to be carried out in any depth at all. Moreover while the more obvious sequential processes of DNA replication (initiation, elongation, joining) have been investigated experimentally, there are processes, such as the swivel, which are largely inferred from theory, and probably other mechanisms no one has yet thought of. In the following sections the replication of R-factor DNA is considered under conditions in which multiple copies are being generated, where DNA is being transferred at conjugation, and where the R-factor is duplicating in reasonable synchrony with the host. Not surprisingly, more than one basic mechanism of circular DNA replication appears to be operative. Furthermore the biochemical events taking place are, at best, only partially understood. While considering these data it may be useful to follow the suggestion given in 1968 by Rollin D. Hotchkiss that 'sophisticated' chromosomes of bacteria and plasmids having coordinate replication may have a regulated orderly system of replication along the lines of a symmetrical Cairns model, while DNA species specializing in 'mass production' of genomes may replicate more in line with an asymmetrical rolling circle model. *The important point is that the same molecule may well be able to follow fundamentally different modes of initiation and replication depending upon the biological circumstances under study.*

6.5.2 Replication in *P. mirabilis*

The replicative behaviour of R-factors has been examined both in *Proteus mirabilis* and in *Escherichia coli*. In *Proteus*, because of the density difference between the R-factor and the chromosome, the data have been obtained by density labelling, primarily in the laboratory of Robert Rownd and his collaborators. These experiments have shown that the number of R-factors per *Proteus* chromosome varies in a systematic way with the cell doubling time. For example, host cells with a thirty-minute doubling time possess approximately eight R-factor copies during the logarithmic phase of growth, whereas there are an estimated 32 copies of the R-factor per chromosome in cultures having a doubling time of two hours. The percentage of R-factor DNA is relatively constant through the exponential phase, which implies that there is a doubling of the multicopy pool of R-factors for each duplication of the bacterial chromosome. Thus, even though there is more than one round of R-factor replication for each division

cycle, the number of rounds is controlled. After entry into the stationary phase the subsequent dissociation and 'relaxed' replication may increase the number of R-factors per chromosome from two- to ten-fold, depending upon the nature of the growth medium.

With the basic recognition that there are multiple R-factors in *Proteus mirabilis*, Rownd and his associates examined the nature of the replication process of the multicopy pool. The phenomenon was observed in experiments in which cells, cultured for many generations in a ^{14}N-containing medium, were transferred to a ^{15}N-containing medium and growth then followed for several generations. Under these conditions the replication of the host chromosome and R-plasmids proceeds semiconservatively. The replication of the R-factors is in substantial agreement with a random model of replication, by which individual R-factor copies are withdrawn at random from the large cytoplasmic pool of copies. After replication the two copies are returned to the general pool. For the next round of replication another R-factor is selected at random, and this process is repeated n times during each generation. Thus it was shown that, after one cell generation, for a pool greater than six copies about one-half have been replicated once, about one-quarter have replicated more than once, and the remaining quarter have not replicated at all.

These findings effectively rule out alternative replicative models in which all plasmids replicate once and only once during each generation, as presumably is the case for the limited copy pool of R-factors in *E. coli*. Similarly a model in which one of the R-factor copies in the multiple pool duplicates n times each generation is ruled out. Rownd's studies further show that the initiation of the replication of the entire multicopy pool of R-factors in *Proteus* does not occur simultaneously and that the initiation of R-factor replication is not in synchrony with the initiation of the host chromosome.

Other experiments by Rownd have employed auxotrophic mutants of R-factor-infected *Proteus*, which have been starved of their required amino acids. There is only a small amount of R-factor replication after protein synthesis has been arrested. This finding is interpreted to mean that one or more proteins are required for the initiation of R-factor replication and that this protein is present in only limited amounts within any cell. The implication is that R-factor replication is controlled by a positive regulation system in which the number of rounds of replication is regulated by a determined quantity of initiator proteins during each bacterial division cycle. These putative initiator proteins presumably interact randomly with individual R-factor copies.

More recently (1971–1972) Dennis Kopecko and James D. Punch have examined the regulation of R-factor replication in *Proteus* with respect to the differential sensitivity of R-factor subunits to inhibitors and cultural conditions which interfere with protein and DNA synthesis. Every

investigator who has studied the behaviour of R-factor DNA in *Proteus* has noted that the addition of antibiotics, especially chloramphenicol, to the growth medium results in a much larger proportion of R-factor DNA, particularly the 56% G + C molecular species. Kopecko and Punch present evidence that these findings may reflect that, although the cells are resistant to chloramphenicol with respect to cell viability, the drug is nonetheless acting in low concentration to inhibit or reduce cellular protein synthesis. It is important to re-emphasize that increased replication and the dissociation of the R-factor molecule into subunits occur in drug-free medium. The addition of antibiotics amplifies this effect and this amplification was the phenomenon examined. After addition of chloramphenicol (25 μg/ml) to a *Proteus* R-factor culture, it was observed that there was an increase in the replication of all R-factor components by almost twofold, with the 56% G + C replicon showing a somewhat larger proportional increase. The addition of a higher concentration of chloramphenicol (100–150 μg/ml) resulted in a sharp reduction of protein synthesis and was observed to be associated with a concomitant sharp reduction in the replication of the 49% G + C transfer replicon and the composite molecules. Meanwhile, replication of the 56% G + C replicon was scarcely affected. Kopecko and Punch interpret these, and similar findings with streptomycin, puromycin, and at low temperature to mean that a repressor is situated on the 49% G + C transfer replicon (and on the composite molecule), which acts in a negative way on both itself and on the 56% G + C replicon. Low concentrations of chloramphenicol are presumed to inhibit synthesis of this repressor, leading to an increase in all R-factor components. The inhibition of the replication of 49% G + C composite molecules by higher concentrations of chloramphenicol is taken to suggest, in agreement with Rownd, that these molecules require protein synthesis for replication and are under the positive control of a self-coded initiator. In contrast the 56% G + C replicon is derepressed and continues to replicate for a significant period of time in the presence of chloramphenicol, as evidenced by the large 56% G + C satellite component observed in caesium chloride gradients. Kopecko and Punch further present the hypothesis that the replication of the transfer and composite replicons requires a specific 'maintenance site' for replication whereas that of the 56% G + C replicon does not but follows a strictly cytoplasmic replication. The lines of evidence used to support this latter hypothesis come from the observation that phenethyl alcohol and nalidixic acid (which are reported to affect DNA-membrane association and initiation of DNA synthesis respectively in *E. coli*) inhibit replication of the transfer and composite replicons, while, again, the 56% G + C replicon escapes unscathed.

The results of Rownd and his associates and of Kopecko and Punch, although agreeing in many substantial points, are in disagreement in other aspects. Both groups have presented models with testable predictions, so

that further experiments will be most enlightening. These studies emphasize, even at this early stage, the many advantages offered by the *Proteus* R-factor system for the study of DNA replication and its control.

6.5.3 Replication in *E. coli*

The stability of both *fi*$^+$ and *fi*$^-$ R-factors in *E. coli* attests to a functioning replication apparatus that effectively duplicates the R-factor molecule, and a segregation mechanism that provides for at least one copy of the plasmid in each of two daughter cells at cell division. The biochemical details of R-factor replication and segregation are poorly understood. Since most *fi*$^+$ and *fi*$^-$ R-factors are present in *E. coli* as only one to three copies per chromosome the obvious inference is that they are ordinarily duplicated only once during the bacterial division cycle. Preliminary evidence has been obtained that *fi*$^+$ R-factors in *E. coli* replicate at a fixed time during the *E. coli* cell cycle, although R-factor initiation does not necessarily coincide with that of the bacterial chromosome. Moreover, as will be pointed out in the following sections, in accord with the replicon hypothesis the bacterial membrane appears to play a direct role in the replication of R-factors. All in all, the known facts support the notion that, in the autonomous state, R-factors (as well as F and other plasmids) behave as small supernumerary chromosomes which are nonessential but stably maintained and replicated more or less synchronously with the chromosome. The replication of these autonomous plasmids in *E. coli* seems to be closely akin to the mechanism regulating the bacterial chromosome itself.

The nontransmissible plasmids such as Su–Sm and Ap have not yet been examined extensively in *E. coli*. The data available (J. H. Crosa and S. Falkow, unpublished work) show, however, that the small plasmids replicate randomly, as already described for the multicopy Col E1 and the replication of the 56% G + C drug-resistance replicon in *Proteus*. Thus some of these plasmid molecules replicate twice during the cell cycle and an approximately equivalent number do not replicate at all. There are only limited data on the control of replication, but even these data permit one to state that those small R-factors are not uniform with respect to their initiation of DNA replication. For example, the Ap plasmid RSF1030, like Col El, replicates in the presence of chloramphenicol, and about 30% of the molecules covalently incorporate RNA into their structure. In contrast the superficially similar Su–Sm and Tc plasmids show little replication in the presence of chloramphenicol. Clearly then, the Su–Sm and RSF1030 plasmids have rather different control mechanisms with respect to the initiation of DNA replication in the absence of host protein synthesis.

While density differences suffice to separate R-factors from the host chromosome in *Proteus*, this is usually not practical for replication studies in *E. coli*. Most molecular studies in *E. coli*, therefore, have of necessity dealt primarily with the isolation of twisted closed circular molecules.

Since this form is the end product of replication, there are very few direct studies on replicating R-factor DNA once it is established in *E. coli*. The separation of transient noncovalently-closed plasmid forms from the mass of host chromosome has been simply too great an experimental burden to overcome. The very recent finding of R-plasmids which can replicate when the chromosome has ceased to replicate, because of an antibiotic such as chloramphenicol or owing to a temperature-sensitive host mutation (see chapter 7), will probably hasten the determination of the number and nature of gene products responsible for the replication of R-factors, as well as the biochemical reactions involved in the control and initiation of the duplication process itself. For the present, however, we must be content to concentrate on several experimental approaches which permit one to follow R-factor replication in *E. coli* immediately after conjugation and after segregation into anucleate bacterial cells.

(a) R-*factor replication during conjugation*. Despite the voluminous information available on the genetics of bacterial conjugation, there has been until recently little information on the mechanism of transfer. Jacob, Brenner and Cuzin proposed in 1963, as part of the replicon hypothesis, that gene transfer was a specialized form of DNA replication that occurred in the male cell. These authors, as well as Gross and Caro in 1966, showed that the transferred DNA found in a recipient cell consisted of one strand synthesized during mating and one strand that existed in the male before mating, implying indeed that DNA synthesis was involved in conjugational DNA transfer.

There has been an interesting controversy concerning the relationship of DNA synthesis to gene transfer. Various investigators have proposed different models to explain transfer replication: it has been proposed that the entire thrust of replication occurs in the donor, with the female showing complete passivity; models have been proposed advocating that the female plays the active role, while DNA replication in the donor is not directly involved in transfer; finally, it has been suggested that DNA synthesis by both the donor cell and the recipient cell is necessary to consummate the conjugal act. Most recent studies favour the last view, that DNA synthesis in both the donor and recipient is required.

It now seems well established, particularly from the work of Vapnek and Rupp, that only a single strand having a 5′-phosphate end at the origin is preferentially transferred from the donor to the recipient and that the complementary strand is synthesized in the recipient. The single strand remaining in the donor likewise has a complement synthesized so that it is preserved rather than discarded by the donor cell. Thus, as noted earlier, conjugal DNA synthesis represents a remarkable and startling spatial and enzymatic separation of the replicative fork. It may also be noted that the single rolling circle model makes the prediction that the strand transferred to a female cell would be the one that is peeled off the

circle first, which, in accord with the experimental evidence, would enter with its 5′ end first. Ordinarily one might have expected the opposite result, namely that the 3′-hydroxyl would have entered first, so that a continuous smooth synthesis on it would drive the transfer. At any rate, both F-like and I-like R-factors are transferred from *E. coli* as single strands, which enter the recipient with the 5′-terminal first. The replicative steps that subsequently befall this single strand have also been sketched in broad outline in my laboratory.

The replication of R-factor DNA within a recipient has been followed in a system developed by the Freifelders, which employs a male cell that contains a genetic block preventing thymine incorporation, together with a female cell which cannot replicate its own DNA as a consequence of its inability to repair lesions produced by a heavy dose of ultraviolet light.

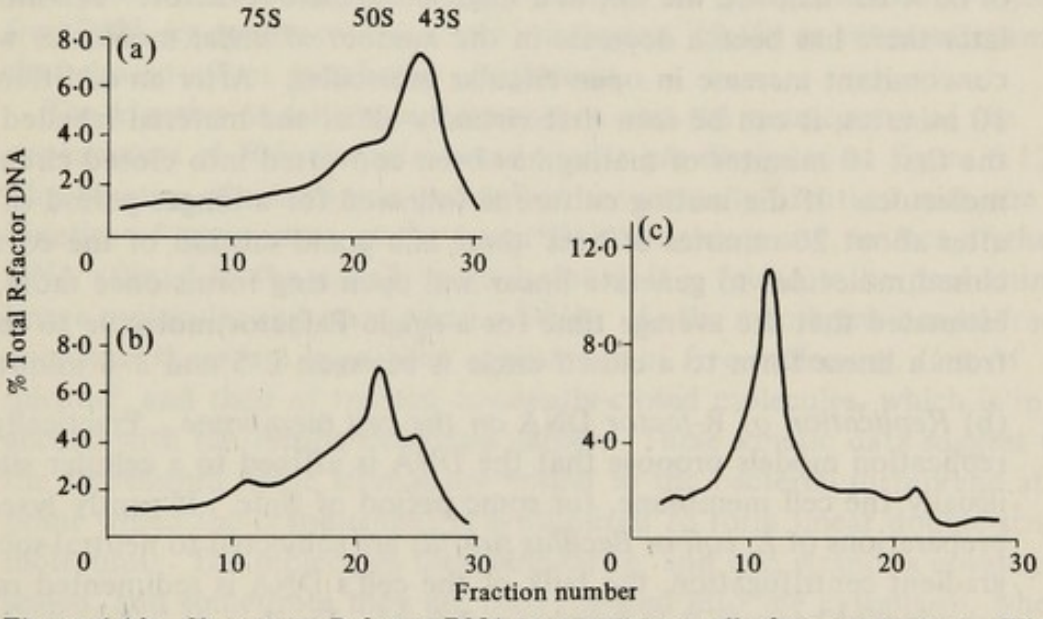

Figure 6.10. Changes in R-factor DNA in a recipient cell after conjugation. When mating is performed under appropriate conditions in the presence of radioactive thymine, the only net incorporation of label is found in the R-factor DNA within the female cell. In this experiment the cells are mated in the presence of [^{3}H]thymine for 10 min (pulse), at which time an excess of unlabelled thymine is added (chase). This procedure permits one to follow any changes subsequently occurring to the DNA labelled during the first 10 min of mating. (a) Sedimentation in a linear neutral sucrose gradient of R-factor DNA synthesized during the pulse period. The predominant species of R-factor DNA is a 43S linear double-stranded DNA monomer (65×10^6 daltons). It is known from other studies that the R-factor DNA actually enters the recipient cell from the donor as a single-stranded linear molecule. Thus during the first 10 min the complementary strand has been synthesized within the recipient cell. (b) 10 min later (20 min after mating) the linear molecules have been largely transformed into a DNA species which sediments at 50S, indicating that a conversion into 'nicked' ring forms has occurred. (c) After an additional 10 min (30 min after mating) it can be seen that the R-factor DNA has been virtually completely converted into a twisted covalently-closed ring, which sediments at 75S.

The irradiated female is still capable of performing as an active sexual partner with the male cell, so that replication and even subsequent transfer can occur. Thus, when a mating is performed with this system in the presence of radioactive thymine, the major net incorporation of the label is found in the R-factor DNA. Since sex factor function of most R-factors is generally repressed, only de-repressed R-factors have been examined thus far by this method.

Figure 6.10 shows the molecular transformations undergone by R-factor DNA after cells are mated in a short pulse of [^{3}H]thymine followed by a 'chase' of a large excess of nonradioactive thymine. Under these conditions the fate of molecules labelled early in the mating can be followed. At the time of the chase, 10 minutes after conjugation was initiated, the predominant species of R-factor DNA are linear double-stranded molecules corresponding to approximately a unit length in this case of 65×10^6 daltons, the size of a single composite R-factor. Ten minutes later there has been a decrease in the number of linear molecules with a concomitant increase in open circular molecules. After an additional 10 minutes, it can be seen that virtually all of the material labelled during the first 10 minutes of mating has been converted into closed circular molecules. If the mating culture is followed for a longer period of time, after about 20 minutes of 'rest' there is a grand scission of the covalently closed molecules to generate linear and open ring forms once more. It is estimated that the average time for a single R-factor molecule to replicate from a linear form to a closed circle is between 2·5 and 3·0 minutes.

(b) *Replication of* R-*factor* DNA *on the cell membrane.* Practically all replication models propose that the DNA is affixed to a cellular site, usually the cell membrane, for some period of time. If gently lysed cell preparations of *E. coli* or *Bacillus subtilis* are subjected to neutral sucrose-gradient centrifugation, the bulk of the cell's DNA is sedimented rapidly, presumably because it is membrane-bound. Further, it is found with this technique that a pulse of labelled thymine added to cells growing at an exponential rate appears first as DNA in the membrane fraction, suggesting that newly replicated DNA or the replication points are specifically membrane-bound. The attachment of the DNA to the membrane is a comfortable way of explaining both the regulation of replication and the separation of daughter replicons.

The association of R-factor DNA with the cell membrane during bacterial mating has been determined in an experimental mating system identical, except in one respect, with that discussed above. The difference is that the method of cell lysis employed is less disruptive to the cellular architecture. Furthermore the sedimentation of the cell lysates in sucrose gradients is performed over a dense shelf of sucrose admixed with caesium chloride. Membrane-bound DNA forms bands directly above this shelf

and is clearly separated from the cellular debris, which pellets at the bottom of the tube, and from the free 'cytoplasmic' DNA, which sediments near the top of the gradient. When this method of separation is applied to DNA pulsed early in a mating it is found, as shown in figure 6.11, that initially most of the DNA entering a recipient cell is indeed attached to a cellular structure. However, as replication proceeds in the recipient, it is found that the proportion of membrane-attached DNA decreases significantly while the proportion of cytoplasmic unbound R-factor DNA accumulates. An important reservation in interpreting these results is the nature of the cellular material to which the DNA is attached. The attachment is specific in the sense that added DNA does not simply adsorb to cellular fragments. However, although membrane fragments are present in abundance on the shelf, it has not been positively shown in these experiments that the DNA is attached actually to membranous structures. The bound DNA can, however, be converted into cytoplasmic free DNA by treatment with ionic detergent, alkali, or other substances that should affect membrane attachment.

The kinetics of membrane association and the time-course of the appearances of R-factor molecular species are displayed in figure 6.12. The kinetics of membrane association are virtually identical with the kinetics of appearance of the linear DNA duplexes and, in fact, if the DNA affixed to the membrane is isolated, it is found to be predominantly linear molecules and open circular DNA. As the membrane-bound fraction decreases there is a sequential appearance of free molecules, first of 'nicked', and then of twisted covalently-closed molecules, which is in accord with the results described earlier. These kinetic data suggest that the transferred single strands are bound to the bacterial membrane at the point where the complement is synthesized to form linear double-stranded molecules. The molecules then circularize, and one strand is covalently sealed, and sometimes they are then released into the cytoplasm, where they are completely covalently sealed to form a twisted supercoil. One must specify "... sometimes released", for some R-factor DNA (about one-third) remains on the membrane throughout the entire process. An alternative therefore is that the molecules fixed to the membranes serve as templates for the synthesis of new R-factor copies, which appear in the cytoplasm.

These results are in accord with the idea that, rather than staying strictly associated with the membrane, R-factor DNA, although apparently replicating upon the cellular membrane, need not have a stable relationship with this cellular structure between rounds of replication. In fact if exponentially growing cultures which harbour R-factors, F-factors, or Col factors are gently lysed and separated into membrane-bound and free DNA fractions, the bulk of the plasmid DNA is found as covalently closed molecules in the cytoplasmic fraction.

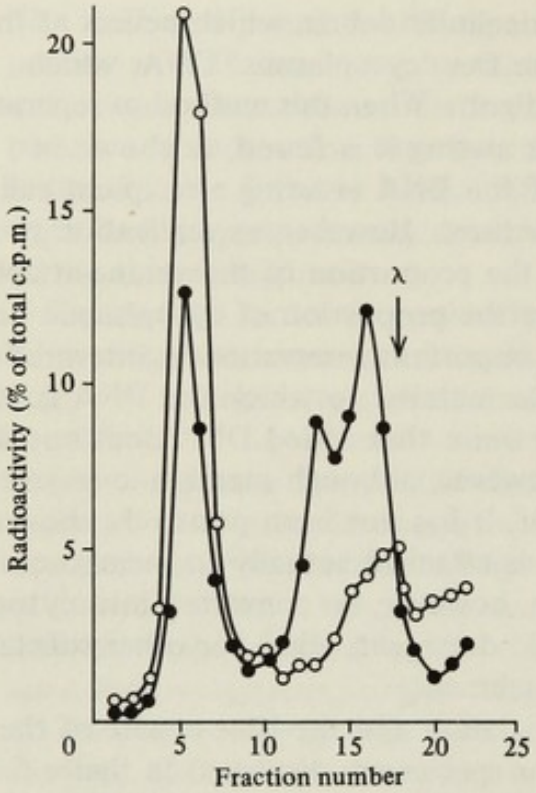

Figure 6.11. Association of R-factor DNA with the bacterial membrane after conjugation. A mating was performed under appropriate conditions in the presence of radioactive thymine, so that label is found only in the R-factor DNA transferred to the recipient. The mating mixture was lysed with the surfactant Brij 58, and run on a 5-20% linear sucrose gradient layered over a dense shelf of sucrose + caesium chloride. Membrane-bound DNA bring bands directly over this shelf (around fraction 5), and 'free' cytoplasmic DNA sediments near the top of the gradient according to its sedimentation properties. It can be seen that the proportion of R-factor DNA bound to the bacterial membrane changes dramatically over a relatively short time-span. ○, 10 min after mating; ●, 30 min after mating.

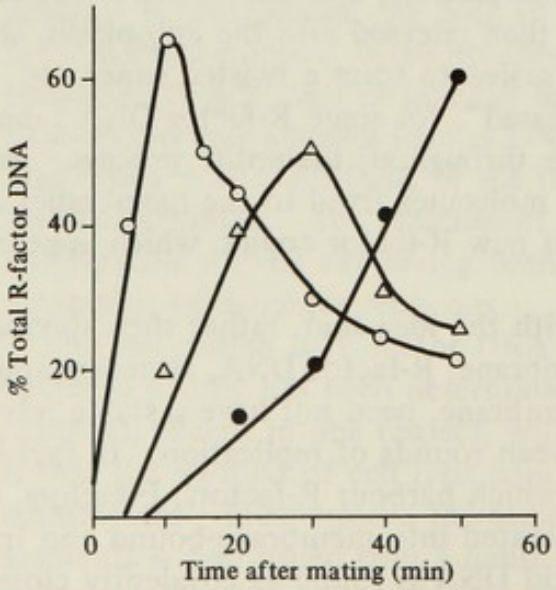

Figure 6.12. Time-course of the appearance of R-factor molecular species after conjugation. ○, Membrane-bound DNA; △, 'nicked' ring; ●, closed ring.

This latter observation has been exploited in a practical way as a method to obtain plasmid nucleic acid. Thus, whereas most replication models envisage linkage of the chromosome to the membrane because segregation at cell division is automatic, the experimental evidence gives a somewhat contrary view. At face value it would seem that replication models for plasmids must be made more complicated by postulating enzymatic steps that cut the DNA off the membrane and close the circle. On the other hand, as one example, the experimental findings enable one more easily to envisage the mechanism of F-integration to form an Hfr strain (chapter 2). It should be emphasized that the data still support the notion that the replication point is a membrane-bound function. Of course, it is rather uncomfortable to think of circular DNA just 'floating around' aimlessly in the cytoplasm. The data do not imply this but only that a 'stable' relationship between the plasmid DNA and the membrane does not exist under the experimental conditions. In the following paragraphs a phenomenon is described which also suggests that R-factors and other plasmids may be cytoplasmic- as well as membrane-bound.

(c) *The kinetics of protein and* RNA *synthesis in a recipient cell following conjugation.* Fenwick and Curtiss have investigated the effect of inhibitors of RNA and protein synthesis on the conjugal DNA-synthesis of an *fi*$^-$ R-factor. They found that the initial conjugal events in a donor cell are sensitive to rifampicin but are insensitive to chloramphenicol. These data are interpreted to mean that initiation of the transfer process is inhibited owing to the failure to make an untranslated RNA species (perhaps a 'primer'). The data also indicate that whichever proteins are required for conjugal transfer are present in cells containing a vegetatively replicating plasmid. Vickers Hershfield, working in my laboratory (unpublished studies), has also examined RNA and protein synthesis subsequent to conjugal transfer of the *fi*$^+$ R-factor, R1*drd*19. To accomplish this end she employed rifampicin to inhibit RNA and protein synthesis in donor cells while recipient cells, blocked for chromosomal DNA synthesis but resistant to rifampicin, were employed. When these cells were mixed in the presence of rifampicin + radioactive leucine or uracil, the net incorporation of the labelled precursors could be used as a measure of R-factor protein and RNA synthesis within the recipient. Whereas the addition of rifampicin has a rapid effect on donor cells, so long as they are used immediately they may function efficiently as donors for five to seven minutes.

Under these experimental conditions, Hershfield found that R-factor DNA could be successfully transferred, become established, replicate, and produce specific R-factor proteins. The relative rate of incorporation of labelled precursors into R-factor DNA, RNA, and protein appear to be correlated with the time-course of R-factor replication in the recipient cell. The entire active transfer process takes only about 5 minutes and, as indicated earlier, at the earliest times measured (10 minutes after

conjugation) the large proportion of R-factor DNA in a recipient was found as double-stranded linear molecules attached to a cell membrane component. During this time period there was relatively little net DNA synthesis, but there was a significant synthesis of R-factor-specific RNA and protein. Thus during the first 15 minutes after receiving an R-factor, the recipient cell synthesizes only 12% of the final level of DNA that will be made, but one finds that over 40% of the total protein and over 60% of the RNA have been synthesized. The RNA synthesized was about 9S–12S and could be specifically hybridized to R-factor DNA. A somewhat unexpected finding was that virtually all of the R-specific messenger RNA (mRNA) synthesized during the first 15 minutes was hybridizable with the RTF portion of the R-factor molecule, but not with the drug-resistance determinant (that is, the 12×10^6 dalton replicon). This result is interpreted as indicating that this initial burst of mRNA and protein synthesis reflects the expression of RTF genes that may be required for plasmid replication and establishment. Between the 15th and 30th minute after receiving an R-factor, the rate of DNA synthesis increased sharply and roughtly 62% of the total R-factor DNA was synthesized. During the same time period, the relative rate of synthesis of RNA and protein declined sharply. Yet at this time over 50% of the hybridizable mRNA forms specific duplexes with genes of the drug-resistance determinants, rather than with the RTF portion of the molecule. Concommitant with this change in transcriptional specificity one begins to see a very rapid synthesis of R-specific drug-inactivating enzymes (see chapter 9). By 40 minutes after conjugation, the rates of DNA, RNA, and protein synthesis remained at a fairly constant level. There was a low level of mRNA synthesized continually that was directed both at the RTF and drug-resistance replicons, particularly the latter. Moreover, between the 30th and 40th minutes after conjugation one could show, for the first time, retransfer of the R-factor from the converted recipient cell—that is, the recipient cell had now been converted into an effective R-factor donor.

Hershfield feels that the burst of DNA synthesis during the 15th to 30th minutes after entering a recipient cell represents at least an R-factor doubling, which is coincident with the previously observed change in topology from the linear double-stranded form to the open circular and covalently-closed forms. Although one cannot precisely identify the number of replicative rounds which the entering R-factor may undergo, the level of R-specific drug-inactivating enzymes displays a fourfold increase in activity compared with normal established cell lines. This suggests that up to two or three rounds of replication take place in a recipient cell. These data are of course by no means definitive and, since inhibitors were employed to examine R-specific products, may not be a reflection of normality. Nonetheless the data are consistent with a temporal sequence of replicative and transcriptional events that do not seem unreasonable.

(d) *Segregation of* R-*factor* DNA *into chromosome-less E. coli cells.* Mutants of *E. coli* K-12 have been isolated that possess a remarkable cell-division lesion. At cell division small anucleate spheres are produced in addition to normal-sized cells. These minicells, which occupy about one-tenth the volume of a normal cell, are produced under a variety of growth conditions and are from one to three times as numerous as their normal-sized sisters. Because of their small size, minicells can be easily separated from normal cells by differential centrifugation or by taking advantage of the fact that normal cells are sensitive to penicillin-induced lysis while the minicells are totally resistant. Isolated minicells from an F^- strain are almost entirely devoid of DNA, although chemical analysis shows that they contain RNA and protein in a ratio similar to that of normal-sized cells. It is noteworthy that certain enzymes having DNA as their substrate (for example, DNA methylase and DNA-dependent RNA polymerase) do not appear in minicells. This has been interpreted to mean that practically all molecules of these enzymes are constantly bound to their DNA substrate. Yet the enzymes DNA polymerase and polynucleotide ligase are found in minicells. Their presence raised the interesting possibility that, if DNA could be introduced into a minicell, it might serve as a template for further DNA synthesis. This idea was tested by employing the minicells as recipients with radioactively labelled F^+, F-merogenote, and Hfr donors. Such experiments showed that a single strand of DNA was transferred into the minicells, which, upon further incubation with exogenously supplied bases and nucleosides, was converted into double-stranded material on the cell membrane.

A number of investigators have presented both biochemical and biological evidence for the segregation of F, Col, and R-factors into minicells during the bacterial growth cycle (see table 6.4). Sex factors within the minicells are functional, since they may be donated to suitable normal recipients by conjugation. These results were unexpected, since several lines of previous experimental evidence suggested strict cosegregation of the sex factor, F, with the bacterial chromosome. Leon Kass and Michael Yarmolinsky, who studied the segregation of F-merogenotes into minicell lines, considered two explanations to account for the appearance of F (and other plasmids) in these lines: first, F is not free in the cytoplasm but is joined by a common element to the bacterial chromosome with which it is cosegregated. Occasionally F and the chromosome are separated by the plane of abnormal cell division, so that F enters the minicell but remains bound to its membranous (or other) site. Second, F and other plasmids exist both free in the cytoplasm and bound to the cell membrane. When truly cytoplasmic, F will be occasionally trapped in a newly forming minicell. It is interesting that the evidence for strict cosegregation of the sex factor with the chromosome came from experiments, which, for technical reasons, employed a sex factor whose

replication had been blocked either by a temperature-sensitive mutation or by the dye acridine orange. Thus the findings of strict cosegregation may apply only to a nonreplicating plasmid because it remains bound to the membrane. The minicell data and the data obtained from the conjugal studies described above are in accord with the view that actually F, R-factors, and other plasmids are membrane-bound only temporarily. Obviously the minicell system holds considerable promise in providing a means of purification of the plasmid–membrane attachment site.

The minicell method offers an easy and reasonably efficient way to isolate pure plasmid DNA and permits one to focus on plasmid interaction with the cellular environment without concern for the host chromosome. It is well established that both F-like and I-like R-factors can segregate into minicells. Indeed preliminary evidence suggests that R-factors are segregated more efficiently into the anucleate cells than are F-merogenotes.

Table 6.4. Properties of *E. coli* minicells and a summary of types of plasmids segregated into *E. coli* minicells[a].

Distribution of stable macromolecules and some enzymes in minicells

Component or enzyme	$\frac{\text{Minicells}}{\text{cells}}$[b]	Component or enzyme	$\frac{\text{Minicells}}{\text{cells}}$[b]
RNA	0·9–1·1	DNA-dependent RNA-polymerase	0·012[c]
DNA	<0·01–0·029		
Putrescine	0·84	RNA methylase	0·77
Spermidine	2·8	DNA methylase	<0·04
5′-Nucleotidase	2·3	DNA ligase	0·77
Ribonuclease I	0·7–1·5	DNA polymerase I	0·45
β-Galactosidase	0·7	DNA polymerase II	0·5
Ribonuclease II	1·0	DNAase (ATP-independent)	0·125

Types of plasmids segregated into minicells of E. coli

F	R1 (*fi*$^+$ Cm-Km-Ap-Su-Sm)	Col E1
F′-*lac*	R222 (*fi*$^+$ Tc-Su-Sm-Cm)	Col B
F′14 (F-*ilv, met, arg*)	RTF	Col V
F′-*gal*$^+$	R64-11 (*fi*$^-$ Sm-Tc)	Clo DF13
F′λ*gal*$^+$	R6K (*fi*$^-$ Ap-Sm)	
	N-3 (*fi*$^-$ Su-Sm-Tc)	λ*dv*
	219 (nontransmissible, Tc)	
	Su Sm (nontransmissible)	

[a] Data from Frazer and Curtiss (1974).
[b] The numbers refer to the ratio of the specific activity or quantity found in minicells to the activity for normal cells.
[c] This figure refers to the activity found in minicells not containing plasmid. The presence of this enzyme in plasmid-containing minicells is inferred from their ability to incorporate uracil into RNA, and amino acids into protein.

The significance of this latter finding is not altogether clear, although it is my own opinion that it may be associated with the relative size of F-merogenotes and R-factors. Whereas most R-factors are in the size range $45-70 \times 10^6$ daltons, F-merogenotes are more commonly 100×10^6 daltons or greater. The physical structure of R-factors within 'minicells' has been found to be that of a single molecular species, which does not dissociate into replicon subunits. The absence of the host chromosome therefore does not apparently affect the control mechanisms which hold the transfer and drug-resistance replicons into a single composite structure. Recent experiments by Ron Sheehy, Roy Curtiss III, and their collaborators have focused on the molecular nature of R-factors isolated from *Salm. typhimurium* minicells. One rather significant finding here was that an F-like R-factor dissociated into a transfer replicon and a drug-resistance replicon within the *Salm. typhimurium* minicells (as it does in *Proteus*), although, it is perfectly stable in *E. coli* minicells. Here again one sees the importance of the cellular environment in determining the replicative behaviour of R-factors.

Replication of R-factors within the minicell lines occurs, but thymine-incorporation data indicate clearly that this is less than would be expected if all the DNA were actively replicating. In fact density labelling experiments demonstrate that R-factor DNA is not undergoing more than one full round of replication in two hours. By way of contrast, Col E1 replicates most efficiently within minicells. Since the Col E1 plasmid is of the 'multicopy' type, compared with the composite R-factor type of *E. coli*, there may be some fundamental importance in this. R-factors do transfer from minicells so some replication must be taking place. If radioactively labelled thymine is incorporated into the R^+ culture during the growth cycle, the analysis of DNA subsequently found in minicells shows that it is largely in the form of twisted covalently-closed and 'nicked' circular molecules. An interesting sidelight here is that studies from Cohen's laboratory reveal that about 10% of the total DNA isolated from R^+ minicells is found as dimeric catenated forms. Catenanes, consisting of closed circular molecules which are topologically linked to open circular molecules, have previously been found in mitochondrial DNA from various sources and from intracellular replicative forms of the bacteriophage P-22. Joseph Inselburg has also shown that if purified minicells are incubated with [^{3}H]thymine, the newly labelled DNA comprises the usual R-factor molecule forms as well as catenated dimeric forms. Catenation therefore does not appear to be associated solely with the segregation process but continues even after segregation of the R-factor into the minicell has taken place. Kontomichalou, Mitani and Clowes (1970) demonstrated catenated R-factor DNA in lysates of normal *E. coli* cells harbouring the R-factor R6K. You may recall that this R-factor displays relaxed replication in *E. coli*. Catenanes of R6K likewise

comprised about 10% of the total DNA, and while most were catenated dimers, higher catenates were also found occasionally (for example, a pentamer composed of three 'nicked' circular monomers interlocked with two covalently-closed molecules). Although some workers have considered that catenation had its origin through a recombinative rather than a replicative mechanism, Kontomichalou *et al.* point out that the number of catenated dimers averages about one per chromosome under all phases of growth and may therefore be in some way related to R6K replication. Recent pulse–chase studies by Kupersztoch and Helinski have provided further evidence that the catenated forms of R6K DNA represent a replicative intermediate rather than a replicative end-point.

Perhaps the most exciting ramification of the minicell investigations has been the discovery that the segregated plasmid transforms the inactive anucleated cell into one that can also synthesize RNA and protein. Stuart Levy, who has studied R-factor segregation into minicells extensively, ascribes this synthesis to RNA polymerase carried into the minicell bound to the R-factor DNA. He asserts that the polymerase is of bacterial origin but does not rule out the possibility that a small amount of polymerase (less than 10%) could also be of R-factor origin. Compared with a normal viable cell, there is about 2–5% incorporation of radioactive uracil ('RNA') and labelled amino acid ('protein') per minicell. The RNA transcribed from plasmids is predominantly between 4S and 9S (presumably mRNA). No 16S or 23S ribosomal RNA is found, indicating that the plasmids (F, R, and Col factors) do not code for these components and further demonstrating the degree of freedom from cellular contamination possible with the minicell system. Disc-gel electrophoresis of the soluble proteins isolated from R^+ minicells indicates the presence of at least fifteen different protein bands. Some of these, it is supposed, represent the drug-inactivating enzymes associated with the drug-resistance replicon, which will be considered in a later chapter. More recently, Levy further separated R-specific labelled protein made in minicells into membrane and soluble fractions. Electrophoresis of membrane protein showed that some components were specific for different R-plasmid mating types (that is, fi^+ and fi^-), and one major component was synthesized by all of the R-factors examined. About 2% of the soluble proteins synthesized have some affinity for DNA.

The kind of experimental approach that may be taken to identify clearly the proteins involved in replication and the control of R-factor function can be seen from some preliminary studies reported by Richard Silver and Stanley Cohen. They have isolated R-factor mutants which synthesize a normal repressor of F-pili at 30°C but which do not produce this protein at 42°C. When such R-factors are segregated into 'minicells' at the nonpermissive temperature there is a change in the specific pattern of protein synthesized, as determined by gel electrophoresis. The specific

protein implicated, the putative repressor of F-pili, binds to R-factor DNA specifically and is at present being more thoroughly characterized. Thus one would expect that a series of similar conditional mutants or deletion mutants, each deficient in a well-defined function, could be admirably exploited to identify and/or isolate specific protein products or RNA intermediates. Of course, one cannot expect that all genes may be expressed or are detectable in minicells. The small nontransmissible plasmids such as Col E1, Tc, etc. seem particularly well suited for studies of plasmid-mediated macromolecular biosynthesis, however. One striking example is provided by a study by A. J. Kool and associates on the polypeptides synthesized by minicells carrying the small bacteriocin-producing Clo DF13 plasmid. A total of eight polypeptides, accounting for 85% of the coding capacity of the mRNA synthesized by these minicells, was identified. The potential usefulness of plasmid-containing minicells for the study of plasmid-specific transcription and translation is clear. On a negative note it should be explicitly pointed out that not all plasmids segregate into minicells and those that do, are not always replicated nor conjugally transferred. As we learn more about the 'normal' behaviour of plasmids in minicells, such exceptions will undoubtedly become increasingly important.

It appears that DNA-less cells are not as uncommon as one might have expected. A number of growth phenomena which interfere with normal bacterial growth or macromolecular synthesis lead to bacterial 'snake' formation and ultimately to the budding-off of anucleate cells. A minicell containing a sex factor is a nonviable vector for genetic recombination. Kass and Yarmolinsky point out that although it is fanciful to consider this phenomenon as a primitive analogue of a spermatozooan, nonetheless it does supply an illustration of how mutation and selection could lead to the formation of specialized nonviable units for promoting genetic exchange. Thus the analogy between a fertile minicell and a bacteriophage is somewhat striking and certainly provides an agreeable mental distraction. Intellectually, at least, one can play with the progressive simplification of a minicell into a bacteriophage or, alternatively, with the progressive accretion of genetic material (since minicells mate) to re-form a more complicated structure.

6.6 Some concluding remarks about plasmid replication

In this chapter I have attempted to give a broad overview of structural properties of R-factors and related plasmids and the way(s) they may replicate. The experimental data provide no evidence for choice between the Cairns, rolling circle, or the covalently-closed nick–reseal modes of replication—nor were they designed to examine this question. Of course, the differences between the models are often difficult to study at the experimental level. There are several instances in which the requisite for more than one mode of replication seems to be indicated. The clearest

example of this is seen at conjugation in which the asymmetric transfer of a single strand and the presence of a linear duplex in the recipient cell fits the rolling circle model quite well; yet the subsequent replicative behaviour of the linear duplex in a recipient cell seems more in accord with a symmetrical mode of replication. This points out the clear distinction between transfer replication and the vegetative replication of a transmissible plasmid. They appear to be quite separate processes under the control of separate gene clusters on the plasmid chromosome. At present (and probably for some time) it would seem to be difficult to formulate a single replication model to adequately explain replications so dissimilar as that observed for a single R-factor in *E. coli* as contrasted with *Proteus*. A corollary to this point, of course, is that some R-factors are assemblages of more than one replicon and each may have inherently distinctive modes of replication and replication control.

There is little one can say at present concerning the enzymes which are actually involved in the replication of R-factors. At conjugation one can generalize that a linear duplex molecule is converted into an open circular form in which one strand is first covalently sealed and then, in turn, is completely sealed to form a twisted supercoil. In vegetatively growing cells the only data available indicate that covalently-closed molecules are replicated semiconservatively and appear to be dependent upon RNA synthesis for initiation. The whole mechanism of duplication takes only a few minutes and the isolation of replicative intermediates is difficult. As will be pointed out in the following chapter, R-factors and other plasmids depend upon the cell for most of their replication enzymes, and their own replication genes appear to be concerned with the control of the timing of the initiation process as well as plasmid apportionment at cell division.

A major concern of those who study plasmid replication has been with respect to the mechanisms which ensure plasmid continuity throughout host cell division. Most prophages have solved the problem by evolving specific mechanisms to permit integration into the host chromosome. Most other plasmids lack this ability; yet, despite their nonessentiality, they are quite stably maintained within the host even though they may replicate under stringent control—hence only a few plasmid copies are present per cell. Thus, there has been a consistent experimental theme to provide a structural cell component which functions to coordinate plasmid replication with that of the host chromosome. The association of R-factor DNA with cell membrane has been confirmed and, although it appears that this association is required for replication, the plasmid is not apparently required to maintain this association at all times to ensure its retention. Nevertheless it does seem likely that ultimately it will be found that a mechanism has been evolved such that the replication of the plasmid and apportionment of the replicas is closely attuned to the cell division cycle. Indeed, for the larger R-factors which replicate under stringent

control, the control mechanisms for replication and segregation are like those of the bacterial chromosome itself, so that any inhibition of chromosomal synthesis leads to inhibition of plasmid replication.

The molecular examination of R-factors and other plasmids also provides insight into a possible alternative maintenance mechanism—one that is not necessarily keyed to the host cell division cycle nor necessarily dependent upon a structural component of the cell. This alternative is found in the plasmid class characterized by relatively small size and relaxed replication, as the 56% G + C replicon of *fi*$^+$ R-factors in *Proteus* and the Su–Sm and Ap plasmids observed in *E. coli*. One can visualize here that the apportionment of replicons—like their replication mode—is random in nature and that segregation of these factors at cell division operates automatically because of the sheer weight of numbers. The small multicopy class of plasmids is emerging as possibly the most common plasmid class in enteric bacteria and *other bacterial genera*. One now sees almost monthly reports of plasmids in *Streptococci, Bacillus, Neisseria*, and in other species. A large number of these reports describe the predominant plasmid class as the small multicopy type. (Indeed, in some species, strains without this plasmid class may be exceptional; for example, in my laboratory Leonard Mayer has found that all but one of eleven strains of *Neisseria gonorrhoeae* harbour a $2{\cdot}9 \times 10^6$ daltons multicopy plasmid pool.) The multicopy plasmids are excellent experimental material for the study of replication because of their 'simple' genetic structure, the presence of a large number of copies, and the ability of some to continue their replication in the absence of net host cellular DNA and protein synthesis. The Col El plasmid has been the most widely used example of this class in recent years, but the abundance of analogous plasmids also carrying drug resistance should add new dimensions to the experimental possibilities. At any rate, while the larger plasmids probably provide a good model for chromosome replication, the smaller multicopy type provides a useful, interesting, and rather different way to think about plasmids and their evolution.

One of the difficulties encountered in studying the small multicopy types of plasmid is that they are nontransmissible. This can be overcome, of course, by mobilization with a transfer factor, but in some instances this is not possible or even desirable. For example, the many questions about the 56% G + C replicon observed in *Proteus* can only be answered by getting this plasmid alone in other host bacteria. Stanley Cohen and his associates have largely solved this problem by developing a system whereby calcium chloride-treated *E. coli* cells may be transformed with isolated plasmid DNA. Covalently-closed circular DNA, 'nicked' rings of DNA, and linear DNA are all effective. The general usefulness of this system for the studies of bacterial properties of plasmids is fairly obvious. The method should be particularly useful for the investigation of nontransmissible plasmids and should serve as a marvelous tool for the study of plasmid

structure. For example, the DNA isolated from a 65×10^6 dalton R-factor has been mechanically sheared and the fragments used to transform *E. coli*. A small, 5×10^6 dalton, nontransferring Tc plasmid was isolated which appeared to be similar to the naturally-occurring Tc plasmid described earlier. The development of the transformation method, coupled with advances in the use of restriction enzymes (described in the following chapter) has provided a means of reducing R-factors and other plasmids to their 'simplest' form and has opened the way for the concentrated study of the 'essential' plasmid functions of replication and the maintenance of the autonomous state.

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6.5 The replication of R-factors

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6.5.3 Replication in *E. coli*

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6.6 Some concluding remarks about plasmid replication

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7

The genetics of R-factors: the maintenance of the autonomous state

The replication of any genetic entity has the basic goal of selfperpetuation. Coupled with the actual mechanism of DNA duplication is the elaboration of specific mechanisms which ensure the faithful distribution of daughter replicas. In R-factors the latter problems are presumably more complex because plasmids are ordinarily not essential to cell viability and replicate independently of the host chromosome. As noted in the previous chapter, theoretical considerations and experimental observation both suggest that genetic continuity is achieved by attachment of a replicon (be it a 'big' or 'little' chromosome) to the cell membrane. This 'maintenance site' can be visualized as providing the site of replication as well as being necessary for replica distribution at the time of cell division. The precise nature of specific R-factor functions that control autonomy, replication, and maintenance is almost totally unknown in the biochemical sense. Similarly, except for postulating the actual maintenance 'site', the host's contribution to plasmid replication and stability has, until recently, been more speculative than real. However, one can describe for plasmids and their hosts certain genetic and replicative properties which do provide some insight into the effectiveness and specificity of plasmid autonomy and maintenance. These genetic properties are significant not only in a basic biological context, but also in a very practical way from the standpoint of the clinician.

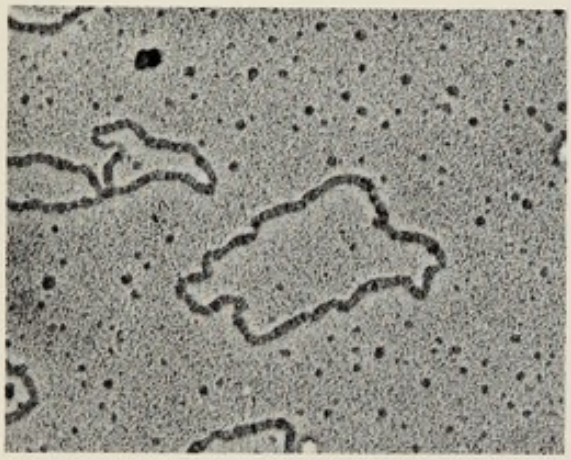
7.1 R-factor segregation

7.1.1 The stability of R-factors *in vitro*

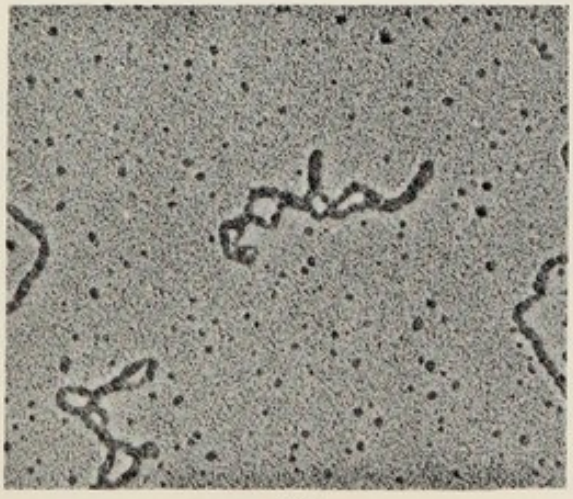
Although R-factors are not essential for the survival of a host in an antibiotic-free environment, they are ordinarily quite stable. In most enteric strains spontaneous loss of the whole R-factor or some or all of its resistance markers occurs at a frequency of 10^{-4} per cell generation or less. This intrinsic stability underlies the concept that special mechanisms are available to ensure the genetic continuity of R-factors.

When R-factor loss does occur, it probably happens at the time of cell division, because of some failure in the mechanism for the faithful distribution of daughter replicas to cell progeny or because of a replication defect. Similarly a mutation in the host or plasmid could affect stability. The spontaneous loss of some or all resistance genes is most likely to occur as a result of deletion (physical loss) of R-factor DNA. Deletion is suspected to be the result of an intramolecular recombinational event that involves the breakage and rejoining of R-factor DNA. One assumes that most deletions remove a single continuous segment of DNA, and that the DNA removed is usually nonviable or, in other words, that it is incapable of autonomous replication unless it has the requisite genes.

(a)

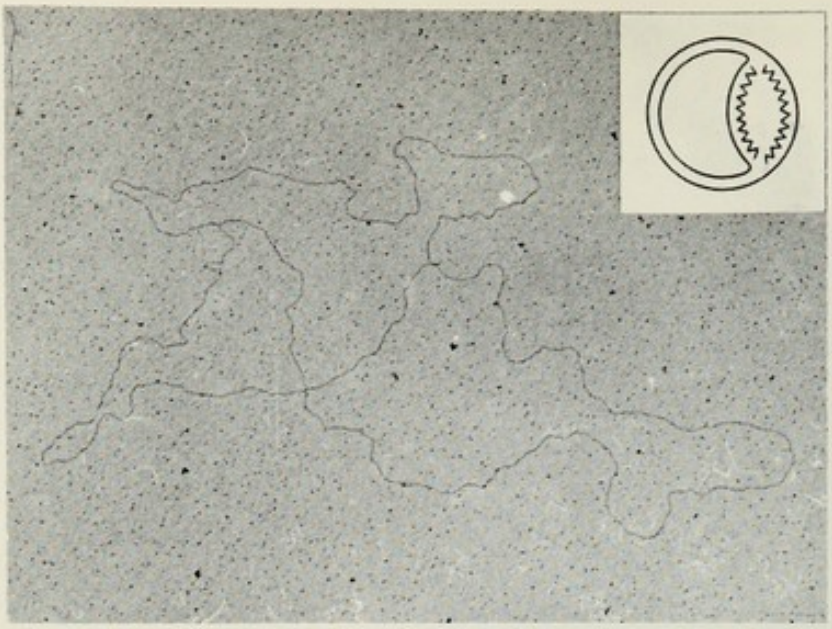


(b)

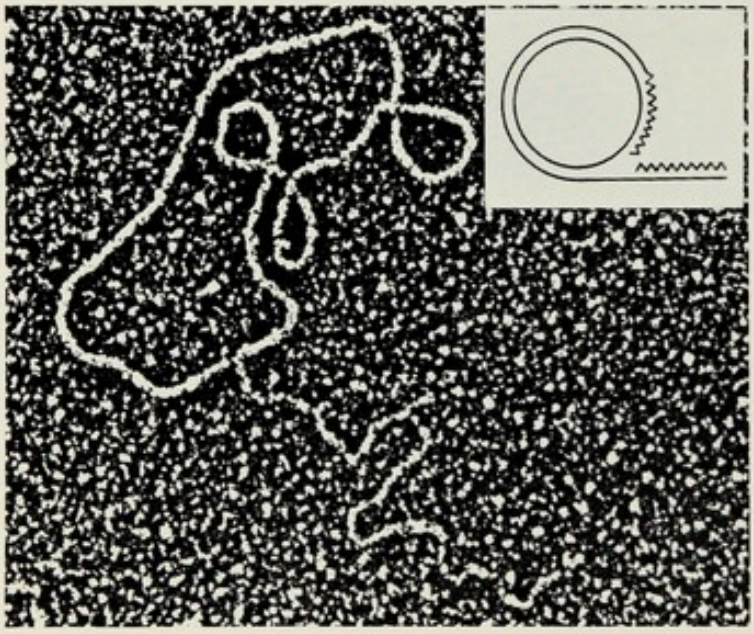


(c)

Plate 1. Electron micrographs of (a) linear, (b) open or 'nicked' ring and (c) twisted covalently-closed ring forms of DNA. Photographs by Claude G. Garon (National Institutes of Health, USA).



(a)



(b)

Plate 2. Two models for DNA replication. (a) A partially replicated λ chromosome illustrates the Cairns configuration for replicating DNA. The accompanying line diagram shows a plausible strand substructure for the molecule. Both strands of the parental chromosome may be circular, although at least a temporary 'nick' must be put into one strand to allow strand separation. Both daughter polynucleotide chains (zig-zag lines) are shorter than unit length. There are two forks in the partially replicated molecule; one or both forks can be growing points when the parental strands are separating, and new DNA is being laid down. (b) The 'rolling circle' configuration for replicating DNA is illustrated by a φX-174 duplex ring, which is generating material for a single-stranded circle. The line diagram shows the strand substructure of this actively replicating DNA molecule. Synthesis occurs by direct elongation of the open + strand, the circular − strand being used as an 'endless' template. In systems where double-stranded DNA is the product of replication, the 'tail' becomes duplex. When the tail becomes longer than unit length, and contains homologous base sequences one genome-length apart, a recombination event presumably detaches a progeny chromosome. (Taken, in part, from a paper by John Wolfson, David Dressler and Marilyn Magazin, *Proc. Nat. Acad. Sci. USA*, **64**, 499, 1972, and reproduced with permission.)

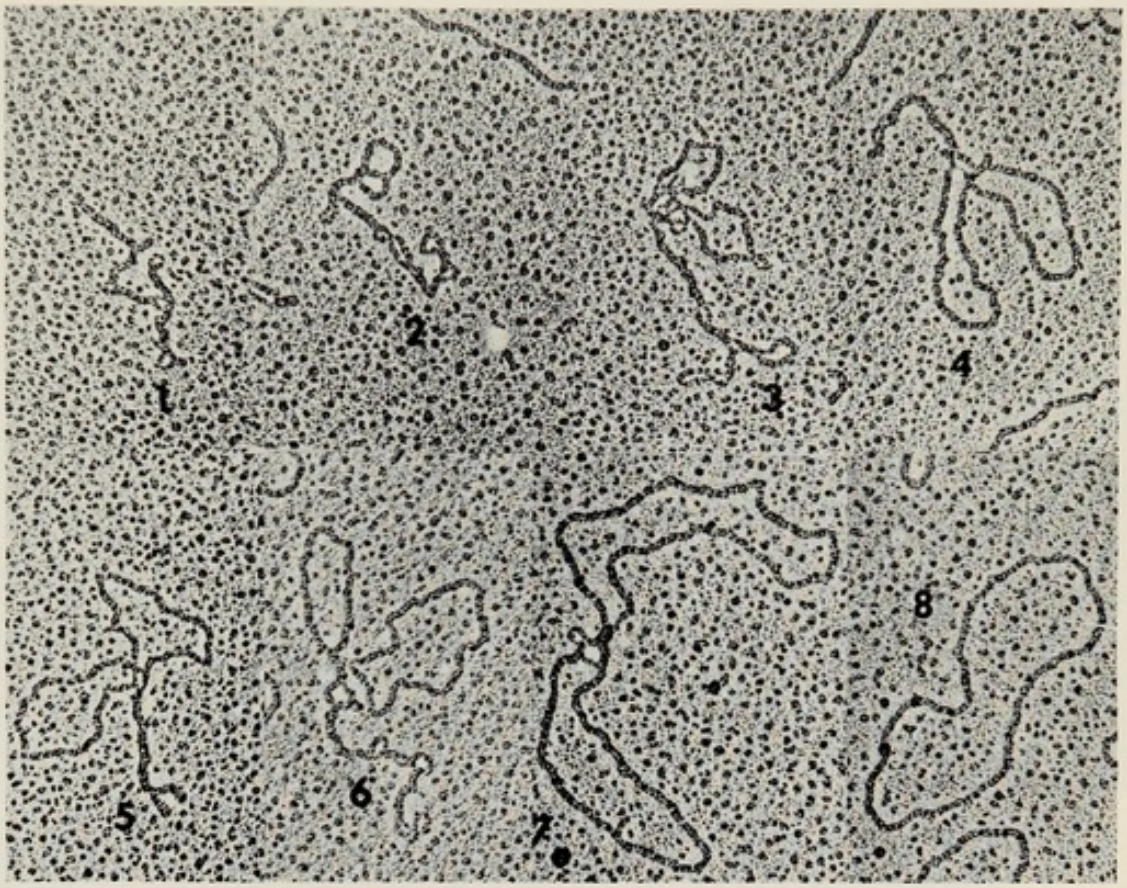


Plate 3. Replicative forms of Col E1 DNA. This figure is a composite of Col E1 DNA molecules in the process of replication in *E. coli* cells, as seen by Leonard Katz, Peter H. Williams, Ronald W. Leavitt and Donald R. Helinski (unpublished observations, 1974). Starting with a covalently-closed twisted molecule (1) it can be seen that replicating molecules (2-6) appear in the electron microscope as partially supercoiled structures containing two open circular branches of equal size. Analysis of similar structures in neutral and alkaline sucrose gradients indicate that at least one strand in the newly-synthesized DNA in the loops is being synthesized in short, discontinuous fragments. The unwinding of the covalently-closed template strands, accompanying replication, is assumed to be a transient nicking-local unwinding-resealing process which reduces the overall superhelical structure of the replicating molecules until (7) two equivalent daughter molecules are produced. An open circular form of a monomeric Col E1 DNA molecule is shown for comparison (8). Photographs generously provided by Leonard Katz, Department of Biology, New York University.

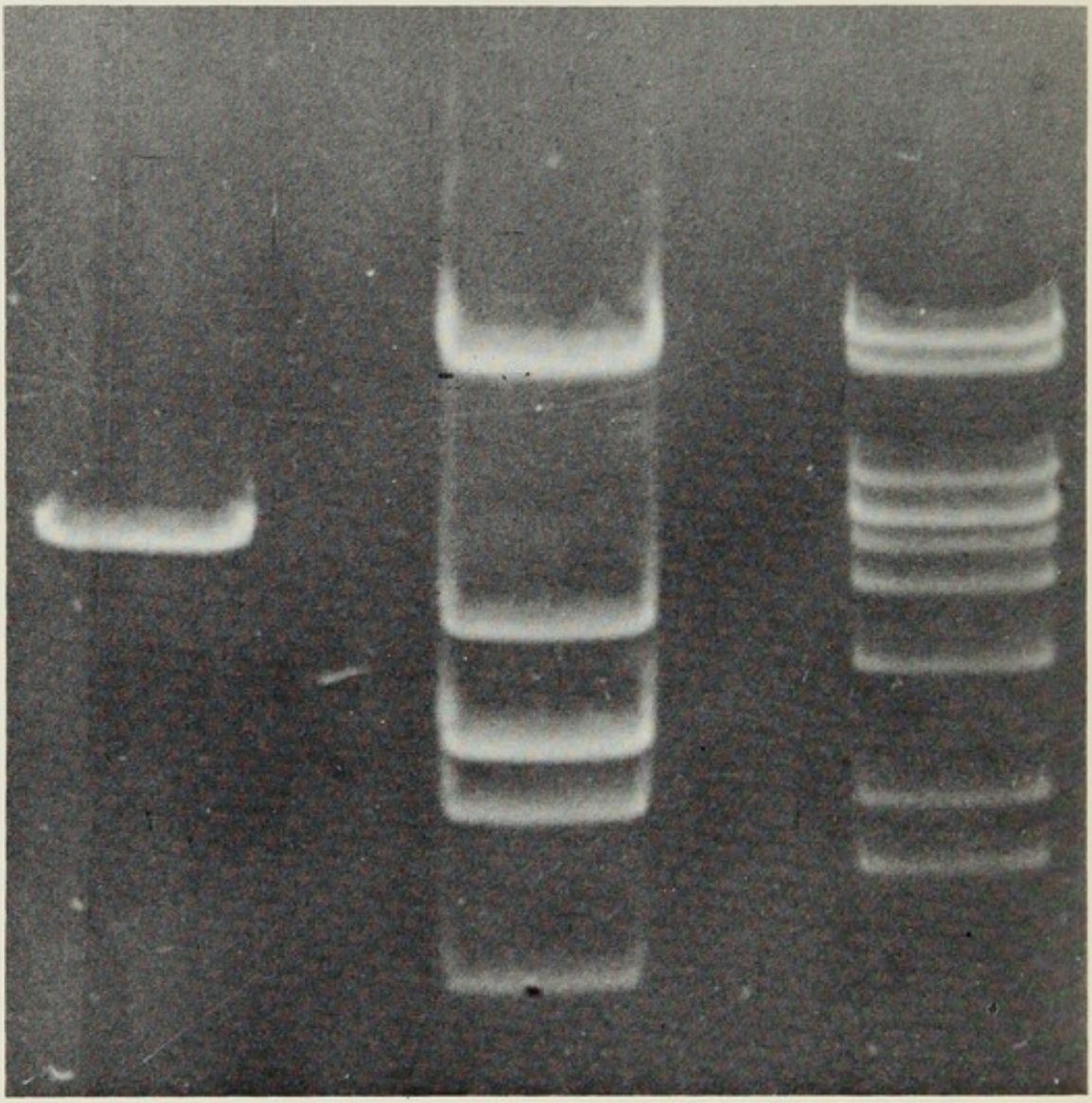


Plate 4. Agarose gel electrophoresis Eco R1 endonuclease-generated fragments of pSC101 plasmid DNA, bacteriophage λ DNA, and the DNA of the transmissible plasmid B41. Electrophoresis was carried out in a 0·7% agarose gel slab and the DNA stained with ethidium bromide and photographed under a long wave UV lamp. It can be seen that the $5{\cdot}8 \times 10^6$ dalton pSC101 plasmid (left) is cleaved to only a single linear fragment. The 30×10^6 dalton phage λ DNA (middle) is cleaved into 6 fragments ranging from $5{\cdot}7 \times 10^6$ to $2{\cdot}09 \times 10^6$ daltons (note two fragments 3·56 and $3{\cdot}7 \times 10^6$ appear as one band here). The 65×10^6 dalton transmissible plasmid B41 is cleaved into at least 11 distinct fragments ranging from 17×10^6 to 1×10^6 daltons (not all fragments are apparent here but can be resolved if electrophoresis is carried out for a longer period).

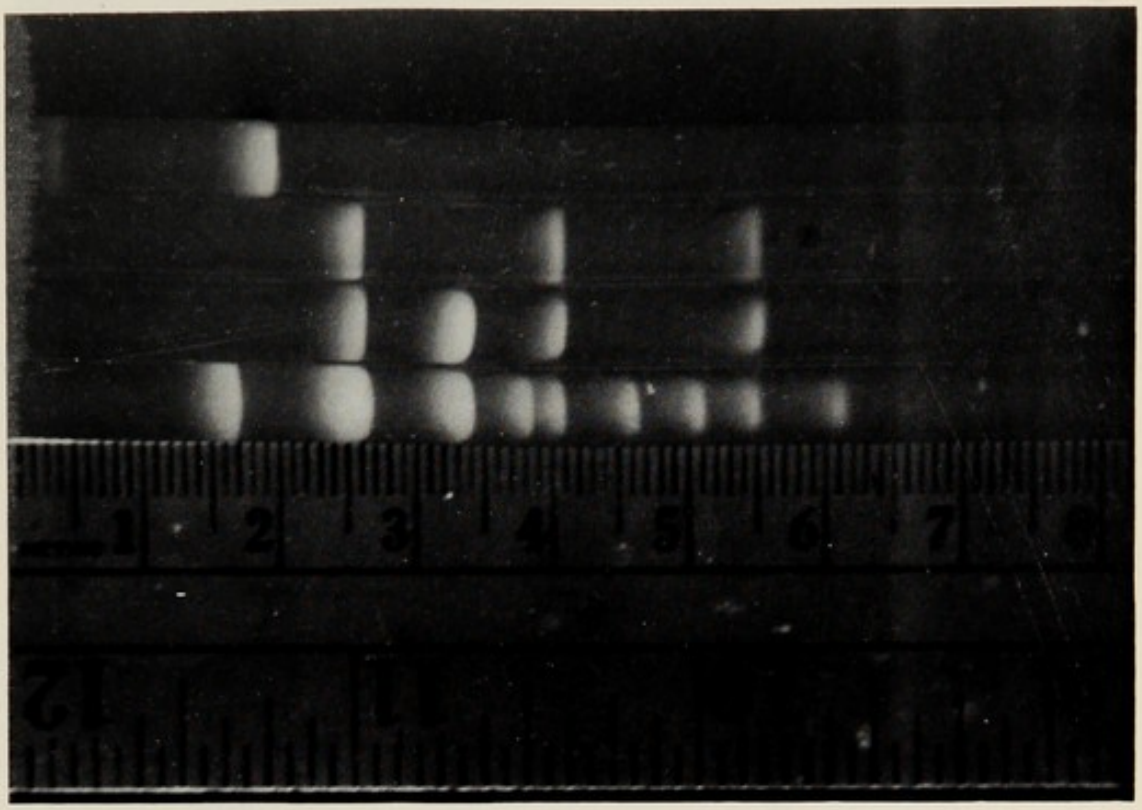


Plate 5. Agarose gel electrophoresis of parental R6-5 DNA and replicons derived from R6-5 DNA by transformation of *E. coli* by Eco R1 endonuclease digests or sheared R6-5 DNA. Electrophoresis runs from left to right. From bottom to top of the figure shows: the Eco R1 endonuclease digests of R6-5 (DNA); a mixture of the DNA derived from the replicons pSC102 (SuKm) and pSC101 (Tc); pSC102 (Su-Km) DNA and untreated pSC102 DNA. The component fragments of pSC102 derived from R6-5 are evident. See text for details. Photograph provided by H. W. Boyer.

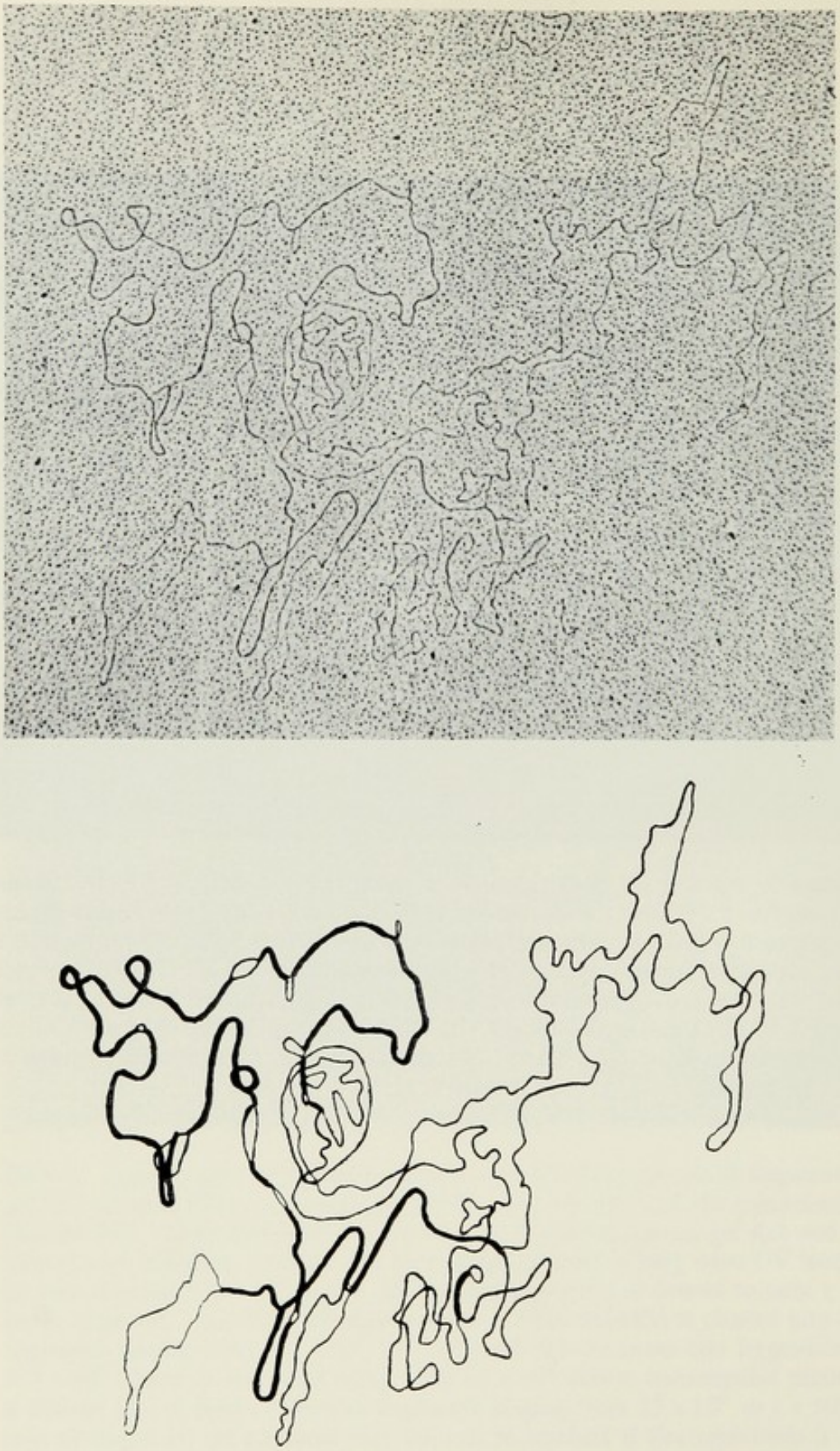


Plate 6. Electron photomicrograph (top) and tracing (bottom) of a hybrid (heteroduplex) DNA molecule formed by an R-factor (R-Su-Cm-Km-Ap) and the classical F-factor. The tracing of the heteroduplex shows the double-stranded region of homology as a heavy line and the nonhomologous single-stranded region of nonhomology as a light line. (From Cohen *et al.*, 1971: reprinted with permission of the authors and The New York Academy of Science.)

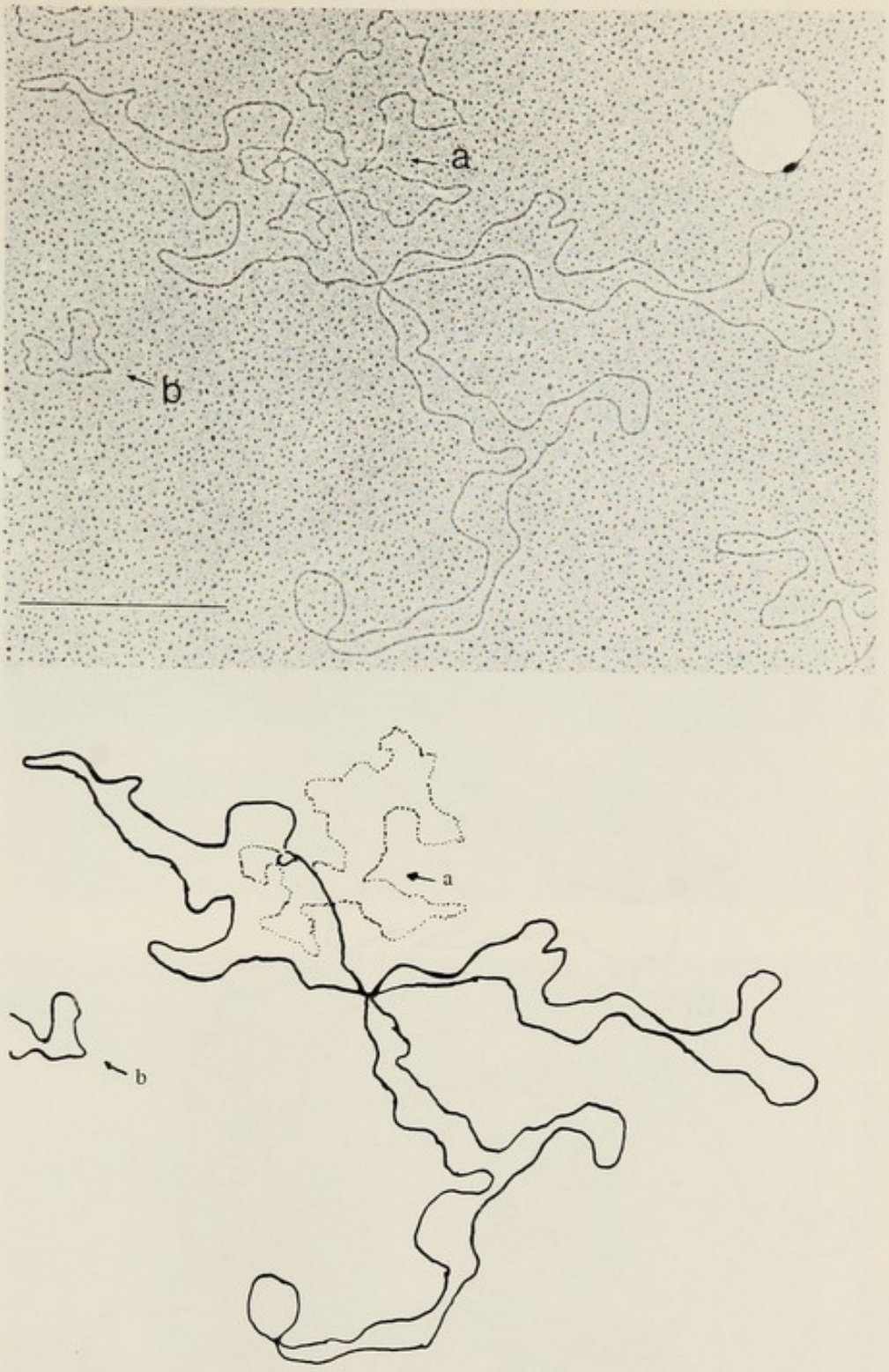
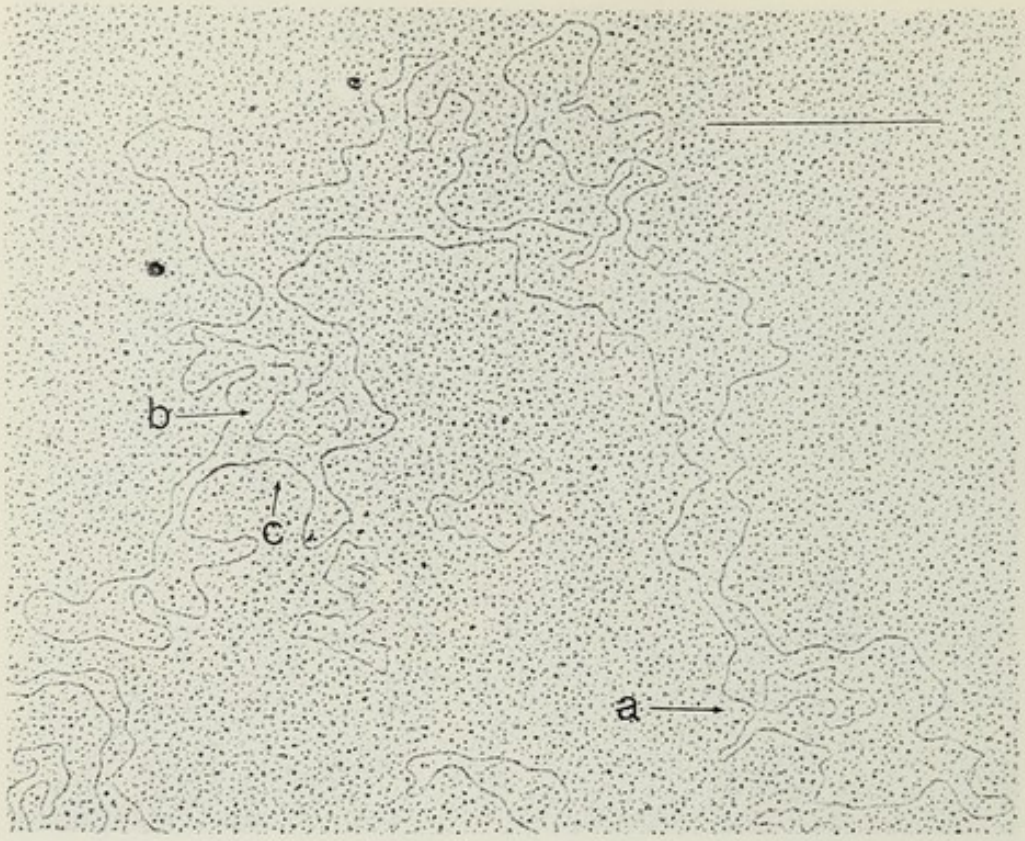


Plate 7. A heteroduplex between the R-factor, R1 (R-Su-Sm-Cm-Km-Ap) and a mutant derivative of R1, RTF1, which has lost all drug-resistance genes. The large deletion-type loop denoted by 'a' (the dotted line in the tracing) corresponds to the drug-resistance genes. The remaining double-stranded part of this heteroduplex (solid line in the tracing) corresponds to the transfer segment of the R-factor and contains all the DNA sequences in R1 homologous to F. The small circular structures, 'b', in the field are single-stranded monomers of phage ϕX added as an integral control for measurement. (Photograph provided through the courtesy of Philip A. Sharp; see Sharp *et al.*, 1973, for details.)



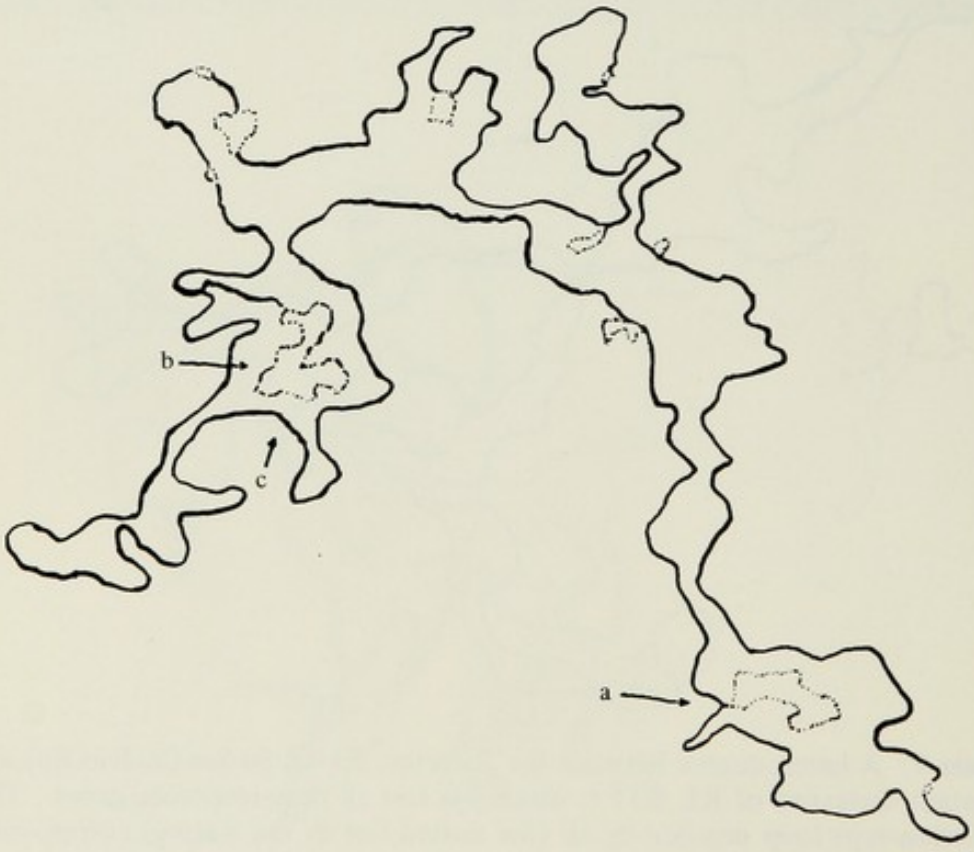


Plate 8. A heteroduplex between R1 (R-Su-Sm-Cm-Km-Ap) and R-100-1 (R-Su-Sm-Tc-Cm). R1 was isolated in England and R-100-1 was found in Japan. Both are *fi*$^+$, F-like R-factors and it can be seen that homology (the solid line in the tracing) extends over the entire length of the R-factors. The loop denoted by 'a' probably contains the Ap, Km drug markers of R1. The large loop denoted by 'b' contains the tetracycline marker of R-100-1. The double-stranded DNA between 'a' and 'c' contains the Cm, Su and Sm markers of R1. This DNA segment was absent in RTF1 (see plate 4). The bar represents 1 μm. See the text for further discussion. (Photograph provided through the courtesy of Philip A. Sharp; see Sharp *et al.*, 1973, for details.)

Plate 9. The effect of *E. coli* stable enterotoxin on the bowel of suckling mice. Two day-old suckling mice were injected intragastrically with 75 μl of sterile supernatant fluid from a 24 hour culture of a nontoxigenic (left) or toxigenic (right) *E. coli*. After four hours the mice were sacrificed and the intestinal tract exposed. The accumulation of fluid within the bowel of the mouse injected with the culture supernatant from the toxigenic strain is apparent.

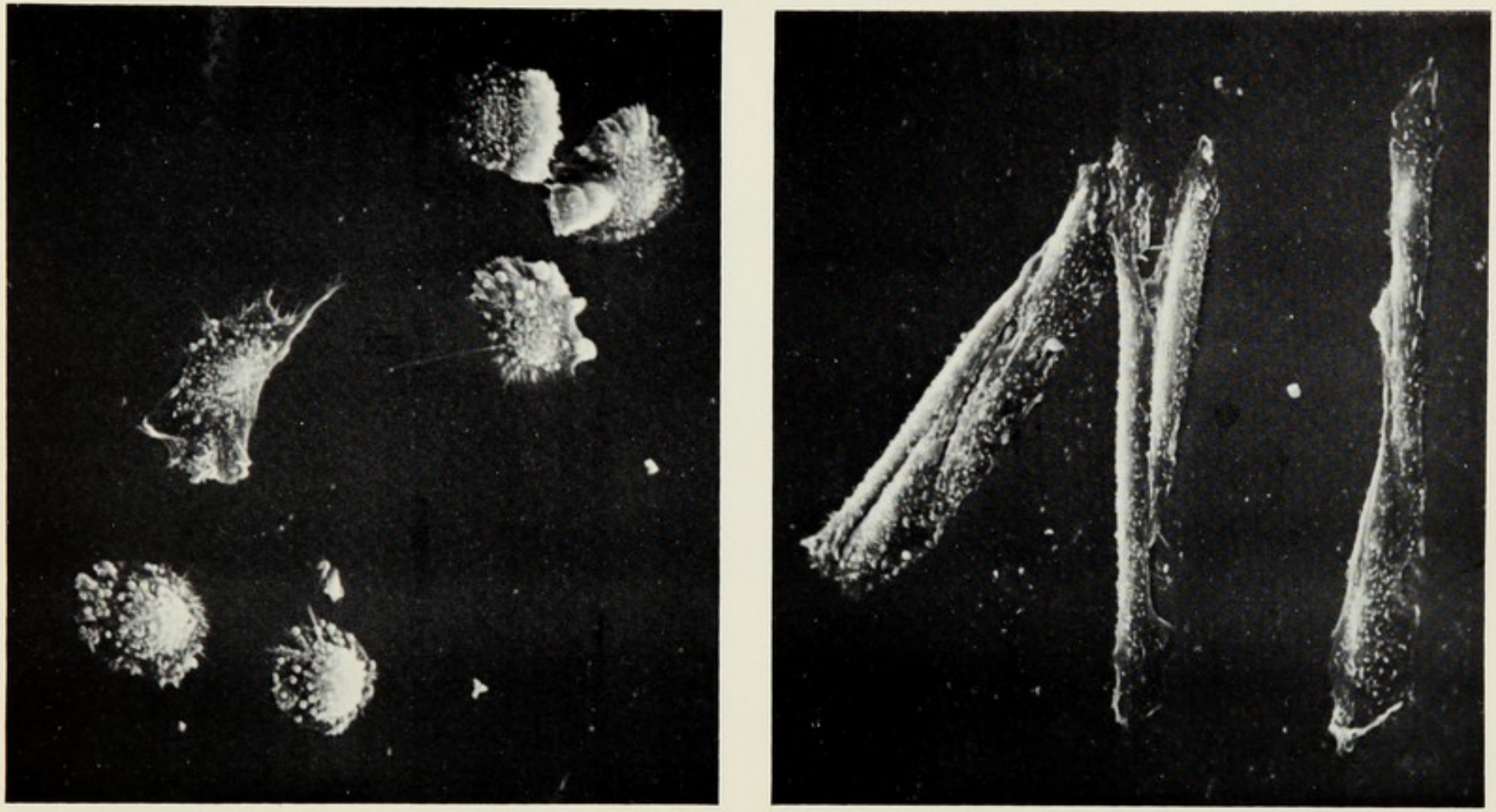


Plate 10. Scanning electromicrographs of Chinese Hamster ovary cells incubated with 20 μl/ml of sterile supernatant fluid from a 24 hour culture of a nontoxigenic (left) and toxigenic (LT) *E. coli* (right). The cells incubated with the supernatant fluid from the nontoxigenic culture maintain their normal morphology, whereas roughly 80% of the cells incubated in the fluid from the toxigenic culture undergo a striking elongation. This morphological transition is presumed to reflect the stimulation of the adenyl-cyclase-cyclic-AMP system of the cells by the labile toxin.

Neither deletion of R-factor components nor spontaneous loss of the whole R-factor occurs at an increased rate in bacterial hosts that have lost, via mutation, a functional host recombinational system. The stability of R-factors in such recombinationless (*rec*$^-$) hosts shows that their survival is independent of the host recombination system and, furthermore, stresses the fact that they are autonomously functioning entities. Of course, a cell which spontaneously loses an R-factor may reacquire it once more by conjugation. Since the transfer frequency in most R$^+$ cultures is relatively low (repressed), reinfection probably does not occur too commonly. The major point, however, is that in most hosts R-factor loss occurs in less than 0·1% of the population after overnight growth (about twenty generations). Thus it can be seen that the sum of all possible segregation mechanisms is relatively rare under ordinary laboratory circumstances.

R-factors are not equally stable in all bacterial hosts. Whereas *fi*$^+$ and *fi*$^-$ R-factors are ordinarily stable in *E. coli, Shigella,* and *Klebsiella,* individual R-factors of both classes are often lost at high frequency (>1% per generation) from *Serratia, Proteus,* and *Vibrio.* The most striking effect of the host on R-factor stability is the observation that many *fi*$^+$ R-factors, in the absence of strict antibiotic selection, are extraordinarily unstable in *Salmonella typhimurium* and other *Salmonella* sp. In many instances it has been reported that the entire R-factor is eliminated from a population of *Salm. typhimurium* after four to ten subcultures in drug-free media. The pattern of segregation in *Salmonella* is not as simple as might be thought at first view. The Tc marker, when present, is reasonably stable and transmissible, whereas a very high frequency of segregation is observed for the R-factor determinants Su, Sm, Cm, and Km. In a sense the segregation of this block of resistance determinants from Tc and transfer function is most reminiscent of the pattern of segregation achieved by phage P-22 transduction described earlier (chapter 5). The implication seems to be that the transfer component of the R-factor is stable but that the resistance determinants (resistance replicon?) are not.

The mechanism of the rapid loss of the Su, Sm, Cm, Km genes in *Salm. typhimurium* is not presently known. Watanabe and Ogata considered the possibility that host recombination enzymes might play some role in the instability of *fi*$^+$ factors in *Salmonella.* Accordingly *rec*$^-$ mutants of *E. coli* and *Salm. typhimurium* were employed as hosts for an *fi*$^+$ R-factor (Su-Sm-Tc-Cm). The R-factor was found to be stable in both *rec*$^-$ hosts. So, whereas this R-factor is markedly unstable in *rec*$^+$ *Salm. typhimurium*, it is stable in the *rec*$^-$ derivative. The authors suggest that the loss of drug markers in the *rec*$^+$ host might be due to recombination of the R-factor with the *Salmonella* chromosome, and that its stability in a *rec*$^-$ host supports this assumption. This explanation seems unlikely to me, since one should not expect drug resistance simply

to disappear after recombination with the host chromosome, unless some very different control mechanisms were operative after integration. Rather the phenomenon may be related to the finding that many R-factors are recombinational assemblages, as discussed in the previous chapter. In the absence of host recombination enzymes the R-factor is stable; in the presence of host enzymes some additional property of the *Salmonella* host might encourage an intramolecular recombinational event leading to the physical loss of the block of drug genes. The 'additional' property may be related to a recent finding that the *Salm. typhimurium* strain LT-2, employed in this and most other genetic investigations, harbours a cryptic nontransmissible plasmid. In a later section of this chapter it will be shown that resident plasmids may indeed have a profound effect on another recently introduced factor. Interestingly enough, I-like and other *fi*$^-$ R-factors carrying genes which specify resistance to the same antibiotics as *fi*$^+$ R-factors are quite stable in the *Salmonella* strain LT-2.

7.1.2 The stability of R-factors *in vivo*

The host-associated stability or instability of R-factors *in vitro* may give some insight into differences in the relative incidence of *fi*$^+$ and *fi*$^-$ R-factors in naturally occurring strains of *E. coli* and *Salmonella*. Table 7.1 shows that *fi*$^+$ R-factors are more common in *E. coli*, whereas *fi*$^-$ R-factors clearly predominate in *Salmonella*. The incidence of *fi*$^-$ factors in *E. coli* and *Salmonella* may actually be higher than appears in the table since a strain carrying an *fi*$^+$ plasmid could harbour a covert *fi*$^-$ plasmid, which would not have been detected at the time of this survey. The isolation of donor-specific phages now make this detection fairly easy and in fact Romero and Meynell found that 16% of *E. coli* and 41% of *Salm. typhimurium* initially thought to harbour only F-like factors have, in addition, an I-like or other *fi*$^-$ factor. For comparison table 7.1 also shows that twelve R-factors isolated from *Proteus rettgeri* were all *fi*$^-$ and none were of the 'classical' I-like group. This point, which will be discussed more fully later on in this chapter, simply shows that certain host–plasmid combinations are more advantageous than others and that

Table 7.1. Relative frequency of *fi*$^+$ and *fi*$^-$ R-factors in naturally occurring *Salmonella, E. coli,* and *P. rettgeri.*

Organism	% R-factors which are:	
	fi$^+$	*fi*$^-$
E. coli[a]	77	23
Salmonella[a]	32	68
P. rettgeri[b]	0	12

[a] Meynell *et al.* (1968).
[b] Coetzee *et al.* (1972).

this is best reflected in the incidence with which certain classes of R-factors are isolated from naturally occurring strains. Of course it is also necessary to take local antibiotic selective conditions into account before generalizing about stability *in vivo*. An R-plasmid which might be lost from an enteric species rather quickly in an antibiotic-free environment (*in vivo* or *in vitro*) may nonetheless be maintained so long as the proper selection is maintained.

Actually it is difficult to assess the stability of R-factors *in vivo*. This requires long-term epidemiological studies, and few of these are available even for drug-sensitive strains. Anderson cites two examples which indicate that R-factors are indeed quite stable in Nature, in the absence of drug selection. One example is an R-Tc factor, which was still prevalent among animals in a herd eighteen months after the use of tetracyclines as a feed supplement had been abandoned. The other example is the phage type 29 of *Salm. typhimurium* (Ap; Su-Sm; Tc) strain, which is still prevalent in Great Britain even though the use of antibiotics to eliminate this organism has become less common. In Washington we have seen patients with recurrent urinary-tract infections who have retained the same R-factor and serological *E. coli* type over four years without any intervening chemotherapy. A recent study followed infants whose intestinal tracts were colonized during their stay in an intensive-care nursery by enteric organisms which carried R-Km factors. Forty-six percent of the infants remained carriers of these R-Km strains twelve months or longer after discharge. In the main, then, it seems fair to conclude that many R-factors, once acquired, become a reasonably stable part of the host's genetic complement. It may be emphasized, however, that in the absence of continued antibiotic selection, the incidence of R-factor-containing bacteria usually diminishes noticeably. The stability of R-factors *in vivo* will be considered once more in chapter 10.

7.1.3 Plasmid 'curing'

Although cells carrying R-factors can spontaneously throw off drug-sensitive (plasmid-negative) variants as a result of segregation or replication errors, the rate of R-factor loss can be greatly enhanced by exposure to a growing list of chemical or physical agents.

The aminoacridine dyes, acriflavine, and acridine orange, in relatively low concentrations eliminate quite readily the classical sex factor, F, from *E. coli*. In addition, other flat *N*-heterocyclic substances such as chloroquine, miracil D, quinine, and berberine have been employed with varying degrees of success to eliminate plasmids from plasmid-bearing strains. The experimental procedure ordinarily employed is to inoculate about 1000 host cells into broth containing varying amounts of the compound under study. If the substance is effective one finds, after overnight incubation, that anywhere from 1% to 100% of the cells have been 'cured' of their plasmid. The assessment of curing is generally based

(sometimes erroneously) upon the loss of some phenotypic property. For example, if one is examining the effect of a compound on the elimination of F-*lac*$^+$ or an R-factor, the disappearance of the *lac*$^+$ property or of antibiotic resistance is assumed to signal that the entire plasmid has been eliminated. Curing must, of course, be distinguished from other genetic phenomena such as mutation. An important point to consider is that a mutation is often reversible whereas plasmid loss is not. Partial deletion of genetic material would be more difficult to distinguish because it is also nonreversible. However, the rate of loss of a genetic character comprising more than several per cent of the population makes mutational considerations essentially irrelevant.

The aminoacridines are quite efficient (up to 100%) in removing the classical F-factor (table 7.2) but, under similar conditions, only about 4% of *fi*$^+$ R-factors are eliminated, although these low frequencies can be considerably increased by exposure to small doses of ultraviolet light. Several investigators have made the observation that only *fi*$^+$ R-factors, but not *fi*$^-$ R-factors, are susceptible to aminoacridine curing. This seems interesting since Col I also is not effectively eliminated by either acridine orange nor acriflavine but can be efficiently removed by thymine starvation. Very preliminary studies from the author's laboratory suggest that thymine starvation is effective in curing both *fi*$^+$ and *fi*$^-$ R-factors from host cells. Moreover representatives of both classes of R-factors can often be eliminated after treatment with acridine-nitrogen-half-mustards and the phenanthridium derivative, ethidium bromide (table 7.3). At best, the elimination of R-factors *in vitro* is a 'may be' event. In instances where an R-factor is spontaneously unstable (>1% loss in an overnight culture), curing is generally easily accomplished. More often (at least in my experience) curing effects are marginal, and in many instances it requires a concerted effort to find even a single R-negative variant, be it spontaneous or chemically eliminated. Ethidium bromide, in general, has the greatest likelihood of curing R-factors. The situation is still quite complex. In some cases the original clinical isolate is refractory to all

Table 7.2. The elimination of F-*lac* from *E. coli* K-12. *E. coli* K-12 containing the F-*lac*$^+$ plasmid was cultured in the presence of graded subinhibitory doses of the indicated compound and plated after overnight growth on an indicator medium for *lac* fermentation. The loss of *lac*$^+$ was equated to plasmid loss. The concentrations are presented as ED_{50+}, the concentration for 50% effective elimination. (Adapted from Hahn and Ciak, 1972.)

Compound	ED_{50^+} (M)
Acridine orange	$8{\cdot}2 \times 10^{-5}$
Ethidium bromide	$7{\cdot}5 \times 10^{-5}$
Chloroquine	$9{\cdot}0 \times 10^{-4}$

curing agents and is rarely lost spontaneously. When the same R-factor is transferred to a new host, the stability and the sensitivity to curing agents appears changed.

The mechanism of the 'curing' effect remains largely unknown. Practically all curing agents are mutagens and, in addition, are bactericidal, so that plasmid loss may involve a different underlying mechanism for different agents. In some cases it seems likely that curing may actually be due to selective sensitivity of R^+ cells to the agent. For example, in some cases the curing of R-factors by aminoacridine dyes at very low concentrations appears to be associated with a plasmid-linked gene which confers dye sensitivity upon the host cell. Similarly sodium dodecyl sulphate, urea, and some atabrine–chloroquine derivatives can 'cure' R-factors, but the curing effect appears to be due largely to the fact that R^+ cells are, on the whole, more sensitive to inhibition by these agents than pre-existing plasmid-negative segregants. In each case it is the R-factor itself that appears to be responsible for this sensitivity. In many instances the sensitivity appears to be correlated with the presence of pili. That is cultures, carrying derepressed plasmids, in which most of the cells are piliated are markedly more sensitive to curing with agents such as urea and sodium dodecyl sulphate than are cells carrying the same repressed R-factor. In specific instances therefore the epithet 'resistance factor' may be a misnomer.

Selective sensitivity is not the only mechanism of curing. For example, when many R^+ bacterial strains are grown for 30–40 minutes in ethidium bromide (approximately 10^{-4} M), the percentage of drug-sensitive cells increases from practically nil to 10% (or more) while viability remains unchanged. It is unlikely that selection is operative here and it can be assumed that there is some specific effect of the agent on plasmid replication or distribution. Since curing agents such as acridine orange

Table 7.3. Elimination of R-factors by ethidium bromide. (Adapted from Bouanchaud *et al.*, 1969.)

Strain	R-factor	Type	R-factor loss[a] (%)		
			−ETBr	+ETBr	Concentration 10^{-6} M[b]
E. coli K-12	R-Sm, Cm, Tc, Su, Ap	*fi*$^+$	0·7	32	1300
Shig. flexneri	R-Sm, Cm, Tc, Su, Ap	*fi*$^+$	2·9	79	60
Salm. panama	R-Km	*fi*$^-$	7·9	15[c]	625
Salm. panama	R-Tc	*fi*$^-$	0·9	1–2	625

[a] A small inocolum of bacteria (10^4 bacteria/ml) was grown overnight in nutrient broth containing the indicated amount of ethidium bromide (ETBr). The culture was plated on agar and isolated colonies were tested for antibiotic resistance.
[b] Subinhibitory concentration giving maximum curing effect.
[c] Difference from control is significant, $P = 0{\cdot}01$.

and ethidium bromide show strong binding to double-helical DNA, it is generally held that the elimination occurs through this direct molecular action. Lerman presented evidence that acridine and other flat *N*-heterocyclic substances become intercalated (inserted) between the levels of base pairs into the double helix. The spaces for these insertions were proposed to occur through a local untwisting of the DNA, which caused a distinct separation between normally adjacent base pairs, although the normal pattern of hydrogen bonding between the paired bases remained unchanged. Intercalation into DNA produces, as noted in chapter 6, characteristic topological changes. Fred Hahn and Jennie Ciak proposed that intercalation eliminates closed circular plasmid DNA because of the progressive unwinding and decrease in the number of (normal) 'right-handed' superhelical twists, and the production of 'left-handed' unnatural supercoiled molecules which are unable to replicate. Although intercalative agents do cause such an effect on isolated DNA, it is still not certain that the same mechanism, no matter how attractive, occurs within the bacterial cell. Curing need not be directly aimed at the DNA of the plasmid. Studies by Riva and collaborators have shown that subinhibitory concentrations of rifampicin, which specifically interacts with RNA polymerase, can eliminate F from 10% of *E. coli* cells after one or two duplications. Because mutants of *E. coli* which are rifampicin-resistant are not cured, it appears that curing is due to an inhibition of transcription. Presumably there is either a preferential inhibition of the transcription of F DNA as compared with the host, or a certain RNA species required for F replication is particularly sensitive to rifampicin.

In most studies of curing, investigators have not performed kinetic studies on the rate of plasmid loss, so that a conclusion regarding selective effects, mutagenic effects, or replication effects remains open. Even though precise mechanisms are unknown, it is important to see that, although all plasmids cannot be cured, demonstrable curing is good presumptive evidence for a nonchromosomal element.

A natural question to ask is whether curing could be exploited in an animal infected by an R^+ bacterium? In 1971, D. H. Bouanchaud and Y. A. Chabbert examined this possibility in experimental infections of mice. In fact they could show rather good curing effects in the intestinal tract of mice infected with *E. coli* and *Salmonella* followed, after three days, by 2 mg of ethidium bromide or acriflavine given *per os* per day. Despite this success it is unlikely that any of the presently known curing agents will be practically effective. Even where the curing agent was not toxic for the mammalian host, a significant proportion of resistant bacteria still remained and would still be selected in patients receiving antibiotics. The fact that curing was seen *in vivo* was somewhat surprising. Curing *in vitro* seems very dependent on drug concentration, pH, inoculum size, and so on. Nonetheless the percentage of cured bacteria in some animals was

very high indeed. Again one does not know whether curing *in vivo* reflects selection, replication inhibition, or a combination of these and other mechanisms. However, it may be assumed that the mechanisms involved are similar *in vivo* and *in vitro*.

7.2 Specificity of plasmid duplication and maintenance

7.2.1 The host

Although the high degree of stability usually exhibited by R-factors *in vivo and in vitro* points out their inherent control of replicative autonomy, it must not be simply assumed that the host cell merely provides a 'warm body'. In this vein David T. Kingsbury and Donald R. Helinski have isolated mutants, both chromosomal and plasmid, that are temperature-sensitive (*Ts*) for biochemical steps which affect plasmid duplication and maintenance. Such mutants show normal duplication and stability at a permissive temperature (30°C), whereas at a restrictive temperature (43°C) the plasmid is lost at high frequency.

The chromosomal genes affecting plasmid maintenance could be functionally divided into three groups with respect to their plasmid specificity. Mutants of group I could not replicate or maintain any plasmid tested at the restrictive temperature. These included the temperate phages λ and P1, as well as Col E1, F-*lac*$^+$, Col V, Col Ib, and representative F-like and I-like R-factors. Group II mutations specifically affected the maintenance of the Col E1 and F-like plasmids (F, Col V, and the *fi*$^+$ R-factors), but not the I-type plasmids (Col Ib and the I-like R-factors). Group III mutations were essentially specific for the replication and maintenance of the Col E1 plasmid; all other plasmids replicated normally in this mutant class at the nonpermissive temperature. These chromosomal mutant classes were mapped at five genetically distinct loci on the *E. coli* chromosome; but the gene products, except in one case, could not be readily identified. Certain mutants in each of the three groups exhibited a temperature sensitivity for cellular growth in the presence of deoxycholate, suggesting a thermosensitive membrane defect. Several of the group III mutants were found to possess a temperature-sensitive DNA polymerase I (the well known Kornberg enzyme), which was in agreement with earlier experiments which showed that the Col E1 plasmid could not be stably maintained in *pol* A 1 mutants of *E. coli*, which are deficient in this enzyme. It is well established that DNA polymerase I alone is not ordinarily required for cell growth or division, and certainly most bacterial plasmids other than Col E1 replicate normally in its absence. Yet these studies establish a role for the DNA polymerase I enzyme in the replication of the Col E1 plasmid. Recently we have also observed that a nontransmissible Ap plasmid (RSF1030; F. Heffron, J. H. Crosa and S. Falkow, unpublished observations) also has an absolute requirement for DNA polymerase I for its maintenance and replication.

The mutants isolated in this study, and others like them isolated in other laboratories, promise to give a new perspective to the investigation of the control as well as to the mechanism of plasmid duplication and maintenance. In a slightly different vein numerous workers have isolated and characterized temperature-sensitive mutants of *E. coli*, whose primary defect is in DNA synthesis. Although the isolation of these *dna* mutations is with respect to chromosomal synthesis, investigators have been naturally curious to see how these defects in host chromosomal DNA synthesis at a nonpermissive temperature affected a carried plasmid. The *dna* mutants have been classified into those specifically deficient in chain elongation, initiation of replication, or blocked in precursor synthesis or in replication *per se*. The usefulness of these *dna* mutations cannot be overstated. Their analyses have directly or indirectly led to the discovery of two unique DNA polymerases, DNA polymerase II and DNA polymerase III, in addition to the classical Kornberg DNA polymerase (polymerase I). As noted above, DNA polymerase I is not essential for bacterial replication, although it seems essential for replication of Col E1 and the Ap plasmid, RSF1030. DNA polymerase II, has been shown by Hirota, Gefter and Mindich not to be essential for the maintenance or replication of a number of R-factors, F, or temperate phages. On the other hand, DNA polymerase III (*dna* E mutants) appears to be *the* polymerase which is indispensable for DNA replication in *E. coli* and for temperate phages. Goebel has shown that Col E1 does replicate in *dna* E mutants, but that an Hly and F-like R-factor did not. In my laboratory, Frederick Heffron, Miguel Ortiz and Jorge Crosa (unpublished observations) have shown that most R-plasmids do not replicate to any significant degree in *dna* E mutants, except for the small Ap plasmid, RSF1030 and the nontransmissible $5{\cdot}0 \times 10^6$ daltons Su–Sm plasmid. Unlike RSF1030 the Su–Sm plasmid did not appear to require DNA polymerase I for replication. We have subsequently found that the Su–Sm plasmid is lost from *E. coli* mutant sublines which are deficient in the Kornberg enzyme. It appears, therefore, that this plasmid has a partial dependency upon DNA polymerase I for maintenance, and that it can employ it for replication in the absence of DNA polymerase III. Thermosensitive mutants of *E. coli* defective in DNA initiation (*dna* A) have also been examined in terms of the replicative behaviour of harboured plasmids at the nonpermissive temperature. When shifted to a nonpermissive temperature *dna* A mutants do not stop DNA synthesis immediately, but rather continue to complete rounds of replication already in progress. Once completed, however, new rounds of replication are not initiated. During the initial period of residual DNA synthesis all plasmids continue to replicate normally. Upon cessation of further chromosomal synthesis replication, typical F-like and I-like plasmids simultaneously cease replication (Miguel Ortiz, Maria Luz Ortiz and Stanley Falkow, unpublished observations). Presumably in this case plasmid replication is 'tied' to

continued replication of the host genome. However, some R plasmids, both transmissible *fi*$^-$ R-factors and nontransmissible plasmids (such as Su–Sm) continue their replication for up to five hours or longer (M. Ortiz, M. L. Ortiz and S. Falkow, unpublished observations). In these cases either the host *dna* A initiation product is not required because it is replaced by a plasmid-specific initiator, or some other host product (perhaps the *dna* C initiator product) can suffice. The majority of temperature-sensitive *dna* mutations in *E. coli* have been mapped at a locus called *dna* B. These mutants exhibit an immediate cessation of growth when shifted to the nonpermissive temperature. James Wechsler has shown that *dna* B is a single gene. The precise nature of the *dna* B product is not known, although there is some evidence that it is complexed or must interact with one or more products to be active. Phage λ cannot replicate in *some dna* B mutants, and the single-stranded DNA phages φX174 and M13 have been unable to multiply in *any dna* B mutants tested thus far. On the other hand, the T even phages and phage P1 replicate in *dna* B. Indeed some mutants of P1 suppress all *dna* B mutations. Hence the alteration of a phage protein can moderate the phenotypic consequences of a bacterial mutation, and conversely, in the case of phage λ, an altered *dna* B product can affect the action of a phage protein (that is, the P gene product of phage λ required for DNA replication). Insofar as I am aware, the only plasmid which can undergo vegetative replication in a *dna* B mutant at the nonpermissive temperature is Col E1 (the nontransmissible R-plasmids have not yet been tested to my knowledge). Interestingly enough, F′- and R-factor *transfer* synthesis occurs normally in *dna* B mutants at the nonpermissive temperature, indicating either that replication is not required for transfer or that the mode of replication that occurs at transfer does not require the missing *dna* B function. Obviously the results obtained with the various *dna* mutants will be as useful in dissecting the replication requirements of plasmids as they have been for the host chromosome. One thing is already quite evident from this work, the control of plasmid duplication is a specific process which requires interactions between chromosomal and plasmid products.

7.2.2 The plasmid

If every R-factor or other plasmid must have a corresponding specific 'maintenance site' within the cell to ensure stability, what is the effect when a cell already carrying a plasmid mates and receives another by conjugation or transduction? One obvious outcome would be that nothing happens; the incoming plasmid is simply accepted and replicated along with the other plasmid complement. This in fact is what is most often seen if one infects a cell harbouring an *fi*$^+$ R-factor with an *fi*$^-$ R-factor, or vice versa. The cell is quite hospitable to both, and the replication of each plasmid does not interfere with the other. Both express their biological individuality. On the other hand if a recipient

cell receives by conjugation a plasmid which is isogenic or closely related to one that is already resident, there is a very different picture, which can be understood as a combination of two different effects termed *entry exclusion* and *plasmid incompatibility*.

(a) *Entry exclusion*

R-factors, F, Col I and other plasmids possess a gene(s) which specifies a cell barrier that prevents the transfer and/or replication of the DNA of an isogenic, or very closely related, sex factor. As shown in table 7.4, the frequency of transfer of an R-factor to a host already harbouring a closely related plasmid is reduced dramatically. In contrast a recipient carrying an unrelated plasmid accepts the superinfecting R-factor at a level roughly comparable with an F^- strain. Table 7.4 further shows that the synthesis of superinfecting R-factor DNA within the recipient is sharply reduced in the recipients carrying a homogenic or related plasmid, but is scarcely affected in the recipient carrying an unrelated R-factor. It seems reasonable to conclude therefore that entry exclusion acts either to prevent the physical entry of the superinfecting R-factor DNA or, if the DNA enters the cell, it is usually not replicated. It may be noted that the inhibition of DNA transfer affects a wide category of related plasmids. In the example, an fi^+ R-factor is inhibited by other fi^+ R-factors and even to some degree by F. Thus entry exclusion provides a very effective method for the selective prevention of an intruding DNA.

The biochemical basis of this phenomenon is not known, but entry exclusion seems to be abolished, in part, by alterations in the growth conditions of the host cell. Cells grown to the stationary phase of growth

Table 7.4. Transfer of an fi^+ R-factor to recipients carrying related and unrelated plasmids. (Data from LeBlanc and Falkow, 1972.)

Recipient cell	Plasmid class in recipient	Relative transfer frequency of fi^+ R to recipient[a]	Relative synthesis of fi^+ R DNA in recipient[b]
E. coli F^-	-	1·0	1·0
E. coli R-Su-Sm-Tc	fi^-	0·80	0·75
E. coli F^+	fi^+	0·24	0·16
E. coli R-Su-Sm-Tc-Cm	fi^+	0·024	0·18
E. coli R[c]	fi^+	0·0009	0·11

[a] The donor plasmid is R1*drd*19 (R-Su-Sm-Cm-Km-Ap). The transfer frequency (1×10^{-1}) of this plasmid to the F^- recipient in 30 min mating at 37°C was taken as 1·0.

[b] The relative synthesis of donor plasmid DNA in the recipient was $1{\cdot}54 \times 10^4$ cpm of [^{3}H]thymine after 60 min of mating with the F^- recipient. This was taken as 1·0 and the other values were compared accordingly.

[c] The *E. coli* R recipient was homogenic to the donor plasmid for sex-factor functions but carried no drug-resistance determinants.

with vigorous aeration, cells which are starved of amino acids or, more drastically, cells treated with periodate, all lose by about three- to four-fold their ability to express entry exclusion. None of these treatments alters the cell's genotype but they do remove the entry barrier, whatever it is, to yield an 'F^- phenocopy'. According to Watanabe and his associates the entry exclusion of an R-factor does not occur after the phage P1 or phage P-22 transduction of an R-factor to a recipient carrying a closely related plasmid. The difference here is not attributed to the transduced R-factor having acquired a new genetic peculiarity, but rather that the R-factor DNA enters the cell through a different receptor site at transduction than at conjugation. It is assumed that entry exclusion operates at the cell surface or cell membrane (probably the latter), preventing injection of the superinfecting plasmid DNA. Although entry exclusion is clearly associated with the sex pilus type, in the sense that most sex factors which exclude one another are of the same pilus type, it seems unlikely that the sex pili themselves play the major role. Mutants of R-factors may be isolated which lose their capacity to produce pili but still retain the exclusion property and vice versa. One should be sure to make a distinction here between immunity and exclusion. It is not certain that the intervention of a repressor, as in prophage immunity or in pilus control, is operative in entry exclusion. The major argument here is that one should not expect a transduced R-factor to escape repression simply because it was introduced into a cell via a different cell receptor. On this basis it may be more likely that receptor sites on the cell surface necessary for the donor pili to establish an effective conjugal contact are modified, or their synthesis is prevented, by a gene product of the resident plasmid. In this vein Sheehy, Orr and Curtiss found that entry exclusion was a phenotypic property that was not expressed by 100% of recipient cells carrying a related plasmid, but when it was expressed there was absolutely no DNA transfer. Their results suggest that entry exclusion prevents pair formation between donor cells—a result that has also been inferred from other studies utilizing transfer-defective mutants of F (see chapter 8). Entry exclusion is not absolutely effective, but even if a related superinfecting plasmid can overcome the entry barrier, this is not, in itself, sufficient for it to establish itself in the host.

(b) *Incompatibility*

The difference between entry exclusion and incompatibility can be seen by a further examination of table 7.4. The experiment measures two parameters: the degree of replication of superinfecting DNA within a recipient during the first sixty minutes after conjugation, and the extent to which a superinfecting plasmid can become established within the recipient. It is clear that the decreased extent of DNA replication is basically similar in recipients with resident F^+ or *fi*$^+$ R-factors. Yet there

is a good correlation between the observed level of DNA replication and the eventual recovery of the superinfecting plasmid in the *fi*$^-$ and F$^+$ recipient strains. On the same basis it can be seen that the eventual recovery of the superinfecting plasmid is sharply decreased relative to the extent of DNA synthesis in recipients harbouring an isogenic or closely related plasmid. Most *fi*$^+$ and *fi*$^-$ R-factors can stably exist in the same cell as F—that is to say, F and most R-factors are compatible. Thus the data shown in table 7.4 are interpreted to mean that the moderate reduction in DNA synthesis and genetic transfer seen with the F$^+$ recipient reflects, in part, the phenomenon of entry exclusion. So, whereas the presence of F in a cell sets up some barrier to entry of an *fi*$^+$ R-factor, the superinfecting DNA which overcomes this exclusion barrier has a high probability of successful establishment. In contrast, although recipients harbouring an isogenic or closely related plasmid exert very nearly the same degree of exclusion as F$^+$ on the superinfecting plasmid, the significant difference between the amount of DNA synthesized and the eventual establishment of the superinfecting R-factor in the recipient reflects the phenomenon of plasmid incompatibility. In this example, entry exclusion reduces inheritance by about 10-fold, whereas incompatibility can reduce inheritance by an additional 50- to 10000-fold. Thus it can be seen that exclusion and incompatibility represent two distinct phenomena—a fact attested to by the finding that different genes on the plasmid chromosome are responsible for each effect (see chapter 8). It appears that they are directed against different stages of transfer replication—entry exclusion at a very early stage and incompatibility at some stage subsequent to physical entry. Although exclusion and incompatibility are distinctly separable, on a practical basis it may be difficult to distinguish between the two. Indeed most investigators do not (or experimentally cannot) distinguish between them, but loosely classify both phenomena under the general category of 'superinfection immunity'. This term is certainly descriptive, but, as pointed out by Richard Novick, the analogy with prophage-mediated superinfection immunity may possibly be misleading.

It should be noted that even the combination of exclusion and incompatibility, both reasonably efficient mechanisms, is not complete, for rare cells may be recovered in which both the resident and the closely related superinfecting plasmid are present. The pairs are still incompatible, and this continuing incompatibility may be experimentally studied by choosing pairs with suitable combinations of drug resistance (for example, one R-Tc and the other R-Su-Sm-Cm). If one of the pairs employed is a derepressed R-factor, so much the better, since this serves as a further identifying feature that can be followed by male-phage sensitivity. Experiments of this kind, with different pairs of related F-like R-factors, have revealed some common features of the incompatibility process. No pair of F-like R-factors were stably inherited together, and

segregation occurred on drug-free media until one or the other (often, but not always, the superinfecting R-factor) was rejected. If repeated cultivation was carried out in the presence of drugs selective for both R-factors, both were maintained simultaneously, presumably by constant reinfection within the culture. Ultimately, however, a stable culture resistant to all the drugs appears. Thus the outcome of continuing selection for the resistance genes of both factors is the emergence of a single recombinant R-factor. This recombinant R-factor carries the selected resistances as a single-linkage group and can be transduced as a single unit by phage P1. In crosses between a derepressed and a wild-type (repressed) R-factor, it is interesting to see that so long as both factors were present the culture was insensitive to male phage, indicating that the derepressed R-factor was being subjected to repression of pilus production by the wild-type factor. Once segregation had ceased and the recombinant form emerged, however, the entire culture became male-phage sensitive, indicating that the wild-type sex-factor-carrying repressor had disappeared. The fact that the derepressed sex factor was always retained is attributed to its high efficiency as a donor. If the unstable state is characterized by repeated segregation of one or the other of the two elements, the R-factor able to reinfect most efficiently would be the one most likely to predominate. It should be emphasized that the drug-resistance determinants from both elements can appear in the recombinant plasmid, but only one set of sex-factor genes; it follows that incompatibility must be generally exerted between the RTF region of the two plasmids.

In general, then, the establishment of a superinfecting plasmid, after the inhospitability of exclusion, involves segregation into a separate cell line, or the 'rescue' of some of its markers by recombination. In most cases the superinfecting plasmid is on a quite different footing from the resident one. On the other hand, several authors point out that there may be a hierarchy of preference for one plasmid over the other. The existence of incompatibility (or exclusion) between two plasmids indicates a close relationship between them. As Hayes points out, it is perhaps paradoxical that two plasmids which can coexist, because they are different, are referred to as 'compatible', whereas similar or identical plasmids which do not, are said to be 'incompatible'.

Superinfection immunity is a very sensitive measure of plasmid specificity. As such, it is one way in which plasmids can be classified. The initial division of transmissible R-factors into two main classes, fi^+ and fi^-, according to their effect on F-mediated conjugation, was subsequently linked to the type of pilus specified by the plasmid. When it was observed that all fi^+ R-factors examined determined production of an F-type pilus, and 20 out of 26 fi^- R-factors determined the production of a pilus similar to the one determined by the Col I factor, it was common to equate fi^+ with F-like, and fi^- with I-like.

The connotation that all R-factors belonged to one or another of two groups, as might be expected, was an oversimplification of matters. A number of laboratories have shown by means of male-phage specificities and superinfection immunity that both fi^+ and fi^- R-factors (and other plasmids) are heterogeneous. Datta and Hedges, as well as other workers, have subdivided plasmids into a number of distinct groups on the basis of compatibility (remember a *compatibility group* is composed of plasmids that are mutually incompatible with each other, but compatible with all other plasmids). At the time that this is being written, approximately twenty plasmid compatibility groups have been documented. For example, among the fi^+ plasmids four compatibility groups have been described. FI, the class including the classical F-factor; FII, the class including most of the fi^+ R-factors; FIII, a class including Col B-K99 and Col B-K166; and FIV, a class containing a single fi^+ R-factor, R124. The number of distinct compatibility groups really blossomed upon study of the fi^- factors. Initially, Datta and Hedges divided the fi^- R-factors into four general groups: I (these plasmids, which determine I pili, can now be subdivided into five distinct compatibility groups); N; P (a class found initially in *Pseudomonas aeruginosa*); and W (named for Watanabe, this class is not common but can be isolated from various Enterobacteriaceae and *Aeromonas liquefaciens*). Table 7.5 shows the kind of genetic data that are used to assign R-factors to distinct compatibility groups, as well as a summary of some representative R-factors of different compatibility classes. Since the modest assignment of the I, N, P, and W groups, it now often seems that each new issue of a bacteriological journal brings forth a new compatibility group. This I believe is probably more of a reflection of current intense interest in the plasmid complement of enteric species other than *E. coli, Salmonella,* and *Shigella*, rather than an indication that there are an infinite variety of compatibility groups.

The major question, of course, is whether differences in plasmid compatibility reflect a few gene differences or a more general overall difference in genetic structure. As will be shown in the next chapter, the fine genetic structures of F, I, N, W, P, and other plasmid groups are, in general, very different when assayed for nucleotide sequence similarity. The conclusion seems to be that members of the same compatibility groups (related plasmids) are derived from a common ancestral source, but are almost totally unrelated to plasmids of other compatibility groups. These findings have important theoretical and practical implications. For example, one immediate theoretical question is whether there is a single pool of drug-resistance genes that transfer factors draw upon or whether there are a number of distinct pools. At the practical level the grouping of R-factors by compatibility class may be employed for epidemiological purposes. For example, an outbreak of *Shigella dysenteriae* type 1 in Central America was followed soon after by a massive epidemic of typhoid in Mexico. In both outbreaks R-factors with

similar drug-resistance phenotypes were described. The presence of the R-factors, of course, complicated treatment, and a number of clinicians were convinced that the R-factor of the earlier *Shigella* epidemic found its way into the typhoid bacillus. However, the two R-factors are quite

Table 7.5. (a) An example of transfer and compatibility of an N plasmid with plasmids of the same and different compatibility groups. (b) Some representative plasmids of different compatibility groups.

	Donor[a]	Recipient	Frequency of transfer	Recipient cells tested for presence of each plasmid
(a)		*E. coli* K-12	4×10^{-3}	–
		E. coli (R-FII, Km)	2×10^{-3}	20/20 both present
	E. coli (R-N, Ap-Su-Sm-Cm-Tc)	*E. coli* (R-I, Km)	5×10^{-3}	20/20 both present
		E. coli (R-P, Km)	2×10^{-3}	18/20 both present
		E. coli (R-W, Su-Tp)	3×10^{-3}	20/20 both present
		E. coli (R-N, Km-Su-Sm)	2×10^{-5}	0/20 both present

	Plasmid	Compatibility group	*fi*	Resistance determinants
(b)	R386	FI	+	Tc
	R1	FII	+	Su-Sm-Cm-Km-Ap
	R124	FIV	+	Tc
	R64	I	−	Sm-Tc
	R62	I	+[b]	Su-Sm-Ap
	R483	I	−	Sm-Tp
	R46	N	−	Su-Sm-Tc-Ap
	RP4	P	−	Tc-Ap-Km
	S-a	W	−	Su-Sm-Km-Cm
	R388	W	−	Su-Tp
	R401	T	−	Sm-Ap
	R391	J	−	Km
	R724	O	−	Su-Sm-Tc-Cm

[a] An *E. coli* K-12 strain carrying an R-factor of the N compatibility group was mated with a nalidixic-acid-resistant *E. coli* K-12 strain carrying each of the indicated R-factors of different compatibility groups. Mating mixtures were plated on a medium containing chloramphenicol + nalidixic acid to select for recipient clones which had received the N plasmid from the donor. The frequency is expressed in terms of transfer per donor cell in the mating mixture. Twenty of the recipient clones appearing on the selection plates were reisolated on antibiotic-free medium and tested for the presence of both plasmids (compatible pairs) by their antibiotic-resistance pattern and by genetic transfer to suitably marked recipients. A dramatically reduced frequency of transfer and an inability to stably coexist with the resident plasmid is seen only in the recipient carrying another N plasmid.

[b] The unusual *fi*$^+$ phenotype of R62 is considered later (chapter 8).

different in molecular size and are of different compatibility groups, which suggests that they are of independent origin. Moreover, in line with a theme established earlier, it is interesting that most *fi*⁻ R-factors initially studied in Japan have now been found to belong to *fi*⁻ compatibility group N, whereas those studied most extensively in England were plasmids of group I. One again notes a substantial difference in the ecology and genetic properties of the R-factors found in Japan and England (responsible, no doubt, for some confusion in the literature).

The added complexity of a growing number of compatibility groups of R-factors is mitigated to some degree by the realization of a kind of 'preference' of certain compatibility groups for certain bacterial groups, as well as by a restriction on the part of the bacterial host upon which kind(s) of R-factors it can stably maintain (table 7.6). Thus which R-factor one finds probably depends more on the bacterial genera one examines than on geographical location. As noted earlier in *E. coli* and *Shigella* sp., *fi*⁺ factors (group FII) are in the majority, but in *Salmonella* sp. *fi*⁻ factors predominate, most of which are of the I compatibility group. Among naturally occurring *Proteus* sp., F group and I group plasmids are quite uncommon (I has never been found), but N group as well as W, T, and other *fi*⁻ groups are common. P and C group plasmids are the rule in *Pseudomonas aeruginosa* and this species does not accept FII or I group plasmids. Yet the P plasmids themselves have an extraordinary host range, which can be extended in the laboratory to include such species as *Rhizobium lupini* and even to *Neisseria* (C. C. Brinton, personal communication). Thus as the interest in R-factors in Gram-negative species other than *Salmonella* and *Shigella* has grown (particularly since 1968), a new dimension has been added to our study of the ecology and biology of transmissible plasmids. It may be noted that incompatibility also exists between small nontransmissible plasmids.

Table 7.6. Host range of R-factors of different compatibility groups.

Host bacterial strains	Plasmid compatibility group[a]					
	FI	FII	I	P	N	W
Escherichia, Salmonella, Shigella	+	+	+	+	+	+
Proteus group	+	+	−	+	+	+
Pseudomonas aeruginosa	−	−	−	+	−−	−−
Rhizobium lupini	+	+	−	+	−−	−−
Other *rhizobia* and *agrobacteria*	−	−	−	+	−−	−−

[a] + means transfer was accomplished from an *E. coli* donor;
− means transfer was not detected using an *E. coli* donor;
−− means not done Adapted from Datta and Hedges (1972a).

The study of incompatibility between these plasmids is more difficult, since ordinarily they carry but a single drug-resistance marker which may be scored in genetic transfer experiments. Nonetheless Smith, Humphreys, and Anderson have shown that the Su–Sm plasmid forms a compatibility group with Ap–Su and Ap–Su–Sm nontransmissible plasmids. In a similar vein, J. Inselburg (personal communication) has shown incompatibility between Col E2 and Col E3.

(c) *Molecular basis of superinfection immunity*

To explain incompatibility most authors have called upon the replicon model. It is presumed that the number of maintenance sites in a cell are limited and are replicon specific. According to this model, compatible plasmids would differ in attachment-site specificity whereas related (incompatible) plasmids would compete for the same site(s). A plasmid entering a cell already inhabited by a closely related plasmid has been presumed to be at a marked disadvantage and unable to replicate properly, since the resident plasmid occupies the specific site and is already fully asserting its biochemical 'squatter's rights' to ensure proper attachment and replication. In those cases where the superinfecting plasmid preempts the resident, it has been postulated that it has a greater biochemical affinity for the maintenance site than the resident. It is unfortunate that one cannot speak in more precise biochemical terms when describing exclusion and incompatibility, but the biochemical bases of these phenomena are virtually totally unknown. One can, however, get a more precise idea of the phenomenon by examining the fate of the same superinfecting DNA in hosts harbouring a compatible or incompatible resident plasmid.

Figure 7.1 shows the differences in the replicative picture of a superinfecting *fi*$^+$ R-factor sixty minutes after the beginning of conjugation in a recipient cell containing a resident compatible F$^+$ plasmid, and a recipient cell containing a resident isogenic plasmid. In the compatible recipient cells, the superinfecting DNA is found exclusively as a nicked and twisted covalently-closed molecule—the expected 'normal' products of replication. The DNA extracted from the equivalent incompatible recipient does not show these normal end products but, instead, sediments over a broad region intermediate between the nicked and closed-ring forms. An initial reaction to this finding is that incompatibility is either associated with a mechanism that produces aberrant replicative end products, or that normal replicative intermediates have accumulated because of some barrier to replication.

The experimental choice between these alternatives can be approached by performing a pulse–chase experiment in which the fate of DNA synthesized within the first few minutes is followed over an extended period of time. Such an experiment shows that, shortly after entering the cell, the superinfecting DNA is predominantly present as normal linear

monomeric molecules, suggesting that the initial processing of the DNA is normal. The subsequent picture, however, is one which is interpreted to represent a circular molecule which sediments faster than a usual nicked ring because it is larger than a unit length. This increased molecular mass is thought to reflect either a σ or θ form (described in chapter 6), although direct electron-microscopic confirmation is not yet available. The most significant result of the experiment (figure 7.2) is the finding that, eventually, well over 60% of the superinfecting DNA within the incompatible recipient does become converted into twisted covalently-closed monomeric rings. So, a normal replicative end-product does accumulate, and it may be assumed that the unusual molecular structures seen in the incompatible host represent normal replicative intermediates. As a corollary it can be concluded that the phenomenon of incompatibility is, at least in part, characterized by a significant reduction in the rate of superinfecting DNA replication. This can be more precisely stated in terms of the length of time it takes on the average for a single R-factor molecule to replicate from a linear form to the twisted covalently-closed form. The time in an F^- cell or host carrying a compatible plasmid is 2·5–3·0 minutes. The replication time of the same plasmid in an incompatible host is calculated to be 35–40 minutes. This approximately fifteenfold decrease in replication rate presumably could reflect the action of some resident plasmid-specific repressor substance. This model predicts that the incompatible DNA would be diluted during growth of the culture because it could not keep pace. It seems unlikely, however, that incompatibility can be entirely accounted for by a repressor

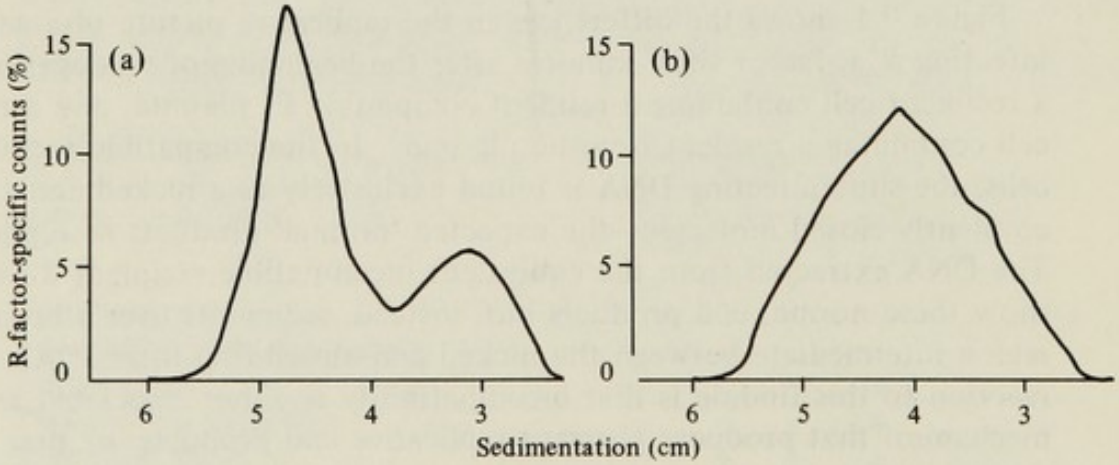


Figure 7.1. Differences in the replicative picture of R-factor DNA in a compatible and incompatible host. (a) In compatible recipient cells the superinfecting R-factor DNA is found exclusively as nicked and twisted covalently-closed molecules 45 min after mating. (b) In an incompatible (R^*) host 45 min after mating, the superinfecting R-factor DNA sediments predominantly over a broad region intermediate between the nicked and closed-ring forms. Sedimentation refers to the number of centimetres sedimented from the top of the gradient. (Data from LeBlanc and Falkow, 1972.)

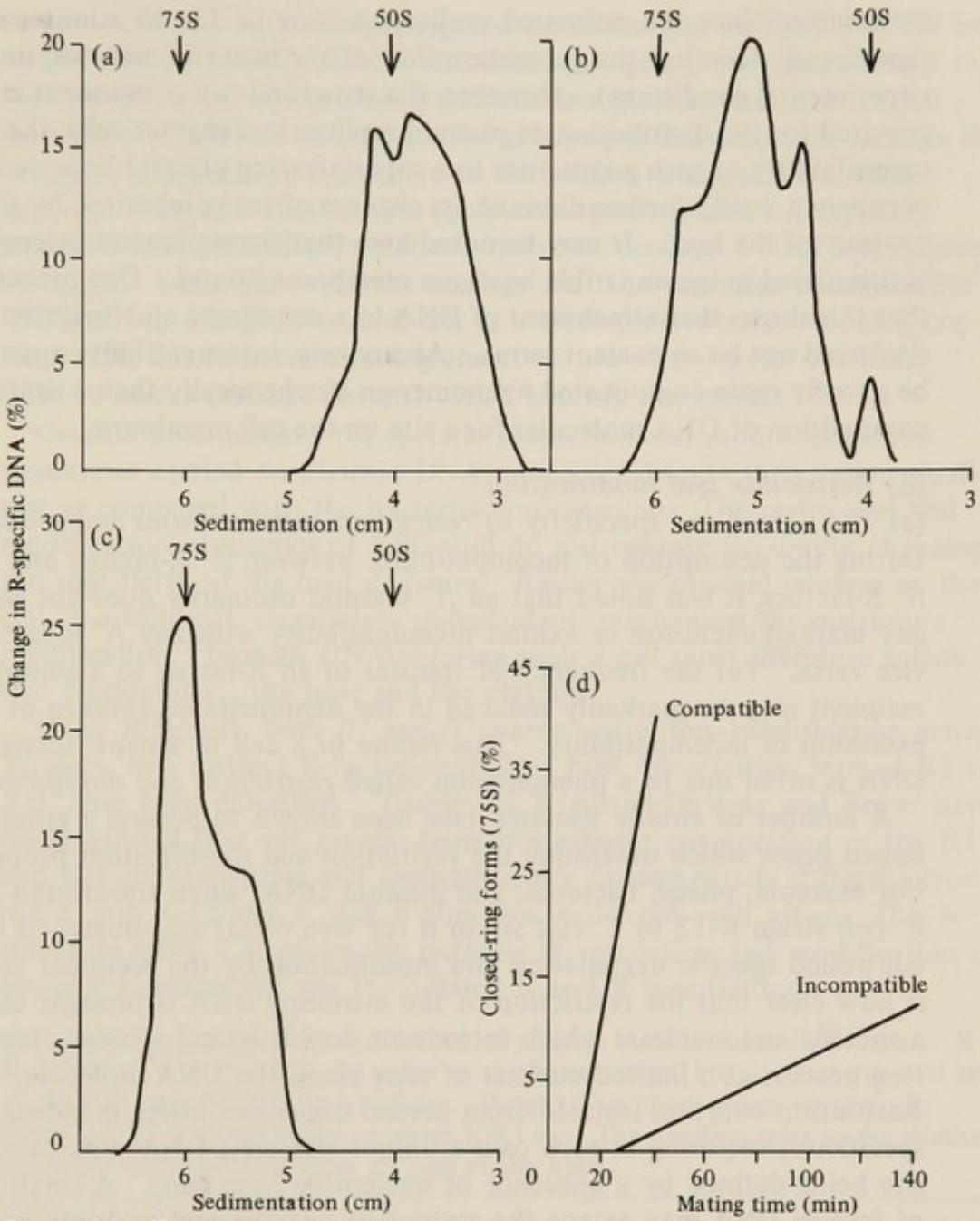


Figure 7.2. Kinetics of replication of R-factor DNA in an incompatible host. This experiment was designed to follow the ultimate fate of DNA labelled for a short time after entering an incompatible host. (a) The DNA, 20 min after entering an incompatible host, is found largely as nicked-ring forms 50S. (b) The DNA seen in the incompatible host between 30 and 90 min after the initiation of mating sediments at a rate faster than a nicked ring, but slower than a twisted covalently-closed ring form. These molecules are assumed to represent replicative intermediates that are larger than a unit length such as σ or θ forms. (c) Between 90 and 150 min after the initiation of mating the superinfecting DNA is finally transformed into the 'normal' replicative end product, a 75S twisted covalently-closed ring. (d) The replication rate of an R-factor, as measured by the rate of appearance of closed-ring forms, is approximately eleven times faster in a compatible host than in an incompatible host. Sedimentation refers to the number of centimetres sedimented from the top of the gradient.

mechanism, since the estimated replication time of 35–40 minutes is still significantly less than the generation time of the host (65 minutes, under the experimental conditions). However, if a structural cell component is required for the distribution of plasmid replicas to daughter cells, the unavailability of such a structure to a superinfecting plasmid because of prior occupancy would further diminish its chances of being inherited by the progeny of the host. It may be noted here that the replicative intermediates accumulated in incompatible hosts are membrane-bound. One presumes that this shows that attachment of DNA to a membrane and 'maintenance site' need not be equivalent terms. At any rate, incompatibility appears to be a vastly more complicated phenomenon biochemically than a simple competition of DNA molecules for a site on the cell membrane.

(d) *Restriction and modification*

(*a*) *The nature and specificity of restriction endonucleases and methylases*

During the description of incompatibility between *fi*$^+$ R-factors and *fi*$^-$ R-factors, it was noted that an *fi*$^-$ plasmid ordinarily does not show any marked exclusion or exhibit incompatibility with any *fi*$^+$ R-factor or vice versa. Yet the frequency of transfer of an R-factor to a compatible recipient may be markedly reduced in the demonstrable absence of exclusion or incompatibility. This failure of a cell to accept 'foreign' DNA is often due to a phenomenon called *restriction* and *modification*.

A number of enteric bacteria have been shown to possess a group of linked genes which determine the restriction and modification property. For example, phage, bacterial, and plasmid DNA, when transferred from *E. coli* strain K-12 to *E. coli* strain B (or vice versa) are subject to host-controlled specific degradation and modification by the recipient cell. It is now clear that the restriction of the intruding DNA is brought about by a specific endonuclease which introduces double-strand scissions in a two-step process at a limited number of sites along the DNA molecule. Restriction enzymes isolated from several organisms differ in substrate specificity, requiring in each case a unique site on a DNA molecule, this site being defined by a sequence of nucleotide base pairs. A small amount of foreign DNA may escape the restriction enzyme and multiply normally, but this DNA has not escaped completely unchanged. Hence when phage λ, initially grown on strain K-12, is used to infect strain B, only one phage particle in about 10000 successfully multiplies and produces λ progeny. The progeny phage now have the ability to grow normally on strain B, but have lost their ability to grow on strain K-12 (except again for a rare phage particle). The phage have been *modified* by growth on strain B and now operate with the same specificity of strain B DNA and, in turn, will now be 'restricted' by strain K-12. Modification is dependent upon methylation of specific sites on the DNA molecule recognized by the restriction endonuclease. In bacteria there are at least three host genes associated with restriction and modification. One of the three is thought

to be concerned with recognizing the particular site on the DNA for both restriction and modification. The other two genes code respectively for the endonuclease which cleaves DNA at the specific site, and for the methylase which inserts two methyl groups per substrate and renders the methylated sequences insensitive to the related endonuclease. It is apparent therefore that certain (or probably all) strains of bacteria possess a well-defined enzymatic system for 'reading' DNA to determine its origin. Luria has made the analogy that the organism examines the DNA, not as a linguist would, but as a bibliophile would: looking not at the script, but rather examining the structure of the script for little signs or marks (methyl groups) which identify the printer[7].

Genetic determinants for specific restriction and modification are sometimes carried on plasmid DNA molecules despite their rather small size as compared with the bacterial chromosome. The restriction and modification properties of a plasmid do not operate by simply changing the specificity of the host enzymes. Rather the plasmid confers on the cell an additional, completely independent, mechanism for restriction modification. Foreign DNA entering such a cell must therefore satisfy two bibliophiles: the host and the plasmid.

Many R-factors, both fi^+ and fi^-, carry restriction–modification genes. To date, two distinct R-factor-controlled host specificities, termed RI and RII, have been described. Yoshimori, Roulland-Dussoix and Boyer have demonstrated that the genetic basis and subunit construction of the RI and RII endonucleases and methylases are fundamentally different from the *E. coli* K-12 and *E. coli* B enzymes, and from each other. This is shown in table 7.7, where the patterns of restriction and modification of phage λ is shown for the R-mediated K and B specificities.

Table 7.7. Restriction and modification of phage λ by R-factors. Host strains: K is *E. coli* K-12, B is *E. coli* B, RI refers to the R-mediated RI host specificity, RII refers to the R-mediated RII host specificity. Phage λ K is phage grown on strain K; phage λ K-RI is phage grown on strain K-RI etc. The numbers refer to the relative efficiency of plaque formation on each of the hosts.

Host strain	Phage λ K	Phage λ K-RI	Phage λ B	Phage λ B-RI
K	1·0	1·0	5×10^{-4}	1×10^{-3}
K-RI	4×10^{-4}	1·0	7×10^{-5}	5×10^{-3}
K-RII	2×10^{-2}	2×10^{-2}	7×10^{-5}	1×10^{-4}
B	2×10^{-4}	5×10^{-5}	1·0	1·0
B-RI	2×10^{-4}	1×10^{-3}	7×10^{-5}	1·0
B-RII	7×10^{-4}	6×10^{-3}	2×10^{-3}	6×10^{-3}

[7] Restrictive bacteria remain unaffected in their growth when infected with unmodified phage λ, but it is reported that they seem to be killed by the invasion of unmodified plasmids such as F and R. This effect still remains unexplained.

The R I restriction endonuclease (Eco RI) and modification methylase have been associated with an *fi*$^+$ R-factor, whereas the R II restriction endonuclease (Eco RII) and modification methylase have been associated with *fi*$^-$ R-factors (particularly members of the N incompatibility group). The Eco RI and Eco RII specificities can be defined by the sequence of base pairs recognized by the restriction endonuclease and modification methylase. An examination of the nucleotide sequence of the site restricted by the Eco RI and Eco RII endonucleases reveals some interesting properties. The nucleotide sequence of the site restricted by the Eco RII restriction endonuclease, as determined by Herbert W. Boyer and his associates, is:

$$\cdots\cdots N \downarrow pC-C-A-G-G-\!\!-N' \cdots\cdots$$
$$\cdots\cdots N-\!\!-G-G-T-C-Cp \uparrow N \cdots\cdots .$$

N is any base and the arrows indicate the positions of the phosphodiester bond cleavages; the remaining double-stranded DNA structure is indicated by the parallel dotted lines.

After methylation by the Eco RII modification methylase the site is modified as follows:

$$\cdots\cdots N-C-m^5C-A-\!\!-G-G-N' \cdots\cdots$$
$$\cdots\cdots N'-G-\!\!-G-T-m^5C-C-N \cdots\cdots .$$

Thus it can be seen that the same nucleotide sequence in double-stranded DNA restricted by the Eco RII restriction endonuclease is methylated by the Eco RII methylase (in this case to 5-methylcytosine). A DNA molecule may be a substrate either for the restriction endonuclease or the modification methylase but not for both, since treatment with one enzyme renders the DNA insensitive to the other.

The DNA nucleotide sequence restricted by the Eco RI endonuclease, as determined by Hedgpeth, Goodman and Boyer, is:

$$5' \cdots\cdots A/T-G \downarrow A-A-T-T-\!\!-C-T/A \cdots\cdots 3'$$
$$3' \cdots\cdots T/A-C-\!\!-T-T-A-A \uparrow G-A/T \cdots\cdots 5' .$$

Clearly the Eco RI and Eco RII have quite different specificities. Indeed the distribution of Eco RI and Eco RII specific sites on any particular genome differs considerably. For example, the small virus genome of SV40 shows about seventeen Eco RII cleavage sites per molecule but only one Eco RI site. Plate 4 shows the effect of Eco R1 treatment on the DNA of a nontransmissible plasmid (5×10^6 daltons), phage λ (30×10^6 daltons), and a transmissible *fi*$^+$ plasmid (65×10^6 daltons). Despite the differences in sequence specificity of Eco RI and Eco RII a careful examination of the specific sequences reveals certain common structural features. The most striking feature is a twofold rotational symmetry. Possibly this substrate symmetry is related to the subunit structure of the enzymes that interact with the sequence. In this

vein it is of interest that the purified Eco RI endonuclease is composed of two subunits of identical molecular weight, presumably coded for by one cistron. The other important feature shared by Eco R1 and Eco RII is that both cleave the double-stranded DNA so as to produce fragments containing short overlapping single-stranded ends. For example, if a covalently-closed circular molecule of plasmid DNA contained but a single Eco RI site, the following reaction would occur:

$$\begin{matrix}\cdots\cdot A/T-G-A-A-T-T-C-A/T\cdots\cdot \\ \cdots\cdot T/A-C-T-T-A-A-G-T/A\cdots\cdot\end{matrix}\ \text{circular DNA}$$

Eco R1 endonuclease

$$\begin{matrix}A-A-T-T-C-A/T\text{——}A/TG \\ \qquad\qquad\quad G-T/A\text{——}T/AC-T-T-A-A\end{matrix}\ \text{linear DNA}$$

Thus the Eco R1 cleavage of the phosphodiester bonds in the circular molecule generates a linear molecule with short cohesive termini of pApApTpT (and pTpTpApA, see also later figure 7.3). Although these cohesive termini could spontaneously reassociate or could be repaired by the DNA ligase *in vivo*, they presumably must be destroyed readily by cellular exonucleases in order to account for the *in vivo* restriction that one observes. Since both Eco RI and Eco RII cleave double-stranded DNA so as to produce short overlapping single-stranded ends, it may be seen that *in vitro* the DNA fragments produced by one of these enzymes can associate, by hydrogen bonding, with other fragments produced by the same enzyme. After hydrogen bonding, the 3′-hydroxyl and 5′-phosphate ends could be ligated. Thus it was recognized that these restriction endonucleases had great potential for the construction of DNA molecules *in vitro* from any two DNA fragments generated by endonuclease digestion. The Eco RI endonuclease seemed especially useful for this purpose because, on a random basis, the sequence cleaved was expected to occur only about once for every 4000 to 16000 nucleotide pairs; thus most Eco RI-generated DNA fragments should contain one or more intact genes.

(*b*) *The use of restriction enzymes for the in vitro construction of novel biologically functional plasmids.*

The observation that restriction endonucleases could cleave unique nucleotide sequences to produce complementary termini was the essential step in the recognition that diverse DNA fragments could theoretically be joined. The fact that restriction endonucleases provide a means for joining molecules from different sources does not automatically ensure that one can create new functional DNA molecules, however. Obviously it is necessary to provide a way to transport the joined DNA into the cell and, once inside, the DNA must be able to replicate, express its genetic traits, and be recognized by the investigator.

Stanley Cohen, Annie Chang, Herbert W. Boyer and Robert Helling have examined the construction of new plasmid species by *in vitro* association of the Eco RI-derived fragments from separate plasmids. Initially they asked whether a small functional plasmid could be obtained by reassociation of fragments of a large replicon. The Eco RI endonuclease products of the 62×10^6 daltons *fi*$^+$ R-factor, R6 (Tc-Su-Sm-Cm-Km) were separated into twelve distinct fragments, ranging in size from 17×10^6 daltons to less than 1×10^6 daltons. This fragmented DNA was then employed to transform *E. coli* (see chapter 6), with selection for each of the antibiotic-resistance markers present on the R6–5 plasmid. Although the frequency was low, clones appeared on plates containing kanamycin. One such clone (which was Su^r but Tc^s, Cm^s, Sm^s) was examined and found to harbour a 17×10^6 dalton covalently-closed circular molecule. This plasmid, pSC102, was nontransmissible and, upon treatment with Eco R1 endonuclease, could be separated into three fragments which were equivalent to three of the twelve fragments seen in the parental R6–5 R-factor (plate 5). Clearly, then, these results showed that at least some Eco R1-generated fragments could transform *E. coli* cells, reassociate *in vivo* and, after ligation, circularize and be recovered as functional plasmids by appropriate selection. Cohen and Chang had previously shown that controlled shearing of the same R-factor, R6–5, could lead to the formation of a fragment which could be taken up by *E. coli* cells, circularize and autonomously replicate. This plasmid, pSC101, had a molecular weight of $5{\cdot}6 \times 10^6$ daltons and carried the genetic information for Tc resistance. The pSC101 plasmid has a single Eco R1 site and is, therefore, cleaved into a single linear fragment. Cohen, Chang, Boyer and Helling could thus see whether these two derived plasmids had a similar replication gene. Covalently-closed circular molecules of pSC101 (Tc) and of pSC102 (Km-Su) were used to transform *E. coli* with selection for Tc + Km. Transformation by this *untreated* DNA was successful and the examination of such Km^r, Tc^r clones revealed two separate covalently-closed circular species corresponding to the parental pSC101 and pSC102 plasmids. The ability of two plasmids derived from the same parental plasmid to exist stably as separate replicons in a single bacterial host suggests, of course, that the parental plasmid contains two distinct replicator sites. This interpretation is consistent with the finding that R6 dissociates into two separate replicons (RTF carrying Tc and the drug-resistance replicon carrying Km-Su) in *Proteus mirabilis*. Another mixture of pSC101 and pSC102 DNA was made, but this time treated with Eco R1 endonuclease before transformation. Transformation of *E. coli* cells with the Eco R1 fragments, with selection for Tc^r + Km^r was again successful. The examination of four of these clones revealed only a single covalently-closed DNA species with a molecular weight of about $10{\cdot}5 \times 10^6$.

When this plasmid DNA was digested with Eco R1 and analyzed, two component fragments were identified. One corresponded to pSC101, whereas the other corresponded to one of the three fragments of the pSC102 plasmid. There are several implications of these findings, but one result stands out above all others. The recovery of a biologically functional plasmid, that was formed by insertion of a fragment of one plasmid into pSC101, indicated that the cleavage of the Eco R1 site on pSC101 did not interrupt the genetic continuity of either the tetracycline gene or the replication machinery of this plasmid. This meant that virtually any Eco R1-generated DNA fragment could be covalently inserted into a pSC101 plasmid, transformed into *E. coli* and recovered either by selection of a function controlled by pSC101 (Tc^r), a function controlled by the inserted fragment, or both. To show that it was in fact possible to join DNA of entirely different origin, Cohen, Chang, Boyer, and Helling joined the cohesive-ended plasmid DNA of the 49% G + C pSC101 and a 56% G + C Su-Sm plasmid. It appeared, therefore, that pSC101 could serve as a molecular vehicle for carrying diverse fragments of DNA into *E. coli* (see figure 7.3 for a diagrammatic representation of the process). How diverse could the fragments be? Chang and Cohen showed that the penicillinase genes carried on Eco R1-endonuclease-generated fragments of *Staphylococcus aureus* plasmid DNA could be joined to pSC101 and introduced into *E. coli*, where they not only

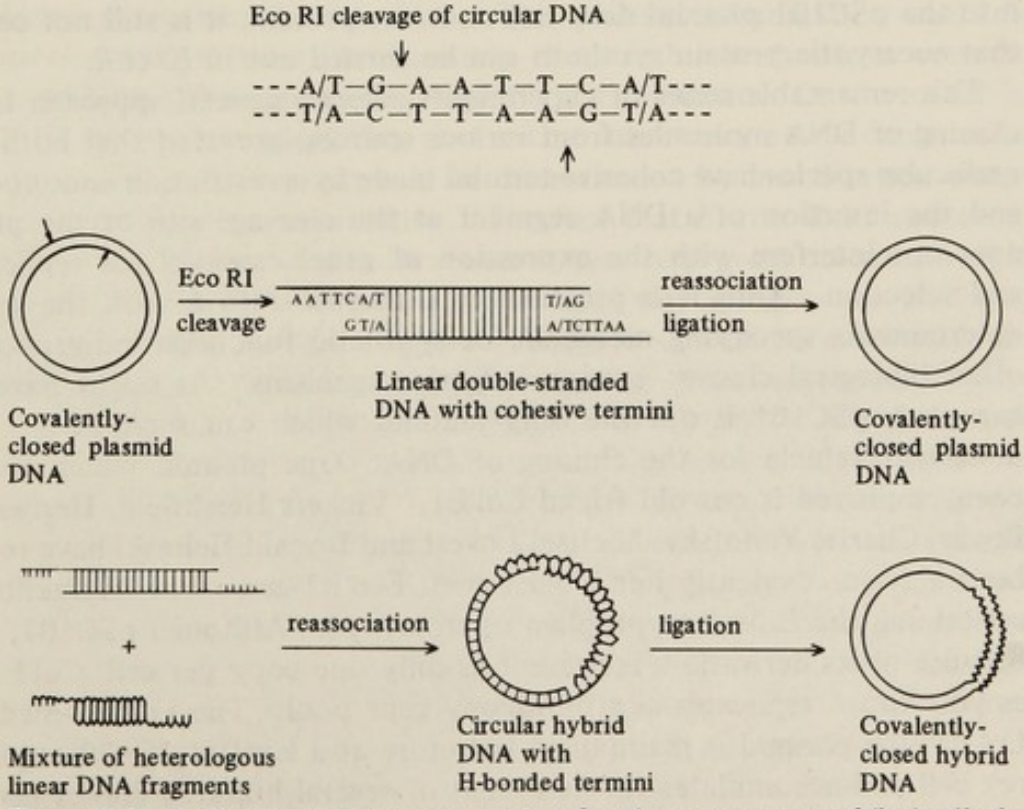


Figure 7.3. A diagrammatic representation for the contraction of 'hybrid' plasmids by the joining of Eco R1 endonuclease-generated fragments.

replicate as part of a biologically functional unit but also express their genetic information. Moreover this nontransmissible *E. coli–Staphylococcal* hybrid plasmid could be mobilized among *E. coli* strains by a transfer factor. On an even more spectacular note, John Morrow, Stanley Cohen, Annie Chang, Herbert Boyer, Howard Goodman and Robert Helling have shown that Eco R1-generated fragments of amplified euaryotic *Xenopus laevis* DNA, coding for 18S and 28S ribosomal DNA (rDNA), could be ligated *in vitro* with pSC101 and the recombinant molecular species introduced into *E. coli* by transformation. Among the tetracycline-resistant transformants about 25% were found to contain hybrid plasmids consisting of pSC101 linked to two or three fragments of *X. laevis* rDNA. These recombinant plasmids, containing both eucaryotic and procaryotic DNA, replicate stably in *E. coli*. The transcription of the *X. laevis* rDNA could be detected in the *E. coli* transformants, although the amount was but a very small fraction of the total labelled RNA to be studied easily. The *E. coli* minicells system proved to be a powerful tool in this case. The pSC101–*X. laevis* hybrid molecules were transformed into a minicell-producing line of *E. coli*, and the minicells, carrying only plasmid DNA, were isolated, incubated with [^{3}H]uridine and the RNA from the minicells was purified. A significant proportion of the RNA synthesized by the plasmid-containing minicells hybridized with purified *X. laevis* rDNA. Hence transcription of cloned *X. laevis* rDNA does occur in a procaryotic organism. However, since the *X. laevis* rDNA inserted into the pSC101 plasmid does not code for protein, it is still not certain that eucaryotic protein synthesis can be carried out in *E. coli*.

This remarkable series of experiments offers a general approach for the cloning of DNA molecules from various sources, provided that both molecular species have cohesive termini made by a restriction endonuclease, and the insertion of a DNA segment at the cleavage site of the plasmid does not interfere with the expression of genes essential for replication and selection. Thus it is practical to introduce into *E. coli* the genetic determinants specifying metabolic or synthetic functions indigenous to other biological classes—even eucaryotic organisms. As might have been expected, pSC101 is not the only plasmid which can serve as a molecular vehicle for the cloning of DNA. One plasmid which also has been employed is our old friend Col E1. Vickers Hershfield, Herbert Boyer, Charles Yanofsky, Michael Lovett and Donald Helinski have recently been able to covalently join Col E1 with Eco R1-generated fragments containing the *E. coli* tryptophan operon (*trp*). Although pSC101, because of its derivation, is present as only one copy per cell, Col E1, as you know, replicates as a multicopy gene pool. The Eco R1-mediated Col E1–*trp* plasmid is maintained therefore at a level of 25–30 copies per cell and accumulates to the extent of several hundred copies per cell in the presence of chloramphenicol (see chapter 6). Thus cells carrying the Col E1–*trp* plasmid can produce elevated levels of *trp*

operon-specific mRNA and tryptophan-biosynthetic enzymes. Hence the Col E1 plasmid can be used as a molecular vehicle both for cloning and amplification of DNA.

The potential use of R-mediated restriction enzymes, and other restriction endonucleases from other sources, staggers the mind. Yet, for all the excitement generated by this approach to the 'dissection' of genetic structure and function, we should do well to concentrate for a moment on the thought that there are potential biological hazards to the use of this method. The DNA of the tumour virus SV40 suffers only a single cleavage to a linear molecule after treatment with Eco R1. It is theoretically possible to insert any number of DNA fragments into the SV40 genome, which could be isolated by subsequent infection of host cells. One could even form a pSC101-SV40 hybrid and transform *E. coli*. Considering that millions of people were infected with SV40 in contaminated polio vaccine without any detectable deleterious effect, it would seem (in retrospect) perfectly safe to put SV40 into *E. coli* as part of a plasmid. But a number of scientists have been reluctant to construct tumour-virus-bacterial-plasmid hybrids because of their concern that this would be an unnecessary risk, creating a new unnatural reservoir for the virus. By the same token, theoretically it would be simple to construct a plasmid consisting of pSC101 (or Col E1) and a fragment of the β-phage of *Corynebacterium diphtheriae*. Once transformed into *E. coli* it is a reasonable assumption that diphtheria toxin biosynthesis would take place. This plasmid combination could be of great potential value for the study of toxin biosynthesis, or even (by amplification) for obtaining higher yields of toxin. On the other hand, even the remote possibility that such a laboratory strain could colonize man, and the hybrid plasmid be disseminated by mobilization with transfer factors, is disquieting. The fact that *E. coli* K-12 and most other laboratory strains of *E. coli* are no longer very good colonizers of the intestinal tract of man and other animals may mitigate the potential hazard to some extent. However, these *E. coli* certainly do survive in the intestinal tract, and gene transfer to and by these strains can occur *in vivo* (see chapter 10). From the standpoint of the bacteriologist, probably the situation is most critical to those who wish to study the genetic determinants of virulence. It is important, I think, not to simply condemn even the idea of examining the determinants of bacterial pathogenicity by these methods. Many determinants of virulence in *E. coli are* plasmid-mediated (see chapter 11) and undoubtedly the use of the restriction endonucleases will be invaluable in determining the structure and function of several plasmid-mediated determinants that contribute to the virulence of several groups of enteric pathogens. The problem would seem not so much a matter of creating a 'super' pathogenic *E. coli* as one of changing the ecology and potential host range of the genetic determinants of virulence once removed from its natural host.

The potential hazards of constructing novel replicons from diverse sources has already been the subject of a dialogue among scientists in several biological disciplines. All concur that it would be prudent to establish reasonable experimental guidelines and acceptable safety standards with respect to the construction of strains bearing novel plasmid or viral combinations constructed *in vitro*. Such standards need not hamper or deter research so much as they will ensure thoughtful experimental design.

(*c*) *Some concluding remarks about superinfection immunity and restriction–modification.*

The potential usefulness of an isolated purified enzyme on mixtures of purified DNA is one thing; the significance of restriction–modification *in vivo* is another. To my knowledge, no evidence has been presented to indicate that the restriction and modification enzymes function to produce new DNA combinations *in vivo*. From a genetic standpoint the restriction–modification properties can add to the definition of an R-factor or any other plasmid. Restriction also has several important practical implications. Anderson has shown that the dissociated R-factor ΔSu–Sm, ΔAp, and Δ alone alter the phage sensitivity (phage-type) of *Salm. typhimurium*. He specifically cites that when the host strain was phage-type 36 of *Salm. typhimurium*, originally sensitive to all of the *Salm. typhimurium* typing phages, it was changed, after acquisition of the R-factor, to phage-type 6, sensitive to only nine of the phages. Thus phage restriction by R-factors may have to be taken into account when studying outbreaks caused by *Salm. typhimurium, Salm. panama, Salm. typhi*, or other organisms for which phage typing is epidemiologically important. Anderson suggests that when two phage types of an organism are found in an outbreak, if one type has a more restricted range of phage sensitivity than the other, it may often be that the more restricted strain harbours an R-factor (or other plasmid). Similarly any marked change in the prevalence of certain phage types during epidemiological surveys would seem to warrant an investigation into their plasmid complement.

It is generally held that restriction–modification offers enteric bacteria and their plasmids protection against the intrusion by 'foreign' DNA. In the same vein, exclusion and incompatibility may also provide protection against unnecessary or spurious genetic recombination since during adaptation of any species, generalized recombination may have only limited value. Under normal circumstances, exclusion and incompatibility prevent intrusion of a related plasmid. Yet, under selective conditions, the net result of shared occupancy of a host by two related plasmids is a genetic recombinant better suited than either parental type for survival. In a general sense, therefore, restriction–modification, exclusion, and incompatibility may be primarily concerned with genetic isolation

against the obliteration of biological individuality. The finding that these isolating mechanisms have evolved independently both on plasmid and host chromosomes indicates their general importance at strikingly different levels of genetic organization. Of course, as discussed in the following chapter, gene flow is not abolished by these mechanisms. It is clear, however, that DNA species *in vivo* are quite specific biochemically about the DNA that they will chose for recombination. Under these criteria it is more likely that useful new gene combinations will be permitted, but major alterations in genetic organization are avoided. Indeed it seems to me that plasmids themselves provide their hosts with a mechanism which facilitates genetic adaptation with minimal disruptive recombination. Plasmids usually replicate independently of the host chromosome. Plasmids are additions to the genetic endowment of the cell but can be lost without involving any host chromosomal changes. The plasmids therefore provide a kind of general gene pool in bacteria, without either the host or the plasmid necessarily altering its individuality.

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Recombination

A recombinant cell contains genetic material from more than one parent. In recent years there has been a growing distinction between recombinant molecules formed by 'ordinary' 'general' recombination and those formed by 'illegitimate' recombination. There are numerous models for general recombination but no single model adequately describes the recombination process in all, or even most, organisms. There is near unanimous agreement about several aspects of general recombination, however. To begin with, general recombination is a relatively frequent process, which is extremely accurate because it depends upon substantial nucleotide sequence homology. It is generally accepted that general recombination occurs by a breakage and rejoining mechanism, and that the primary recombination product contains one or both DNA strands derived directly from the two parental types. In microorganisms several specific genes have been associated with general recombination. The *rec*$^+$ genes of *E. coli* facilitate recombinations in conjugation, P1 transduction and F interaction with the chromosome. Similarly the *red* genes of phage λ, and similar genes in phages T4 and P-22, are involved in promoting efficient generalized recombination. Some of these generalized recombination genes have identifiable functions. Both exonuclease and endonuclease activity are implicated in the action of the *E. coli rec*$^+$ genes; the *red*$^+$ gene of phage λ codes for an exonuclease. These functions are, of course, consistent with a process involving breaking of strands and there are plenty of available polymerases and polynucleotide ligases to account for strand rejoining. Little more can be said here about the mechanism and its specificity.

The most clearcut distinction between general and illegitimate recombination is the lack of dependence of the latter upon the bacterial *rec* genes and nucleotide sequence homology. In the main, illegitimate recombination has the effect of creating new DNA sequences around a point of fusion between different segments of a single genome or between nonmatching segments of different genomes. Thus a deletion can be viewed as an illegitimate recombination in that the loss of a DNA segment is necessarily accompanied by fusion of DNA ends to form new DNA sequences. Similarly, aberrant excision of F leading to an F-merogenote results in the fusion of segments of two nonmatching segments from different genomes. Illegitimate recombination seems to occur at a low frequency and depends upon DNA scissions, exchanges and DNA joining, which are relatively nonspecific compared with the precise pairing between complementary base sequences seen in general recombination.

It is already clear from the preceding chapters that recombinational mechanisms have played a significant role in the evolution of the genetic elements we call R-factors. The discovery that many R-factors are

composites of more than one replicon must suggest that illegitimate recombination has been operative. One would like to examine the juxtaposition of the fused replicons in the composite genome and inquire in a more general way about the role of illegitimate recombination in the creation of novel plasmid types. It is also important to try to study systematically the general recombination of R-factors to deduce their biological organization from the arrangement of functional determinants in relation to one another.

8.1 Attempts to prepare linkage maps by recombination between R-factors

In genetic analysis the function and arrangement of hereditary determinants are inferred from the study of mutants that are detectably abnormal. A linkage map showing the arrangement of mutant determinants in relation to one another can be deduced from the results of genetic crosses, on the assumption that recombination separates continuous segments of the chromosome. The earliest genetic pictures of R-factors came from transduction studies and were useful in establishing qualitative linkage relationships, but unfortunately they gave very little useful data on the detailed organization of R-factors (chapter 5). Subsequently there were several attempts, notably by Hashimoto and his collaborators, to obtain a linkage map by crossing pairs of mutant R-factors. Initially Hashimoto and Hirota isolated independent chloramphenicol-sensitive R-factor mutants by a penicillin screening method. In this method a small amount of chloramphenicol was added to a culture of R-Su-Sm-Cm-Tc-containing bacteria. The growth of bacteria containing Cm^s mutants of the R-factor was inhibited by the chloramphenicol but they were not immediately killed. The later addition of penicillin, which kills only growing cells, removed the majority of cells carrying wild-type R-factors, whereas the R-factor Cm^s mutant bacteria survived and could be easily isolated. Obviously this basic technique could be employed to obtain several different classes of drug-sensitive R-factor mutants. A series of Cm^s mutants of an R-factor were introduced, in pairs, into a single host by both conjugation and transduction and, indeed, it was possible to isolate Cm^r recombinant cells when such strains were plated on a medium containing chloramphenicol.

The difficulty with these experiments, however, was that the two mutant plasmids introduced into the bacterial strain were incompatible. Therefore linkage relationships were extraordinarily difficult to determine because of persistent heterogeneity, and the unequal footing of the superinfecting Cm^s plasmid relative to that of the resident Cm^s plasmid introduced a strong bias. Nonetheless two-factor crosses of pairs of Cm^s mutants showed a specific gene order relative to Tc and a determinant, *m*, which specified mating ability. Similarly several sites in the Cm locus were mapped with respect to Sm. A tentative linkage order Cm (Cm_1, Cm_2, Cm_9)-Sm and Tc-*m* fit the very complex recombination data best

but the authors, despite this valiant attempt, could only conclude that the linkage between Cm-Sm and Tc-*m* was in a very ambiguous state. An extension of this work by Hashimoto and Mitsuhashi employed Tc^s mutants isolated from Cm^s or Sm^s R-factor mutants, which were either m^+ or m^-. The same difficulty with incompatibility was experienced here but the data showed one additional very important point. The order of markers identified in the recombinant plasmids showed in part:

Tc_1-Tc_2-Cm-*m*
Cm-*m*-Tc_1-Tc_2 .

In both recombinant classes a similar linkage relationship appears between Cm and *m* but their position differs with respect to the Tc_1 locus in a striking way. In one case Tc_1 is an inside marker linked directly to the *m* locus; in the other, Tc_1 appears as an outside marker unlinked to *m* and more closely linked to Cm. Examination of further linkage data of this type led Hashimoto and Mitsuhashi to the conclusion that these data could not be explained by a single linear linkage map. Rather they contended that the order of the markers could be explained by assuming that the order was a circular permutation of a single-linkage order, and therefore the R-factor genome was genetically a circular structure. Of course it has already been emphasized that the R-factor DNA molecule is circular most of the time. It should be noted that recombination maps need not be topologically identical with the molecules that generate them. For example, circular molecules may have a linear recombination map if, as with phage λ, a crossover can occur anywhere, but the ring is always cut at a particular point during or after recombination.

Incompatibility has continued to effectively stifle studies of general recombination between isogenic pairs of R-factors. Recombination between compatible plasmid pairs of fi^+ and fi^- is not satisfactory since they rarely combine with one another because of fine structural differences (see below). It would seem fruitful to conduct recombination studies with a mutant R-factor that does not exhibit incompatibility but to my knowledge this has not yet been done. In a more general vein the same difficulty with incompatibility has nullified recombinational analysis of most plasmids. The situation is not totally bleak, however, for several experimental methods have been eminently successful in determining the genetic organization of plasmids and have directly demonstrated plasmid–plasmid relationships.

8.2 Complementation analysis of transfer-defective mutants of sex factors

Several workers have been concerned with the isolation and characterization of transfer-defective (Tra^-) mutants of F′, Hfr and R-factors. Generally such mutants are isolated by selecting for resistance to male-specific

phages or by treatment with a mutagen and subsequent screening for loss or reduction in genetic transfer. Experiments of this type by Eichi Ohtsubo *et al.* and Mark Achtman *et al.* reveal three general groups of transfer mutants on the basis of their response to male phages (table 8.1). Mutants of group 1 do not synthesize any F-pili and this alone seems sufficient to explain their lack of transfer. Group 1 mutants are resistant to all male phages, do not form mating pairs and, additionally, some members of this class do not exhibit surface exclusion. The group 2 mutants are resistant only to RNA male phage f_2 whereas group 3 mutants retain sensitivity to all male-specific phages. Both group 2 and group 3 mutants produce seemingly normal pili, form mating pairs, and display surface exclusion. These data underline the fact that sex-factor functions other than piliation are required for genetic transfer.

The genetic basis for the various phenotypes of the transfer-deficient mutants has been analysed by the fact that Tra^- mutants of F- and R-factors may be able to complement one another and restore transfer ability. Thus the introduction of an F-like R-factor into a Tra^- Hfr cell derivative restores the fertility of the cell, and subsequent chromosome transfer shows the same orientation of genes as the original Hfr strain. Since the F- and R-factors in this case remain distinct, it is probable that the restoration of transfer ability is mediated by diffusible cytoplasmic gene products rather than by recombination. Of course, since F- and R-factors are compatible, one need not worry about recipient bias nor the other problems which plagued workers studying incompatible plasmid pairs. Ohtsubo and his associates tested complementation between mutant Tra^- F- and mutant Tra^- R-factors by introducing both into the same cell by conjugation or transduction. Restoration of donor ability was evidence of complementation, whereas the failure to restore donor ability was evidence that the mutant pair were defective in the same functional group (cistron). Thus whenever a defective mutant of F, with a mutation in one cistron, coexisted with a defective mutant of R, with a mutation in a different cistron, complementation was observed not only for conjugal fertility of F and R but also in sensitivity to male-specific phages, the

Table 8.1. Classes of transfer-defective mutants of the F-factor. R, resistant, or S, sensitive to the specified male phage. + property present; − property absent. (Data from Ohtsubo *et al.*, 1970, and Achtman *et al.*, 1971.)

Mutant class	Transfer	Sensitivity to			Pili	Pair formation	Surface exclusion
		f2	β	f1			
Wild type	10^{-1}	S	S	S	+	+	+
1	$<10^{-6}$	R	R	R	−	−	+
1	$<10^{-6}$	R	R	R	−	−	−
2	$<10^{-6}$	R	S	S	+	+	+
3	$<10^{-6}$	S	S	S	+	+	+

formation of specific pili, and all other sex-factor-specific functions (table 8.2). Although no combination of defective F- and R-factors having a mutation in the same cistron exhibited complementation, such pairs produced a *rare* stable clone active in conjugal ability. These exceptional clones represented recombinant forms between F and R (see below). A second complementation system, employed by Achtman, Willetts and Clark, utilized transient populations of cells carrying two different Tra$^-$ F-*lac* mutants constructed by conjugation or by phage Pl transduction. The systems used by Ohtsubo *et al.* and Achtman *et al.* both measured the transfer abilities of cells carrying two different Tra$^-$ mutants, which allowed the mutations to be assigned to different complementation groups. The order (arrangement) of the cistrons identified in these experiments was accomplished by complementation between a series of Tra$^-$ F-*gal* deletion mutants and Tra$^-$ R100-1 mutants, or by complementation between Tra$^-$ F-*lac* mutants and a series of deletions extending different distances into chromosomally-inserted F.

These experiments, taken together, have identified twelve cistrons required for F transfer. The order of these genes on the F-*lac*$^+$ chromosome have been found to be: Origin (of transfer) ... *tra* J *tra* A *tra* L *tra* E *tra* K *tra* B *tra* C *tra* F *tra* H *tra* G *tra* S *tra* D *tra* I ... *lac*$^+$. As has been noted earlier for bacteriophage (chapter 2) this map shows a clustering of related functions. All mutants in *tra* A, L, E, K, B, C, F, H and some in *tra* G were resistant to all F-specific phages and lacked the F pilus. Thus nine of the twelve cistrons are presumably concerned with the synthesis, modification, and assembly of a phosphorylated and glucoylated single protein subunit into the F pilus. All mutants in *tra* I and *tra* D, as well as some in *tra* G, still synthesized the F pilus and were sensitive to

Table 8.2. Complementation between defective F- and R-factors. Hfr Hayes transfers its chromosome with the orientation *ara*$^+$-*lac*$^+$-*gal*$^+$ at the frequency indicated in line 1. A Tra$^-$ mutant of Hfr Hayes, line 2, shows no gene transfer nor gradient of transfer. The de-repressed F-like R-factor R-100 (R-Su-Sm-Tc-Cm) shows little transfer of chromosomal genes but it transfers Cm at high frequency, line 3. The Tra$^-$ mutant of R-100 shows virtually no transfer activity (line 4). A cell carrying both Tra$^-$ mutants of F and R shows a high degree of restoration for both F and R specific transfer activity (line 5).

		Transfer of genes to F$^-$ recipient cells			
		ara$^+$	*lac*$^+$	*gal*$^+$	Cm
1	Hfr Hayes	8×10^{-2}	4×10^{-2}	2×10^{-2}	-
2	Hfr Hayes Tra$^-$ mutant	$<10^{-7}$	$<10^{-7}$	$<10^{-7}$	-
3	R-*drd*	$<10^{-6}$	$<10^{-6}$	$<10^{-6}$	5×10^{-2}
4	R-*drd* Tra$^-$ mutant	$<10^{-6}$	$<10^{-6}$	$<10^{-6}$	$<10^{-7}$
5	Hfr Hayes Tra$^-$ +R-*drd* Tra$^-$	1×10^{-3}	4×10^{-4}	3×10^{-4}	4×10^{-3}

all F-specific phage (except that RNA phage can absorb to, but not infect, *tra* D^- mutants). Piliated mutants in these three cistrons form normal mating pairs and there has been speculation that their gene products are concerned with the DNA transfer mechanism at conjugation. Since *tra* G mutations can affect both pilus synthesis and DNA transfer (?) it may be bifunctional. Mutations in *tra* S abolished surface exclusion but not incompatibility; the precise mechanism by which the *tra* S product reduces pair formation between two F-containing cells remains unknown. Mutations in *tra* J, located at one end of the Tra region, abolishes F-pilus synthesis and surface exclusion. Since *tra* J is the target of transfer inhibition by *fi*$^+$ plasmids, it seems likely that it plays the role of a control element, with the *tra* J product acting as an inducer for all other *tra* genes or for a particular block of genes.

The I-like plasmids, R64 and Col Ib, do not complement any Tra^- F mutants and therefore have genetically unrelated transfer systems. Two F-like plasmids, Col V2 and Col VB *trp*, fully complement mutants in any of the twelve Tra cistrons of F and so probably have essentially identical systems. The F-like R-factors R100 and R1 fully complement mutations in ten of the twelve *tra* cistrons, the exceptions being *tra* I and *tra* J (the control cistron). Presumably the products of these two cistrons are plasmid specific. Although these *fi*$^+$ R-factors can complement F for missing functions in the ten other Tra genes, there are noticeable differences in specificity. For example, an *fi*$^+$ R-factor cannot restore full RNA phage sensitivity to an F *tra* A^- mutant. This is not a totally unexpected finding since the pili of the F-factor and F-like R-factor may be distinguished by serological and other analytical procedures (see chapter 3). Similarly to F, the F-like R-factors Col V2 and Col VB *trp* have related surface exclusion genes, *tra* S, although each retains its own degree of specificity. The Tra^- mutants of transfer plasmids hold great promise in terms of working out the phenomenon of mobilization of nontransmissible plasmids. F can mobilize Col E1 as well as Su–Sm and other nontransmissible drug-resistance replicons. Col E1 is transferred at high frequency by F-*tra* I mutants even though the F-factor itself cannot be transferred. Mutants in other Tra cistrons, even those retaining the capacity to synthesize F pili, cannot mobilize Col E1. These preliminary data reenforce the view that covalent linkage is not required for plasmid mobilization, and the mobilization phenomenon requires more than simply a random access to a recipient cell.

This very nice research is still in its embryonic stages, but it does seem, at last, that the genetic analysis of plasmid function has been launched. The availability of these mutant classes should permit a firmer grasp of the functional basis of piliation, specific pair formation, surface exclusion, and other functions related to donor ability. The studies reported above are particularly useful in pointing out both the basic functional similarity and yet the degree of individuality retained by both F and *fi*$^+$ R-factor plasmids.

In the following section a physical basis of this functional similarity is also revealed.

8.3 Homology between plasmids

Obviously F-factors and F-like R-factors have sufficient functional similarity that their gene products may correct a mutational defect in transfer in one another. It is just as obvious that an F-factor and an R-factor, although functionally related, are not equivalent structures, if for no other reason than that one carries drug-resistance genes. The functional similarity between F and R is also reflected by their ability to undergo genetic recombination. Several laboratories have reported the isolation of F–R genetic recombinants, which are transferred and transduced as a single unit, and are eliminated simultaneously by curing agents. In the main, investigators have utilized strains carrying Tra^- R mutants or defective P-22 transductants of R-factors as hosts for F or F-merogenotes (usually F–*lac*$^+$). Where they have been searched for, invariably F–R or F–*lac*$^+$ R recombinant plasmids have been found, albeit they are generated at a rather low frequency (about 10^{-6} per mating). Once formed, however, they are reasonably stable and act as efficiently as the wild-type factors. Both structures are usually not conserved. In many instances only one or a few genes of one of the partners is to be found in the recombinant structure. Thus an F-Tc factor may contain no other R-factor genes than Tc, or an R–*lac*$^+$ structure contains only *lac*$^+$ genes, but little else that is recognizable from F. In other cases large segments of both structures may be found in the single recombinant structure. It is interesting to note here that June Pitt and David Smith have found an R-factor carrying *lac*$^+$ determinants in a clinical isolate. It is presumed that this element represents the recombinational product of two plasmids or a plasmid chromosome interaction, so that one should not consider these recombination experiments as simply laboratory tricks without natural counterparts.

The molecular basis for these recombinational events, as well as others to be considered in this chapter, is currently unknown but it involves several basic questions. Clearly the base sequence homology of the plasmid partners and the resulting recombinant structure and their relative sizes and topology are important parameters. If there is some similarity in genetic fine structure, one can consider that general recombination at rather high frequency may be a reasonable mechanism; if two plasmids (which show little similarity in nucleotide sequence) recombine, then perhaps one is more justified in concluding that illegitimate recombination is operative. Similarity in fine structure can be a matter of simple probability. Identical runs of up to seventeen nucleotide pairs may appear on any two DNA structures by chance alone; but obviously the longer a stretch of overall homology between two molecules the higher the probability of a successful crossover. The actual size of two

interacting plasmid pairs will also be critical. To be sure, it can be assumed that most interacting plasmid pairs will be circular structures most of the time. Yet if the two structures have large regions of nonhomology or are grossly unequal in size then undoubtedly pairing difficulties can arise. A direct assessment of the nucleotide sequence similarity between heterogenic plasmids can be made by DNA–DNA reassociation studies. The basis of these experiments is simple denaturation and renaturation of DNA mixtures. Under the experimental conditions one can measure the formation (if any) of heteroduplex molecules in which each strand is derived from a different plasmid. Table 8.3 shows a limited set of such nucleotide-sequence-relationship studies between representative plasmids of several compatibility groups.

Table 8.3. Nucleotide-sequence relationships among R-factors and other plasmids. In each case ^{3}H-labelled denatured plasmid DNA was permitted to reassociate with unlabelled denatured DNA of the indicated plasmid. The degree of duplex formation for the homologous system was set as 100% and all other reactions were calculated relative to this. Under the conditions of these experiments, the measured duplex may contain about 5% mismatched, that is noncomplementary, base pairs. (Unpublished observations from S. Falkow, P. Guerry, R. W. Hedges, and N. Datta, 1972–1974.)

Unlabelled R-factor DNA	C.G.[a]	M.W.[b]	Mol. fraction G + C	No. of copies per cell	Extent of DNA–DNA duplex formation with [^{3}H]thymine labelled DNA of:					
					R144	R1	N-3	RP4	S-a	R724
F	FI	62	0·48	1–3	–[c]	39	–	–	–	–
R1 (Su-Sm-Cm-Ap-Km)	FII	65	0·51	1–3	6	100	5	9	14	10
222 (Su-Sm-Cm-Tc)	FII	78	0·51	1–3	–	82	–	–	–	–
R144 (Tc-Km)	Iα	63	0·50	1–3	100	14	2	1	2	28
R64 (Tc-Sm)	Iα	72	0·50	1–3	78	–	–	–	–	35
Col Ib—P9	Iα	65	0·50	1–3	81	8	3	1	2	–
R62 (Su-Sm-Ap)	Iα	81	0·50	1–3	90	8	60	1	1	–
R483 (Sm-Tp)	Iβ	55	0·50	1–3	66	–	–	–	–	35
R724 (Su-Sm-Tc-Cm)	O	58	–	1–3	20	–	–	–	–	100
R16 (Su-Sm-Tc-Ap)	O	69	–	1–3	16	–	–	–	–	96
S-a (Su-Sm-Cm-Km)	W	25	0·62	3–5	5	4	8	4	100	6
R388 (Su-Tp)	W	21	0·62	3–5	–	–	–	–	79	–
R7K (Su-Sm-Ap)	W	24	0·62	3–5	–	–	–	–	75	–
N-3 (Su-Sm-Tc)	N	32	0·49	1–3	2	8	100	1	10	8
R15 (Su-Sm)	N	40	0·49	1–3	–	–	75	–	–	–
RP4 (Tc-Km-Ap)	P	34	0·58	1–3	3	–	4	100	3	4
R6K (Ap-Sm)	X	25	0·45	15–20	1	8	1	2	1	4

[a] C.G. = compatibility group.
[b] M.W. = 10^{6} × mol.wt.
[c] – = not done.

In general it can be seen that R-factors and other plasmids of the same compatibility group are similar in their size, mol fraction G + C, number of copies per cell, and share a high proportion of their nucleotide sequences in common. Representative plasmids of different compatibility groups share relatively few polynucleotide sequences in common. Thus, for example, the *fi*⁻ W-plasmids react at a high level with each other, but at only a very low level with the *fi*⁻ N, I, P, X, or *fi*⁺ FI and FII plasmid groups—and so on for each of the general classes of compatibility groups. One can only conclude that this reflects profound differences in overall genetic structure, consistent with distinct phylogenetic origins. These results therefore confirm the utility of the use of incompatibility as a means of plasmid classification. Of course exceptions do occur. The R-factor, R62, for example, makes I-pili and is of the I compatibility group; but it is somewhat larger and, unlike most I plasmids, is *fi*⁺ and confers resistance to Ap. The *fi*⁺ phenotype has been found to occur by a mechanism unrelated to that of F-like plasmids, and the polynucleotide-sequence studies reveal that R62 contains both I-like and N-like sequences. R62 can probably be best considered as the product of an illegitimate recombinational event between an I-like and N-like plasmid. Similarly members of the O compatibility group (which do not make detectable I pili) and plasmids of the I group consistently share about 20–35% of their sequences in common. Although O plasmids and I plasmids can usually coexist within the same cell, at least one I plasmid has been recently identified which is genetically incompatible with members of the O group. These genetic and molecular findings suggest, therefore, that the O and I group plasmids share some degree of common ancestry.

These sequence-relationship studies also provide an interesting insight into the evolution of compatibility differences between plasmids. Plasmids determining F-pili constitute at least four distinct compatibility groups, and every F-pilus-producing plasmid tested so far has fallen unambiguously into one or other of these compatibility groups. Simiarly plasmids which determine I-pili show a variety of intricate compatibility relationships and can also be subdivided into different compatibility groups. It can be seen, however, that members of one distinct compatibility group can still share a significant proportion of their sequences in common with members of other groups which determine similar transfer pili. The reasonable conclusion is that these regions of similar polynucleotide sequences are due to phylogenetic homology between genes governing transfer functions, while the genes governing incompatibility may be either entirely unrelated or have perhaps diverged from a common ancestor. A firmer structural basis for this conclusion is presented in the next section of this chapter. In summary, the general rule is that plasmids of the same compatibility group have a significant proportion of their polynucleotide sequences

in common, whereas plasmids of different groups share few sequences in common. The major exception is that any set of compatibility groups whose members determine similar pili may be phylogenetically related at least with respect to the DNA sequences involved in transfer. The other exceptions which occur, such as R62 and the O plasmids (and I am sure many more will be found), often provide evidence of R-factor interactions or a view of pathways of R-factor evolution. One should also not overlook the fact that, despite apparent differences in phylogenetic origin, size, and even in the mol fraction of G + C, virtually all plasmids show some minimal level of nucleotide-sequence relationship with one another. In most cases these levels are of the order of 6–8% (usually equivalent to about 1×10^6–5×10^6 daltons). The significance of these low levels of nucleotide-sequence homology is unknown, although they are reproducible and seem too large to represent just regions of fortuitous matching. Do they represent an evolutionary thread of conserved sequences common to all extrachromosomal elements of enteric bacteria? Do they reflect a current low level of genetic exchange between heterogenic plasmids? Do they possibly reflect that the drug-resistance genes are often common? These low levels of similarity should not be ignored; they may yet provide important information on plasmid behaviour and plasmid–plasmid interactions (see chapter 9 on the origins of drug-resistance genes in this regard).

Relatively few polynucleotide-sequence studies have been carried out with the nontransmissible drug-resistance determinants. In my laboratory we have not been able to detect any significant degree of sequence similarity between Su–Sm, RSF1030 (Ap); Tc or Col E1. Among the Col E1; Col E2; Col E3 complex itself, it has been found that Col E2 and Col E3 share about 80% of their sequences in common but are only slightly related (less than 18%) to Col E1. Peter Barth and Nigel Grinter (unpublished observations) have recently examined twelve representative nontransmissible Su–Sm plasmids isolated from a wide taxonomic and geographical range. Nine of these twelve plasmids were about $5{\cdot}7 \times 10^6$ daltons in size, while the other three were $6{\cdot}3 \times 10^6$, $7{\cdot}4 \times 10^6$, and $9{\cdot}2 \times 10^6$ daltons respectively. All were found as multicopy gene pools. The DNA relationships between these plasmids revealed that ten of the twelve Su–Sm plasmids shared 80–93% of their sequences in common with a labelled reference Su–Sm plasmid, while the $9{\cdot}2 \times 10^6$ daltons plasmid showed 45% homology and the $7{\cdot}2 \times 10^6$ daltons plasmid showed 35% homology. The results are described analogously as the relationship between ten siblings and two cousins. Barth and Grinter suggest that the Su–Sm plasmid evolved once and has spread efficiently, with relatively few modifications, around the world. The data for transmissible plasmids, with respect to geographical and taxonomic range, are also similar. That is, virtually all FII R-factors that have been examined show at least 70% sequence similarly regardless of the geographical source or the strain of isolation.

Whether this reflects the dissemination of a single ancestral plasmid, or independent evolutionary events occurring in response to antibiotic selection, remains to be seen.

The measurement of total nucleotide sequence similarity between heterogenic plasmids is useful in a general way in specifying those plasmids most likely to recombine or those sharing similar origins. By the judicious choice of genetic variants, particularly deletion mutants, one may also specify in a general way the nature of shared sequences between plasmids. For example, *fi*$^+$ R-factors show about 40% heteroduplex formation with the classical F-factor. Does this relationship include any of the DNA associated with the drug-resistance replicon? If one employs only the transfer part (RTF) of an *fi*$^+$ R-factor in DNA-binding experiments, the answer is clear. The relative degree of shared sequences between F and RTF is not decreased as would be the case if some of the shared sequences were part of the drug-resistance replicon.

Although the measurements of total sequences held in common give useful and interesting information concerning the genetic organization and fine structure of plasmids, the method suffers from a major limitation. The data do not permit one to know whether the shared sequences are continuous regions of 'gene blocs' or are scattered over the genome. This limitation has been largely removed, however, by directly examining heteroduplex molecules in the electron microscope.

8.4 Electron-microscope heteroduplex studies of sequence relationships among F, R and other plasmids

In heteroduplex experiments a mixture of two related DNA species, AA′ and BB′, is denatured to separate the complementary strands and then renatured to permit the strands to re-form. The resulting renatured mixture contains occasional unchanged single-stranded molecules, re-formed parental homoduplex molecules AA′ and BB′, and the molecules of interest, heteroduplexes AB′ and A′B. Such molecular mixtures are spread on a basic protein film containing a concentration of formamide that is mildly denaturing. The film is picked up on grids, shadow cast, and examined in the electron microscope. Under these conditions of mounting, both single- and double-stranded DNA appear in the electron microscope as somewhat extended filaments, but the single strands are usually perceptibly thinner and more 'kinky' than the double strands. Thus regions of homology in a heteroduplex appear double-stranded whereas regions of nonhomology appear single-stranded. Not only can one determine the position of a nonhomologous region in the heteroduplex but, additionally, one can often deduce the nature of the genetic modification causing the nonhomology. For example, a single-stranded loop emanating from a point in a duplex and returning to the same point is due either to an insertion or a deletion of a sequence from one of the two DNA molecules (I–D loop, figure 8.1a). Similarly if

regions of the DNA in question are totally nonhomologous, the region is clearly visible as an unpaired region or so-called 'bubble' of unpaired single strands bridging a gap between double-stranded DNA regions (substitution loop, figure 8.1b).

The general principles of the electron-microscope heteroduplex method have been applied to F-, F′-, and R-factor molecules. Mixtures of 'nicked' ring forms are denatured into single-stranded linear and single-stranded circular forms and allowed to renature. Because of topological restraints, two complementary circular strands cannot renature with each other but a linear strand and a circular strand can renature. Complementary linear strands, resulting from random nicking of ring molecules, can also renature and will most probably re-form a ring structure, so long as the two breaks are not close to each other. As might be expected, the number of measurable complete molecules visible on an electron-microscope grid is usually small. These complete heteroduplexes serve, however, to define the overall structure clearly. Length measurements on different single- or double-stranded segments of partial or broken heteroduplex molecules, which occur with a much higher frequency, can be used with confidence to accumulate sufficient data for good statistics on the length of a given feature.

Phillip Sharp, M. Hsu, E. Ohtsubo and Norman Davidson have studied the structure and sequence relationships of heteroduplexes between F- and various F′- factors. It can be immediately appreciated that a heteroduplex molecule formed between an F-factor and an F-factor carrying some segment of the bacterial chromosome will have a region of nonhomology corresponding to the bacterial genes. This feature not only permits one to

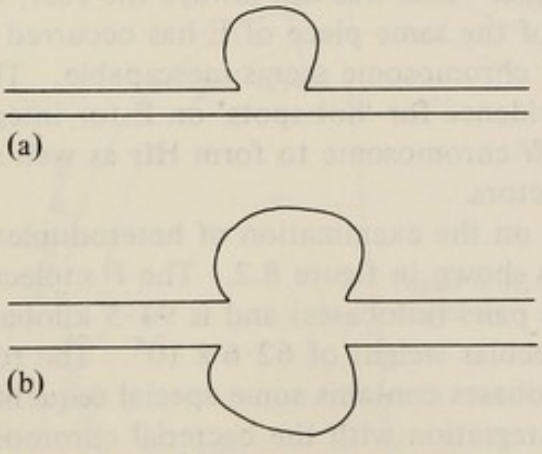

Figure 8.1. Representation of structures seen in heteroduplex molecules. (a) An insertion-deletion (I-D) loop. This type of loop is seen when one complete DNA strand forms a duplex with a DNA strand which is homologus except for an insertion or a deletion. In the case of an insertion, the extra DNA is 'looped out'. In the case of a deletion, the DNA on the complete strand corresponding to the deleted region is 'looped out'. (b) A substitution loop. This loop or 'bubble' is seen when two segments of DNA are nonhomologous. In both cases the loops are composed of single-stranded DNA along double-stranded regions of homology.

have a point of reference when looking at a circular molecule but, additionally, it becomes possible to answer some questions about the recombinational mechanism leading to the formation of the F′-factor. It may be recalled from chapter 2 that F′-factors are formed by the aberrant excision of F DNA from the chromosome of an Hfr bacterium, leading to the subsequent formation of a circular DNA molecule containing the essential genes of F and some bacterial sequences. Two types of aberrant excision have been postulated. It is thought that a type 1 excision results from an illegitimate recombination between an F sequence and a bacterial sequence. The resulting F′-factor will therefore be missing a piece of F and carry either 'early' or 'late' bacterial genes with respect to the origin of transfer by the parental Hfr type. A type 2 excision is visualized as the illegitimate recombination between bacterial sequences on both sides of F. The resulting F′-factor contains all F genes and both 'early' and 'late' bacterial genes. In terms of an F–F′ heteroduplex structure, if the F′-factor was formed by a type 2 process then all F genes are present, and the bacterial genes will be visualized as a region of nonhomology corresponding to an I–D loop similar to the one depicted in figure 8.1a. In contrast, if the F′-factor was formed by a type 1 excision process, the heteroduplex with F will show a substitution loop (figure 8.1b) because some of the F sequences are missing. All of the F′-factors examined by Sharp and his associates were found to be missing a piece of F corresponding to 3–8% of the total molecule. They appear therefore to have been formed from their respective Hfr species by a type 1 excision process. Of particular interest was the finding that the same piece of F was missing in F′-factors derived from different Hfr species in which F had integrated at quite different points on the *E. coli* chromosome. This was not always the case, but the interpretation that integration of the same piece of F has occurred at different points in the bacterial chromosome seems inescapable. Thus these and other findings give evidence for 'hot spots' or. F for integration at different places on the *E. coli* chromosome to form Hfr as well as 'hot spots' for excision to give F′-factors.

A general model for F based on the examination of heteroduplex molecules and genetic studies is shown in figure 8.2. The F molecule is divided into units of 1000 base pairs (kilobases) and is 94·5 kilobases in length, corresponding to a molecular weight of $62{\cdot}6 \times 10^6$. The region of F in the interval of 0–17·6 kilobases contains some special sequences which are the 'hot spots' for integration with the bacterial chromosome mentioned above. This region may be considered 'nonessential' in the sense that segments of it can be deleted without affecting genetic transfer, autonomous replication, or plasmid maintenance. Moving clockwise we find that the segment of F between 17·6–43 kilobases remains largely unknown in terms of function but also appears to be 'nonessential'. The evidence for this conclusion is the occurrence of various F-factors and/or deletion mutants of F which are capable of autonomous replication and

complete fertility. Ming-Ta Hsu has recently found that the region from 0–3 kilobases contains most of adenine + thymine (A + T) sequences of F. Thus, as was the case for phage λ (chapter 2), there seems to be a structural differentiation of F into relatively (A + T)-rich and (G + C)-rich regions. Despite the 'nonessentiality' of the 0–42·9 region, the interval 32·6–42·9 kilobases contains genetic determinants which confer upon host cells resistance to the phages T3, T7, and φII. These sequences may not be essential in the sense of replication or fertility, but it can be seen that they may confer considerable selective advantages.

The Tra genes of F lie in the interval between 62–94·5/0 kilobases. The *tra* F, C, H, G, S, D, I cistrons lie in the 72·9–94·5 kilobase interval, while the *tra* J A L E K B cistrons lie in the 62–72·9 kilobase interval. Since the Tra region may be deleted from F without affecting its autonomous replication, it seems likely (more or less by default) that the segment from 43–62 kilobases (about 20% of the F molecule) contains the genes for autonomous replication as well as those which govern incompatibility. The region between 50 and 94·5 kilobases is of considerable interest to us because it defines the region of DNA homology seen in heteroduplexes of F and fi^+ R-factors, as well as of F and the fi^+ Col V-K-94 plasmid. A heteroduplex molecule between F and an fi^+ R-factor is shown in plate 6. Aside from a few substitution and I–D loops, it is clear that the homology between F and R is limited to continuous stretch of DNA corresponding to about 44% of the molecular length of F.

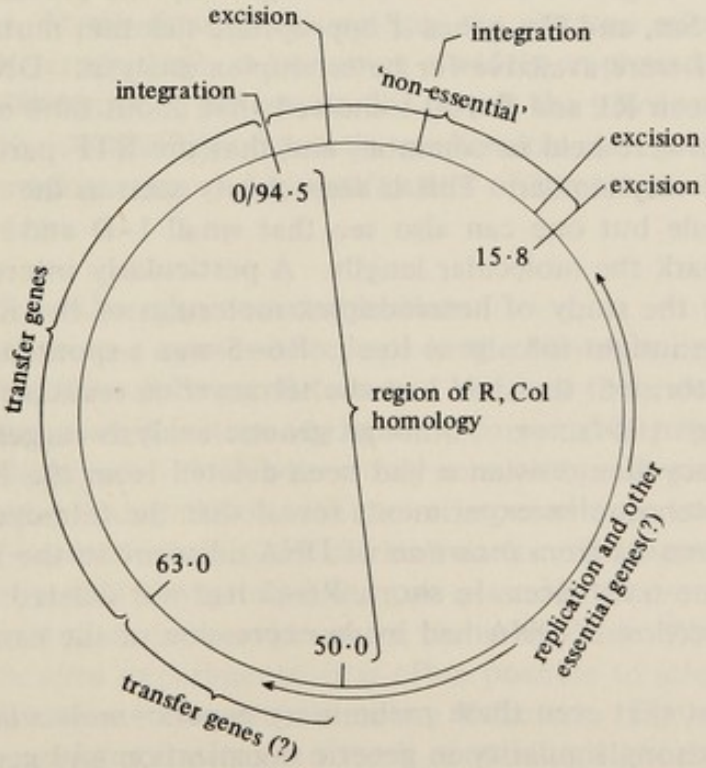

Figure 8.2. Model of F based on heteroduplex studies. The numbers represent units of 1000 bases or base pairs (kilobases). (Adapted from Sharp *et al.*, 1972.)

These data therefore directly establish the inference from genetic analysis and DNA-binding studies that the Tra genes of F and R are very closely related and that the homology may also extend, in part, into the regions thought to be concerned with autonomous replication and incompatibility.

Sharp, Cohen and Davidson have begun an analysis of R-factor heteroduplex molecules. Plate 7, for example, shows a large deletion loop formed in a heteroduplex of an RTF and its parental R-factor. This deletion loop defines the contiguous region occupied by the drug-resistance replicon within the composite R-factor molecule. The double-stranded duplex region defines RTF and contains all of the DNA sequences in the R-factor homologous to F. Plate 8 shows a heteroduplex molecule formed between the R-factor R1 (R-Su-Sm-Cm-Km-Ap) isolated in England, and the R-factor R-100-1 (R-Su-Sm-Tc-Cm) isolated in Japan. This heteroduplex molecule reveals several features of note. To begin with, it can be seen that regions of homology extend over the total length of the molecule. The loop denoted by 'a' probably contains the Ap and Km drug markers of R1, and the loop denoted by 'b' contains the Tc marker of R-100-1. The DNA between 'a' and 'c' contains the genetic material coding for resistance to sulphonamides, streptomycin, and chloramphenicol, common to both plasmids. The 'a–c' segment is seen to be double-stranded, except for one obvious I–D loop, and is completely shared by both plasmids. Since the 'a–c' region is clearly bordered by two readily identifiable loops ('a' and 'b'), the reader can probably imagine how relatively easy it might be to locate unambiguously the precise positions of the Su, Sm, and Cm genes if appropriate deletion mutants of either R1 or R-100-1 were available for heteroduplex analysis. DNA-binding studies between R1 and R-100-1 showed that about 80% of nucleotide sequences were held in common, and that the RTF parts of both molecules were very similar. This is very clearly seen in the heteroduplex molecule but one can also see that small I–D and substitution loops mark the molecular length. A particularly interesting discovery made with the study of heteroduplex molecules of the R-factor R6–5 gives a striking insight into 'gene loss'. R6–5 was a spontaneous mutant of the R-factor, R6, that had lost the tetracycline resistance expressed by the parent R-factor. Although genetic analysis suggested the information for tetracycline resistance had been deleted from the R6 chromosome, the heteroduplex experiments reveal that the tetracycline sensitivity of R6–5 results from *insertion* of DNA adjacent to the region specifying tetracycline resistance. In short, R6–5 had not deleted the Tc genes, but rather insertion of DNA had made expression of the carried Tc genes impossible.

It seems significant that even these preliminary genetic–molecular maps of F and R show a strong similarity in genetic organization and gene clustering rather reminiscent of phage λ and other temperate phages.

Perhaps the most distinctive feature of the heteroduplex data thus far is that three *fi*$^+$ plasmids, F, R, and Col V-K-94 are virtually totally *nonhomologous* except for their transfer genes and possibly a short segment concerned with replication function. Thus if one were to examine the homology between an F-like R plasmid and a mutant of F which had deleted the Tra region, the inescapable conclusion would be that the two plasmids were largely unrelated. The biological significance of this is still a moot point, but one can suppose that it may indicate a tendency for plasmids to more strongly conserve pili and transfer genes (that is sexuality) rather than other nucleotide sequences. But what of the surprisingly large segments of dispensable genetic information, the 'nonessential' regions? Apparently these regions provide a certain amount of genetic plasticity that can be expended to acquire determinants which confer different degrees of selective advantage. In this respect there is a major difference in the nucleotide sequences of F and R that is not apparent from electron microscope heteroduplex analysis, but is seen in DNA–DNA binding studies. That is to say, F shares about 35% of its nucleotide sequences in common with the *E. coli* host chromosome, whereas *fi*$^+$ R-factors at best share only 10–15% of their sequences with *E. coli*. This finding will be examined in more detail later on, but for the time being one may wish to consider that the 'nonessential' regions of F reflect an accumulation of bacterial (*E. coli*-like) sequences, while this is not the case for RTF which has accumulated 'nonessential' drug-resistance genes.

At the time of this writing, the electron microscopic mapping of heteroduplexes is becoming a widely-employed experimental tool. Obviously it will eventually permit the physical location of many genes, the unravelling of genetic mechanisms and, perhaps most important, the confirmation and quantification of hypothesis based on genetic and other data.

8.5 Heterogenic recombination

There are numerous reports in the literature concerning recombination between different R-factors. Many of these deal with recombination between R-factors of the same compatibility type (highly homologous) under conditions where only a recombinant plasmid could survive the selective conditions that were imposed experimentally. In the main, however, when a cell carries two (or more) plasmids of different compatibility groups, the two plasmids retain their biological individuality and there is no evidence of recombination. Nonetheless, in well designed *in vitro* experiments, it is often possible to select for recombinant plasmids between two quite distinctive R-factors. For example, T. Yokota and his associates constructed a cell containing the naturally occurring *fi*$^-$ R-factor Rts-1 (T compatibility group, Km) and the *fi*$^+$ R-factor R100 (Su-Sm-Tc-Cm).

Under certain conditions, recombinant R-factors carrying Km-Su-Sm were isolated which retained all of the other properties of Rts-1. Hence *fi*$^+$ R-factors can recombine with *fi*$^-$ R-factors, and the expected genetic and molecular barriers between R-factors of different compatibility groups need not be operative. Insofar as I can tell, virtually all the reports of R-factor recombination that have been given revolve around the drug-resistance genes and not the transfer or replication regions of the two interacting molecular species. The only exception to this rule is seen when recombination between two R-factors of the same compatibility group takes place, and in which there is a high degree of homology existing between the Tra and replication genes. The impression gained from reading papers on laboratory-constructed recombinant types, and recombinants obtained from plasmids of widely divergent origin, is that most of the time recombination is observed between nonessential regions of the plasmid molecules, but that the essential regions of both the parental partners remain intact. This is also reflected in R-factors isolated from nature which have the properties of recombinant plasmids. The R62 plasmid, as noted above, has gene sequences which can be traced to both the I and N compatibility groups. Yet in this case both the molecular and genetic data indicate that all of the transfer, replicative, and incompatibility functions of the plasmid are I-like, while the DNA segment carrying the drug-resistance genes is N-like. There can be no doubt that heterogenic recombination occurs since there are several reported examples of R-*lac*$^+$ plasmids as well as of plasmids conferring drug resistance and other physiological traits (for example, H_2S production, raffinose fermentation).

The most interesting and instructive example of the recombinational gymnastics possible between heterogenic plasmids comes from the laboratory of Pierre Fredericq. Fredericq started with a Col B plasmid which had, by illegitimate recombination, acquired some chromosomal determinants of *E. coli* K-12. The chromosomal genes carried by Col B included: *ton* B, a gene controlling resistance to phage T1; *trp*, a group of five cistrons determining the enzymes of tryptophan biosynthesis; att_{80}, the attachment site of the lamboid phage ϕ80; *cys* B, a gene employed in cysteine biosynthesis. This plasmid (Col B *trp*, for short) was the equivalent of an F-merogenote. It was transduced and conjugally transferred into a series of recipients carrying different plasmid types. All in all, Fredericq was able to select from an assortment of recombinant-plasmid types resulting from sequential interaction between Col B *trp*, F-*lac*$^+$, Col V, and an R-Tc-Sm-Cm! Table 8.4 lists the genotypes of some of the recombinant types selected in these studies. The largest hybrid plasmid (Tc Col B *ton* B att_{80} *trp cys* B Col V Sm Cm) has been genetically mapped and is a circular element.

These studies by Fredericq show the full genetic potential of heterogenic recombination. Also, the data confirm and broaden the functional and molecular pictures, which show that Col factors, F-factors, and R-factors belong to the same general class of genetic elements.

The only missing link is phage, and I cannot resist the temptation to note that the Col B *trp* plasmid, and many of its recombinant derivatives which carry att_{80}, can be lysogenized by phage ϕ80. Thus a temperate phage can lysogenize a plasmid chromosome as well as a host chromosome. When such a lysogenized plasmid chromosome is transferred by conjugation to a sensitive recipient there is zygotic induction and the release of mature phage ϕ80. This is by no means the only phage-transmissible plasmid interaction that has been described. Eiko Kondo and S. Mitsuhashi were able to isolate a recombinant plasmid from phage P1 and an R-factor. This resulting recombinant phage, P1CM, retained all essential vegetative phage functions but additionally conferred chloramphenicol resistance upon every cell it lysogenized. Genetic analysis of P1CM suggests that a 'nonessential' region of P1 has been deleted and replaced by a 56% G + C, 2×10^6–3×10^6 dalton segment of R-factor DNA, which includes the chloramphenicol-resistance determinants. A similar P1 derivative P1*dl*, in which the identical section of P1 has been deleted and replaced by chromosomal *lac*$^+$ genes, can also be employed to detect transmissible plasmid–phage interactions. For example, if one infects a strain bearing P1*dl* with F or an R-factor, subsequent conjugal transfer and selection for *lac*$^+$ and/or drug resistance occasionally leads to the isolation of a recombinant plasmid which carries *lac*$^+$ and recognizable phage genes. Thus one can weld a recombinational link encompassing phage, F-factors, Col factors, R-factors, and nontransmissible plasmids.

Obviously we need to know a great deal more about plasmid recombination. There have been severe technical restrictions to genetic analysis and only the past few years have seen the development of approaches to the problems on the molecular level. The availability of DNA-binding methods and, particularly, the examination of heteroduplexes

Table 8.4. An assortment of recombinant plasmids derived from Col B *trp*, Col 6, R-(Tc-Cm-Sm), and F-*lac*$^+$. (Modified from Fredericq, 1969.)

Sm	Cm	Tc	Col B	*ton* B	*trp*	*cys* B	Col V	*lac*
+	+	+	+	+	+	+	+	−
+	+	+	+	−	−	−	+	−
+	+	+	+	−	−	−	−	−
+	+	+	−	−	−	−	+	−
−	−	+	+	+	+	+	+	−
−	−	+	−	+	+	+	+	−
−	−	+	−	+	+	−	−	+
−	−	+	+	−	−	−	−	+
−	−	+	−	−	−	−	−	+

in the electron microscope are most encouraging. These methods should prove useful not only from the standpoint of specifically understanding the nature of plasmid recombinant classes but, more significantly, from the standpoint of understanding recombination *per se*. Undoubtedly a systematic genetic and molecular analysis of the parental and issuing recombinant plasmid types should be a most rewarding system for revealing the broad outlines of the steps of recombination. As noted by many authors, the full understanding of recombination will probably have to await the achievement of the reaction *in vitro*, where intermediates and enzymes can be more thoroughly characterized. When the eventuality does become fact, one may presume that plasmids will represent an ideal model system to employ. For the time being, however, we may look at the existing data on heterogenic plasmid recombination as an insight to the potentiality for the creation of novel genotypes within the extrachromosomal gene pool of bacterial populations. The data certainly have evolutionary implications, which are agreeable mental diversions and, in addition, immediately present a reasonable way to think about the accretion of diverse genes into a single-linkage group.

8.6 R-factors and the host chromosome

The only plasmids known to integrate into the host chromosome at a detectable frequency are F, Col V, Col B, and most temperate phages. Integration crossover, like the plasmid recombination mechanism just discussed, may occur by either legitimate or illegitimate recombination. The coliphage λ elaborates a very specific enzyme catalysing the integration reaction and, indeed, can integrate into a *rec*$^-$ host (illegitimate recombination). F, on the other hand, presumably requires a host enzyme system for integration (legitimate or general recombination), since Hfr cells are very rare in F-infected *rec*$^-$ populations. A basic inference of integration into the chromosome is that the plasmid and chromosome share some phylogenetic ancestry. In fact DNA-binding studies (table 8.5) show that both phage λ and F, the classical examples, do indeed share base-sequence homology with an *E. coli* K-12 host. About one-third of the base sequences of phage λ are homologous with *E. coli* K-12. This homology is imperfect in the sense that perfect complementarity of base sequence is ordinarily not found, but probably no more than 13% of the sequences are mismatched. One might suspect that the homology between phage λ and the host might be restricted to a contiguous region solely associated with integration capabilities. As pointed out earlier (chapter 3), this is not the case, however, the sequences involved in integration of phage λ probably span less than two dozen nucleotide pairs. Rather, homologous sequences are found scattered over the phage chromosome. The reasonable conclusion seems to be that phage λ has associated with the bacterial chromosome in the past not only at its present attachment site but probably with other regions of the

chromosome as well. The same can be said of F, of course. F has a minimum of thirteen well-documented attachment sites and it has been found that about one-third of F DNA shows nucleotide-sequence similarity with its nominal host *E. coli* K-12. The regions of homology are scattered about the host chromosome but it is not known whether the regions on F are confined to a separate area of nonessential genes, as suggested by the heteroduplex data, or are scattered over the F genome in many small areas. Despite its homology, F only rarely integrates into the chromosome. Yet F-merogenotes, which have acquired long contiguous stretches of host material, integrate into the chromosome much more frequently. The question of plasmid insertion into the host chromosome, as well as plasmid–plasmid interactions, has taken on a further dimension in the light of some recent experiments by Saedler and Heiss, and others. It has been observed that some mutations in *E. coli* K-12 are due to the insertion of two classes of DNA, IS1 (about 800 nucleotide pairs) and IS2 (about 1400 nucleotide pairs) into the continuity of the chromosome. IS1 and IS2 are distinct DNA species and it has been found that about eight copies of IS1 and about five copies of IS2 are scattered throughout the chromosome of *E. coli* K-12. In addition both IS1 and IS2 have been found in F-plasmid DNA! No small plasmids exclusively carrying these insertion sequences are found in *E. coli* K-12 and, since insertions are found in various chromosomal regions in different strains, IS1 and IS2 probably result from some unknown mechanism of translocation. Nonetheless the presence of these identical insertion sequences in both F and the chromosome suggest a possibility by which IS1 and IS2 could serve as integration sites for the formation of Hfr cells. Similarly one can visualize that recombination between 'unrelated' plasmids could be

Table 8.5. Nucleotide-sequence relationships between several plasmids and the DNA of bacterial species. In each case ^{3}H-labelled plasmid DNA was denatured and reassociated with the denatured DNA of the indicated host. The degree of duplex formation indicated reflects the percentage of the plasmid sharing nucleotide sequences with the total DNA extracted from the cell. Under the conditions of the experiment the DNA duplexes may contain up to 15% mismatch, that is noncomplementary bases. (Unpublished observations of P. Guerry and S. Falkow, 1971.)

Plasmid	Host bacterium		
	E. coli K-12	*Shig. flexneri*	*Salm. typhimurium*
R1 (R-Su-Sm-Cm-Km-Ap)	10·9	12·9	32[a]
F	33	-	-
Su–Sm	<1	-	-
Phage λ	38	25	27

[a] The strain of *Salm. typhimurium* employed, LT-2, was found to carry a cryptic 40×10^{6} daltons plasmid, which is probably responsible for the high reaction.

mediated by these insertion sequences of their analogues. Finally it may be seen that, although no small plasmids exclusively containing the IS1 and IS2 sequences have been found in *E. coli* K-12, their presence on F ensures their transmission.

One should consider this background when discussing the interactions of R with the host chromosome. The insertion of R-factors into the host chromosome of *E. coli* and other enteric species does not usually occur at a detectable frequency except in one very special case involving *fi*$^+$ plasmids. As shown in table 8.5, this genetic observation is reflected by a minimal degree of nucleotide-sequence relationship between the R-factors studied thus far and the *E. coli* K-12 chromosome. Nonetheless there are several studies which clearly demonstrate the interaction of R-factors with host chromosomes. R-factors occasionally bring about the transfer of the chromosomal genes of their bacterial hosts at a frequency of about 10^{-8} per donor cell. The number of recombinant cells, however, reflects the number of organisms that conjugate, and R-factors, as well as Col I (which also brings about low-level recombination), ordinarily conjugate only at low frequency because of pilus repression. A valid study of this phenomenon was not possible therefore until the isolation of de-repressed R-factor mutants, which permitted every cell to conjugate efficiently. When the ability of R *drd* mutants was compared with the classical F$^+$-mediated mobilization of the bacterial chromosome, it was found that the R-factors were often as efficient as F in their transfer of bacterial genes to recipient cells (table 8.6). Remarkably one R-factor, R1*drd*19, not only transferred the bacterial chromosome as efficiently as F, but, indeed, transferred the *pyr-trp-pur* host genes at a fortyfold greater frequency. This was studied in some detail by Pearce and Meynell, who determined that R1*drd*19 was mediating an ordered gradient of transfer analogous to an Hfr donor (table 8.7). In fact, the gradient of transfer was virtually identical to that of a known Hfr strain, Hfr B10. There were, however, two distinct differences in the behaviour of R1-mediated chromosomal

Table 8.6. R-mediated transfer of chromosomal genes. The transfer of the genes *pro, trp,* and *his* (proline, tryptophan and histidine) are presented relative to the results obtained in an F$^+$ × F$^-$ cross, which is taken as 1 (the actual frequencies ranged from 1×10^{-5} to $1{\cdot}8 \times 10^{-5}$ per donor cell). Each R-factor was a de-repressed derivative. (Data modified from Meynell *et al.*, 1968.)

Cross	Relative transfer		
	pro	*trp*	*his*
F$^+$ × F$^-$	1	1	1
R-*fi*$^+$ × F$^-$	0·48	1	0·45
R-*fi*$^-$ × F$^-$	0·7	1·3	2·0
R-*fi*$^-$ × F$^-$	1	6·5	1

transfer and the chromosomal transfer by a bona fide Hfr strain. First, the number of recombinant cells was significantly less than that observed with an Hfr strain. Second, and more important, the recombinant cells almost invariably also received the R-factor, whereas, of course, in Hfr-mediated transfer the recombinant cells ordinarily do not receive F because it is integrated at the chromosome terminus.

Pearce and Meynell point out that these two features are precisely those which distinguish recombination brought about by F′-factors (such as F–*lac* or F_2, see chapter 3) from gene recombination brought about by Hfr cells. Thus the relative frequencies of sex-factor transfer and the production of *pyr-trp-pur* recombinant chromosomes are altogether comparable with those reported for F′-factors in which the sex factor alternates rapidly between the autonomous state and integration at a chromosomal site dictated by the carried bacterial genes. R1 could be considered therefore as a genetic element with a specific affinity for a particular chromosomal site, presumably acquired as the result of a recombinational event with the chromosome of a previous host. If this is the case, however, the amount of host-like material acquired by R1 cannot be very large since it shares less than 10% similar sequences with the *E. coli* K-12 chromosome.

The interpretation of the mechanism of chromosome transfer by R1 and other R-factors is of course coloured by the only other known analogous system, F. Previously, chromosome transfer by autonomous F^+ populations was thought to be wholly due to rare Hfr mutants in the culture. But this is not always the case. Several *E. coli* F^+ strains are known in which chromosome transfer is normal, but no stable Hfr lines can be isolated. In the same vein, Clowes and Moody have found F- and Col I-mediated transfer of chromosomal genes from *rec*$^-$ hosts, and the same result has also been observed for the R1-mediated transfer of chromosomal genes. The mechanism involved here is presumably identical to the mobilization of a nontransmissible plasmid by a transfer

Table 8.7. Chromosomal affinity of an R-factor. The transfer of the indicated genes is presented relative to the results obtained in an $F^+ \times F^-$ cross, which is taken as 1 (the actual frequencies ranged from 1×10^{-5} to 2×10^{-4} per donor cell). R1 is a wild-type (that is repressed) *fi*$^+$ R-(Su-Sm-Cm-Km-Ap) factor and R1*drd* is a de-repressed derivative of R1. *pro* = proline; *lac*$^+$ = utilization of lactose; *pur* B = purine B; *trp* = tryptophan; *pyr* F = pyrimidine F; *his* = histidine. (Data modified from Pearce and Meynell, 1968.)

Cross	Relative transfer					
	pro	*lac*$^+$	*pur* B	*trp*	*pyr* F	*his*
$F^+ \times F^-$	1	1	1	1	1	1
R1 × F$^-$	$<10^{-4}$	$<10^{-2}$	0·14	0·6	0·16	$<10^{-3}$
R1*drd* × F$^-$	1	0·4	42	40	46	0·3

factor. Covalent linkage does not appear to be involved and as is noted below may result from the mistaken initiation of transfer from a chromosomal base sequence similar to the plasmid's origin sequence.

Although it appeared that R1 'recognized' a preferred site on the *E. coli* chromosome, efforts to obtain a stable integration of this R-factor into the chromosome were unrewarding. Recently, however, a powerful method of selection for the isolation of Hfr cells has been developed which has permitted the first isolation of an intact R-factor inserted into the bacterial chromosome. The method revolves around the phenomenon of *integrative suppression*. This phenomenon is reflected in the phenotypic reversion of *dna* A temperature-sensitive mutants of *E. coli* by the integration of a plasmid at any one of many sites on the bacterial chromosome. The *dna* A mutation affects the initiation of chromosome replication. In strains that have integrated a plasmid, it is supposed that the initiation of replication now takes place at a plasmid site, and that the entire chromosome has become part of the plasmid replicon. Integrative suppression does *not* occur with all plasmids. It does occur, however, with F, *fi*$^+$ R-factors, and F-like Col factors, but not with I-like or other *fi*$^-$ R-factors tested thus far. Nishimura, Nishimura and Caro have found that the *fi*$^+$ R-factor R100, as well as the Col V2 plasmid, can integrate into the chromosome of *dna* A hosts to form stable Hfr strains. The R–Hfr strains are as stable as F–Hfr strains and transfer chromosomal genes with high efficiency if a derepressed (for pilus synthesis) R-factor is employed. Transfer is much lower if an isogenic repressed R-factor is employed to suppress the *dna* A mutation. In all, six different classes of R–Hfr cells have been found which have integrated at different sites on the chromosome. In one such R–Hfr cell line the integration site was found to be closely linked to the *lac*$^+$ operon. Phage P1 was propagated on this strain, and the phage used to transduce a normal *E. coli* with selection for *lac*$^+$ transductants. Some of these transductants were found to contain autonomously replicating R–*lac*$^+$ plasmids. Thus, if one can obtain a sensitive enough selection method, the *fi*$^+$ R-factors can be shown to behave very much like F indeed. Undoubtedly such *fi*$^+$ R–Hfr derivatives will be of enormous value in delineating the genetic map of R-factors, using the methods already established for the mapping of the Tra mutants of F. In terms of considering the behaviour of R-factors *in vivo*, however, one should remember that both F- and R-factors integrate into the bacterial chromosome only very rarely, and that the transfer of host genes by these plasmids occurs at only very low levels. Moreover most R-factors do not mediate any detectable level of chromosomal transfer, let alone integrate *in toto* into their host's genome.

Although integration of intact R-factors into the host genome is a rare event, there are numerous reports that fragments of R-factors may become stably integrated into the host chromosome. In some cases this

integration is clearly mediated by part of a temperate phage genome, as was the case for the integration of Tc by phage P-22 (chapter 5). Other reports, such as one by Iyobe, Hashimoto and Mitsuhashi on the integration of Cm genes, appear to describe another mechanism, which is consistent with the view that illegitimate recombination between R-factor chromosome and host genome can take place. These events appear *not* to involve true matching between homologous sequences since the same R gene can be localized at several points on the host. Here again, one can only speculate that special sequences such as IS1 and IS2, common to both plasmid and host, may be the critical factors involved in both plasmid–host and plasmid–plasmid recombinational mechanisms.

When considering plasmid–host interactions, inevitably the question of evolutionary origin arises. This can be discussed in only the most speculative way since there is obviously no fossil evidence on which to build an evolutionary record, nor can we ever claim to have a detailed knowledge of present-day plasmid populations outside the laboratory. One 'standard' theory of plasmid origin holds that they arose as 'break-away' chromosomal regions.

The experimental observation made in 1971 by Ronald Leavitt and his associates could be taken in support of this theory. It has been recognized for some time that when *E. coli* Hfr cells transfer their genes into the diverse species *Salmonella typhi* the vast majority of recombinant cells are partial diploids for the transferred *E. coli* segment. Leavitt showed that these *E. coli* genes are present as independently replicating covalently-closed ring molecules. This was a surprising finding, since no well-defined replication genes of *E. coli* were known to be among the genes present in the ring molecules, and different genetic segments were involved in different recombinant cells. These ring forms are stably inherited, can be mobilized by sex factors and only occasionally interact with the host chromosome (*E. coli* and *Salm. typhi* have about 38% nucleotide sequences in common). Thus these *E. coli* chromosomal fragments in *Salmonella* conform quite well to the definition of a plasmid. Presumably in time they could show pronounced genotypic variation, and their initial origin would become more obscure. An alternative hypothesis would be that bacterial chromosomes themselves were formed by the recombinational coalescence of many small replicons, and that present-day plasmids are evolutionary vestiges of these, which have taken independent paths of speciality. Indeed many of the experimental observations cited in this chapter demonstrate just how rapidly such a coalescence of replicons would take place under selective conditions. The frustrating question of the origin of plasmid genes will come up again, in the following chapter on the drug-resistance determinants of R-factors.

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The biochemical basis of R-factor-mediated drug resistance and its origin

The majority of drug-resistant enteric bacteria isolated clinically owe their resistance to R-factors. Most previous investigations on the antibiotic resistance of enteric organisms had concentrated on chromosomal mutations obtained by the passage *in vitro* of drug-sensitive bacteria on an antibiotic medium. The biochemical basis of antibiotic resistance determined by R-factors is therefore of far more clinical importance than the study of bacterial mutants. Obviously the definition of the mechanisms of R-factor-mediated drug resistance will permit a more intelligent approach to the choice and synthesis of antibacterial agents which can overcome or circumvent the extrachromosomal resistance mechanisms. On a less practical level, one is curious about the mechanism of R-factor resistance as a molecular clue to their origin.

The earliest ideas about the biochemical basis for R-factor drug resistance were influenced by the abrupt appearance of resistance to so many drugs at once. Several workers postulated that the multiple resistance was attributable to a single genetic determinant which conferred a nonspecific permeability barrier on the host. This view was quickly abandoned when R-factor segregants were found which indicated that each drug resistance was specified by a separate gene. It was still considered possible that specific-drug impermeability accounted for resistance. As will be discussed below, some evidence points to this conclusion for tetracycline and sulphonamide resistance. However, resistance to the penicillins, chloramphenicol, and the aminoglycoside antibiotics have been found to be associated with specific enzymes which modify or hydrolyse the drug to a more innocuous form.

9.1 The mechanism of R-factor-mediated chloramphenicol resistance

Enteric bacteria which carry an R-factor specifying chloramphenicol resistance (Cm) contain a constitutive enzyme that catalyses acetylation of chloramphenicol in the presence of acetyl-coenzyme A (acetyl-CoA). The products of the acetylation of the drug have been shown by Y. Suzuki and S. Okamoto, and by W. V. Shaw to be a mixture of 3-acetoxychloramphenicol and 1,3-diacetoxychloramphenicol (figure 9.1). The chloramphenicol molecule is first acetylated at its 3-hydroxyl group to give the monoacetyl derivative, which is sufficient by itself for inactivation of the antibiotic. The molecule is, however, further acetylated to the 1,3-diacetyl derivative, albeit at about 1% of the monoacetyl rate. This discovery raised several interesting questions. First and foremost, was this activity actually the mechanism of drug inactivation? Studies had before revealed chloramphenicol-inactivating mechanisms in cell-free extracts, but they were present in both resistant and sensitive strains of enteric species.

Further, one wished to know if only one enzyme participated in the two steps of chloramphenicol acetylation. Finally, was the Cm resistance gene of the R-factor the structural gene for the acetylation reaction or, as had been speculated, was the product of the plasmid gene some intermediate product which acted to derepress a chromosomal site in sensitive bacteria? All of these questions were effectively answered by the findings of K. Mise and Y. Suzuki. They isolated *E. coli* carrying temperature-sensitive R-factors for chloramphenicol resistance. In the presence of chloramphenicol, the bacteria grew at 34°C but not at 43°C. The mutations did not, however, affect either the stability of the R-factor at 43°C or the other drug resistances specified by the plasmid. The temperature-sensitive Cm property was transmissible by conjugation, which established that it was R-factor-associated rather than a chromosomal determinant. Moreover enzyme extracts were equally temperature-sensitive with respect to both steps of chloramphenicol acetylation. Taken together these data strongly suggest that only one enzyme, chloramphenicol acetyltransferase, participates in chloramphenicol acetylation and that the Cm gene of the R-factor is, indeed, the structural gene specifying this enzyme. The failure of many workers to isolate *in vitro* Cm mutants which specify similar enzymatic inactivation of the drug and the complete abolition of drug resistance in strains harbouring the temperature-sensitive R-factor, also indicates that R-factor-mediated acetylation is the actual mechanism of drug resistance rather than some chromosomal determinant.

Chloramphenicol acetyltransferase has been purified over 100-fold. It has a pH optimum of 7·8, a native molecular weight of 80000 with a single molecular weight subunit of 20000, and an affinity (K_m) for chloramphenicol of 6·1 μM. Fortunately studies of this enzyme are greatly facilitated by a simple and sensitive colorimetric assay, which follows the coincident formation of free CoA. A wide range of chloramphenicol analogues has been examined for substrate activity, and only the natural D-*threo* form of the antibiotic (table 9.1) can be modified by the enzyme. Acetyl-acceptor activity is found for all chloramphenicol analogues possessing the D-*threo* configuration and which contain an unaltered propan-1,3-diol side chain and an acetyl substituent at the

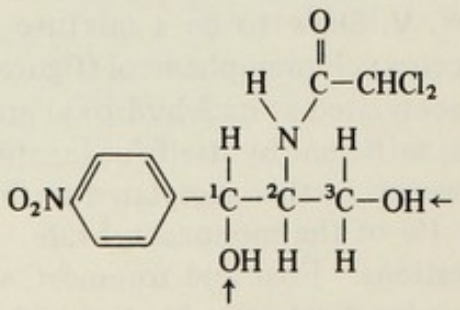

Figure 9.1. Structure of chloramphenicol (D-*threo*-*p*-nitrophenyl-2-dichloroacetamino-propan-1,3-diol). Arrows point to the sites of R-factor-mediated acetylation.

2-amino position. Wide variations in the nature of the substituted 1-phenyl moiety are tolerated without loss of acetyl-acceptor activity.

The vast majority of R-factor clinical isolates that are chloramphenicol-resistant have been found to elaborate chloramphenicol acetyltransferase. Thus far the enzyme has appeared to be reasonably homogeneous in all isolates examined, despite the fact that they are widely divergent with respect to their bacterial host and their geographical location. An important, although unexplained, observation is that R-factors which carry Cm are largely F-like—indeed, in my experience this is true in over 70% of R-factor Cm isolates. The *fi*$^-$ R-factors, of compatibility classes W and C, frequently are Cm^r and also produce a chloramphenicol acetyltransferase. This enzyme appears to have some distinctive molecular properties but nonetheless seems closely related to that found in *fi*$^+$ R-factors. In all reported instances the production of chloramphenicol acetyltransferase is constitutive. It has been recently found, however, at least in *E. coli*, that enzyme synthesis is specifically subject to cyclic 3′,5′-AMP-mediated catabolite repression. The control of R-factor replication in *E. coli* is not subject to cyclic AMP-mediated repression, nor does this observation apply to all R-factor enzymes. In the absence of any known catabolic function of the enzyme, this finding is somewhat surprising and may be an important clue to its origin. There are significant differences in enzyme levels observed among different R-factor-Cm isolates or when the same R-factor is transferred from host to host. There are no definitive data

Table 9.1. Rate of enzymatic acetylation of chloramphenicol and its analogues and isomers by R-factor-mediated chloramphenicol acetyltransferase. All analogues tested were of the D-*threo* configuration. (Data adapted from Shaw and Brodsky, 1967.)

C-1 substituent	Rate of acetylation (% of control)
p-Nitrophenyl (chloramphenicol)	100
p-Acetophenyl	117
p-Iodophenyl	130
p-Methylsulphonylphenyl	67
Steric configuration	
D-*threo* (chloramphenicol)	100
L-*threo*	0
D-*erythro*	0
C-2 substituent	
Dichloroacetamido (chloramphenicol)	100
Formamido	53
Acetamido	121
Butyramido	176
Amino (free base)	0

on the control of enzyme activity but it has become apparent that exponential growth of R-factor-Cm-containing bacteria occurs only after there has been complete inactivation of the drug, and this increase in lag before exponential growth is related inversely to the specific activity of the acetylating enzyme. The host and plasmid variations in enzyme activity may be related to the rate of transcription of the plasmid or to the rate of efficiency of translation of the Cm gene.

9.2 The mechanism of R-factor-mediated penicillin resistance

In both Gram-positive and Gram-negative bacteria, the basis for penicillin resistance has been attributed to the production of inactivating enzymes. Two enzymes have been characterized: an amidase, which hydrolyses the CO–NH bond between the side chain and the 6-amino group in the penicillin acid residue, and penicillinase (β-lactamase), which hydrolyses the CO–N bond in the β-lactam ring of the penicillin molecule (figure 9.2). Enteric bacteria elaborate both enzymes but R-factor-mediated resistance to penicillin has been solely associated with β-lactamase activity. Clinically the penicillins which enjoy the widest use in the treatment of enteric infections are the semisynthetic agents ampicillin (aminobenzylpenicillin) and carbenicillin (α-carboxybenzylpenicillin). Additionally, derivatives of cephalosporin, an antibiotic similar to the penicillins, have proved useful. Notable among these are cephalothin and cephaloridine. Ordinarily, however, when a clinical isolate of an enteric species is characterized as penicillin resistant, the usual implication is that the organism displays resistance to ampicillin. Ampicillin is presently an extraordinarily popular drug—a tribute, I suppose, to its wide spectrum of antibacterial activity, ease of administration, and reasonable freedom of untoward side effects. This increased use has been paralleled by the appearance of the Ap genes in R-factor chromosomes. For example, there was no instance of Ap on R-factors found in *Shigella* organisms isolated at the Children's Hospital in Washington, DC, in 1966. In 1970 (after very extensive use of the drug by the hospital staff and the physicians of the community) 85% of the R-factors found among the *Shigella* isolates in the same institution carried an Ap gene. In every isolate examined, the R-factor-mediated resistance to ampicillin was correlated with the elaboration of a β-lactamase, and this has been the general rule throughout the world since the first description of R-factor-mediated β-lactamases by Naomi Datta and Mark Richmond.

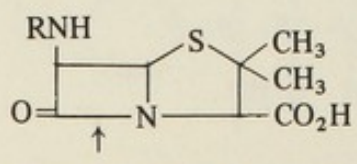

Figure 9.2. Structure of penicillin. The arrow points to the bond hydrolysed by R-factor-mediated β-lactamase.

There are two broad classes of β-lactamase, which are distinguished by their relative substrate specificity. For convenience the two can be designated as penicillinase and cephalosporinase. In the main the β-lactamase elaborated by ampicillin resistant clinical isolates which do not carry an R-factor or by ampicillin resistant mutants selected in the laboratory possess a higher V_{max} for cephalosporins than for ampicillin. These enzymes are often, but not always, inducible. The β-lactamase elaborated by the Ap gene of R-factors displays high activity on both ampicillin and the cephalosporins. All R-factor enzymes described thus far are constitutive and are found in the periplasmic region of the bacterial cell (that is, between the cell wall and cell membrane).

A number of β-lactamases determined by R-factors have been purified and characterized. In terms of physical properties, substrate specificity, and specific activity, the R-factor enzymes fall into two general types: the TEM type (also called the type 1) and the O type (also called the type 2). TEM enzymes show a broad general activity on penicillins and cephaloridine, although their activity on oxacillin is considerably less than that observed with benzylpenicillin. In contrast, the O type enzymes show remarkably high activity on ampicillin and oxacillin, as compared with benzylpenicillin and cephaloridine. A comparison of the properties of both types of R-mediated β-lactamase are listed in table 9.2. Table 9.3 presents a limited view of the molar substrate profile by some representative TEM type and O type R-determined β-lactamases.

A recent study by R. W. Hedges and his associates of 29 ampicillin-resistant R-factors has shown that the TEM type β-lactamase is most common (20 of the 29) and can be specified by plasmids from a variety of compatibility groups from a broad taxonomic range of bacteria. As shown in table 9.3, the TEM type enzyme appears to be uniform with respect to substrate specificity but heterogeneous in absolute levels of β-lactamase activity. Most TEM type enzymes studied have been shown to possess a molecular weight of about 21 000. The O type enzyme is not common and, as illustrated in table 9.3, can be subdivided into those that

Table 9.2. Properties of R-factor-mediated penicillinase.

	$s_{20,w}$	Isoelectric point	pH optimum	Temperature optimum (°C)	K_m [a] (μM)
TEM type [b]	1·43	5·1	6·5	45	27
O type [b]	2·66	8·3	7·6	30	5
E. coli [c]	3·4	-	7·3	-	12

[a] With benzylpenicillin as substrate.
[b] Data from Yamagishi *et al.* (1969).
[c] Properties of penicillinase elaborated by chromosomal mutants of *E. coli* selected for ampicillin resistance by Lindstrom *et al.* (1970).

have a high activity against methicillin, and those with relatively little activity against methicillin. Dale and Smith (1974) have recently shown that the O enzymes with high methicillin activity possess a molecular weight of about 24000, while those O enzymes with low methicillin activity have a molecular weight of about 45000. The hydrolysis of methicillin by the low-molecular-weight O type β-lactamase is a distinctive feature, since methicillin is normally refractory to β-lactamase attack; indeed, TEM type β-lactamase is essentially inactive on this substrate. It should be noted that neither the TEM β-lactamase nor the two subclasses of O β-lactamase are completely homogeneous groups. Each can be divided into subtypes on the basis of their substrate specificity, physical properties, sensitivity to various inhibitors, etc. Presumably as more R-mediated β-lactamases are studied such variations will become even more apparent. As one example, it has been a rather consistent finding that TEM type enzymes possess a considerably higher specific activity than that of O type enzymes. Yet Dale and Smith have now found an example of an O enzyme in which the β-lactamase activity is higher than that of a number of TEM type enzymes.

The properties of the β-lactamase synthesized by an *E. coli* chromosomal mutant present a much more restricted specificity than any of the R-factor enzymes. That is, the *E. coli* enzyme shows essentially identical activity on benzylpenicillin and cephaloridine but rather low activity on ampicillin and oxacillin. The general properties of the *E. coli* K-12 chromosomal β-lactamase appear to be quite similar to those described for many nontransmissible Ap-resistant clinical isolates of

Table 9.3. Levels and specificities of R-determined β-lactamases. [Data modified from Hedges, Datta, Kontomichalou and Smith (1974), and Dale and Smith (1974).]

R-factor	β-lactamase activity[a]	β-lactamase	Molar substrate profile[b] (benzylpenicillin = 100)			
			Oxacillin	Methicillin	Ampicillin	Cephaloridine
R1	121·4	TEM	5·6	–	107	75
R648	134	TEM	5·4	–	107	78
R6K	630	TEM	5·1	–	106	76
R667	1040	TEM	5·4	–	106	75
R455	9·1	O	184	281	161	33
R666a	19	O	198	335	408	31
R16	3·1	O	528	38	133	62
R46	25·2	O	646	23	179	37

[a] Absolute activity expressed as milliunits of enzyme per 10^9 R^+ bacteria.
[b] The molar substrate profile: the absolute activity of each enzyme was measured in milliunits against the different substrates. The values shown were expressed as a percentage of the activity against benzylpenicillin.

E. coli, Citrobacter freundii, Salmonella arizona, and *Proteus.* On the other hand, some nontransmissible Ap-resistant *Klebsiella pneumoniae, Serratia,* and *Proteus* strains elaborate a β-lactamase identical in properties to the R-factor TEM type enzyme. Unfortunately it is not known whether these nontransmissible Ap genes are actually chromosomal or can be mobilized by an appropriate sex factor. It is difficult therefore in some instances to assign a particular β-lactamase to a chromosomal or plasmid origin, although certainly the less common O type β-lactamase has thus far been found only associated with plasmids. Mark Richmond and his associates have concluded that the relative abundance of the amino acids of *all* β-lactamases are extremely similar regardless of their source, distinct substrate specificity, or molecular size. They conclude, therefore, that most of the β-lactamases encountered in enteric bacteria have a common evolutionary origin. The possible origin of the TEM type Ap genes will be considered again in section 9.5.

9.3 The mechanism of R-factor-mediated resistance to aminoglycoside antibiotics

Streptomycin, kanamycin, and other aminoglycoside antibiotics are still commonly employed against Gram-negative infections. In the laboratory, spontaneous mutation to high-level drug resistance occurs at a low frequency and is generally specific for only a single aminoglycoside antibiotic. The mechanism of chromosomal resistance to aminoglycosides has been studied in considerable detail and is associated with specific alterations of the bacterial ribosome. Thus the addition of an aminoglycoside to a sensitive cell is associated with an inhibition of protein synthesis because of a large-scale misreading of the genetic code. Mutation to resistance alters the ribosome in a way that prevents or inhibits binding of the aminoglycoside.

Several early observations suggested the R-factor-mediated aminoglycoside resistance was not associated with a ribosomal alteration. First of all, in *E. coli* strains partially diploid for chromosomal genes, streptomycin sensitivity was dominant over streptomycin resistance, whereas R-factor streptomycin resistance was dominant. Whereas chromosomal-mediated resistance was generally antibiotic-specific, R-mediated cross-resistance to several aminoglycosides was common. And, finally, the level of R-mediated aminoglycoside resistance, but not that of chromosomal-based resistance, varied with inoculum size on plates of antibiotic media. Since an inoculum effect is often associated with drug-destroying enzymes, it was considered conceivable that R-Sm factors produced an inactivating enzyme. Sure enough, the ribosomes of R-Sm strains were streptomycin-sensitive when tested in cell-free systems. A short time later S. Okamoto and Y. Suzuki obtained a supernatant fraction of an R-Sm strain, which was able to inactivate streptomycin in the presence of Mg^{2+} ions and ATP in a cell-free amino-acid-incorporating system.

These initial findings have been expanded by a number of laboratories, and several different mechanisms of R-factor-mediated resistance to the aminoglycoside antibiotics are now recognized. To date, eight enzymes have been characterized which modify different groups of aminoglycoside antibiotics. These are: *streptomycin adenylate synthetase*, which inactivates streptomycin, bluensomycin, and spectinomycin by adenylylation; *streptomycin phosphotransferase*, which inactivates streptomycin, but not spectinomycin or bluensomycin, by phosphorylation; *kanamycin phosphotransferase*, which phosphorylates kanamycin, neomycin, paromomycin, and some components of the gentamicin and nebramycin complexes; *kanamycin acetyltransferase*, which acetylates kanamycin, neomycin, the hybrimycins, and some of the components of the gentamicin and nebramycin complexes; *gentamicin acetyl transferases I* and *II*, which acetylate components of the gentamicin complex and tobramycin; and *gentamicin adenylyltransferase*, which adenylylates kanamycins, components of the gentamicin complex, and tobramycin.

9.3.1 Streptomycin adenylate synthetase and streptomycin phosphotransferase

The two distinct enzymes streptomycin adenylate synthetase and streptomycin phosphotransferase are synthesized constitutively and are, like the R-factor-mediated penicillinases, classified as periplasmic enzymes since they are released by simple osmotic-shock treatment of cells. This latter property provides a very convenient method for isolation and subsequent purification of these enzymes. Both have molecular weights of about 30000 and require ATP and Mg^{2+} ions for drug inactivation. The phosphorylating enzyme can use GTP as an alternative whereas the adenylylating enzyme can alternatively use αATP. Both enzymes are conveniently assayed by measuring the transfer of radioactivity from ^{14}C-labelled ATP or α^{32}P-labelled ATP to unlabelled antibiotic. The modified drug is then measured after thin-layer chromatography or adsorption to cation-exchange paper.

The site of action of both enzymes appears to be the 3′-hydroxyl group of the *N*-methyl-L-glucosamine moiety of the streptomycin molecule (figure 9.3). Inactivation occurs by transfer of either the terminal phosphate or the adenosine monophosphate residue to this site. Despite some similarities, however, there are substantial differences in the substrates that the enzymes can modify. The most obvious difference is that the single adenylylating enzyme inactivates both streptomycin and spectinomycin and, indeed, R-factor strains possessing the enzyme show cross-resistance to both drugs. The phosphorylating enzyme recognizes only streptomycin but not spectinomycin as a substrate. Additionally the phosphorylating enzyme can modify both *N*-methyl- and *N*-dimethyl-dihydrostreptomycin, whereas the adenylylating enzyme cannot. Thus R-strains carrying the phosphorylating enzyme are streptomycin-resistant, but may be spectinomycin-sensitive. A recent observation is that neither

enzyme can modify streptomycin B (mannisidostreptomycin) and, hence, although it is a much less active antibacterial agent than streptomycin, it could be employed against R-factor strains carrying either Sm gene. The two enzymes have never been found to occur simultaneously in the same R-factor genome. Although exceptions occur, it seems a reasonable rule of thumb that R-factors which elaborate the adenylylating enzyme are *fi*$^+$, and the *fi*$^-$ R-factors generally inactivate streptomycin by phosphorylation. This generalization may help to explain an early experimental finding that F-like R-factors generally possess a low level of streptomycin resistance, which does not change the host's permeability to streptomycin, whereas *fi*$^-$ R-factors, which are impermeable to streptomycin, display a higher level of resistance. The biochemical explanation may be that the host bacteria are permeable to streptomycin adenylate but not streptomycin phosphate. At any rate, both biochemical modifications of streptomycin render it inactive as an antibiotic, and studies *in vivo* and *in vitro* reveal that the modified drug can no longer cause misreading of the genetic code, or inhibit peptide-synthetic activity. Thus the modified drugs seem unable to reach or to associate with the ribosome target.

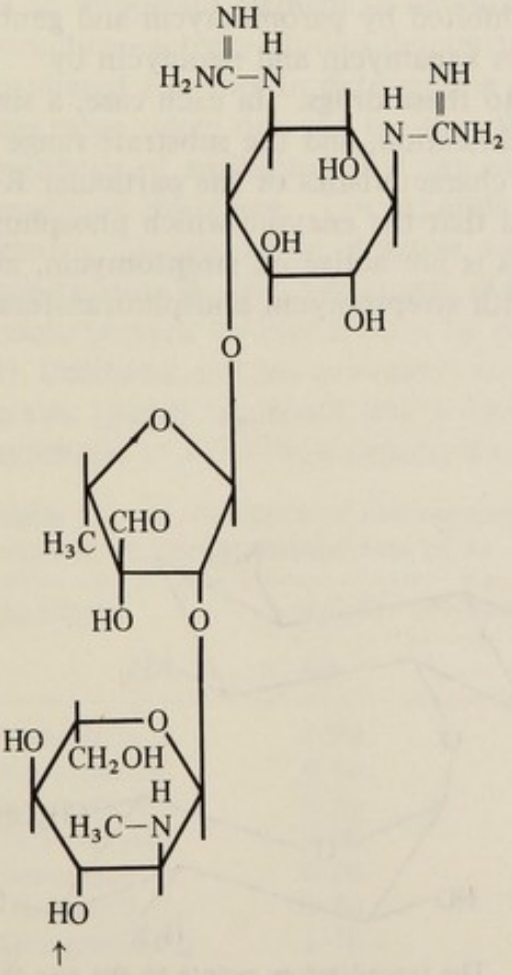

Figure 9.3. Structure of streptomycin. The arrow points to the probable site of adenylylation and phosphorylation by R-factor-mediated enzymes.

To my knowledge no R-Sm strain has been isolated that does not possess one or the other of these enzymes, nor has there been any streptomycin-resistant chromosomal mutant isolated which directs any remotely similar enzymatic modification of streptomycin or spectinomycin.

9.3.2 Kanamycin phosphotransferase and kanamycin acetyltransferase

Most organisms that harbour an R-factor which confers resistance to kanamycin–neomycin, paromomycin, gentamicin A, and nebramycin factor 4 elaborate a specific ATP, Mg^{2+} ion-dependent phosphotransferase, which phosphorylates the 3′-hydroxyl group of the 6-D-glucosamine ring of these molecules. A smaller proportion of R-factors that are kanamycin-resistant elaborate an acetyl-CoA-dependent acetyltransferase, which prefers *N*-acetylation of kanamycin A, kanamycin B, neomycin B and C, the hybrimycins, gentamicin C_{1a} and C_2, and nebramycin factors 4 and 6 (figure 9.4). These compounds all share a 6-amino group on a common hexose ring structure. Raoul Benveniste and Julian Davies point out that one can distinguish between R-factor strains that phosphorylate or acetylate neomycin and kanamycin by examining their resistance profile carefully (table 9.4). A strain harbouring an R-factor which produces the acetylating enzyme will still be inhibited by paromomycin and gentamicin A, whereas a strain that inactivates kanamycin and neomycin by phosphorylation will be resistant to these drugs. In each case, a single enzyme is responsible for the modification, and the substrate range of the enzyme determines the resistance characteristics of the particular R-factor-bearing strain. It should be noted that the enzyme which phosphorylates kanamycin and related compounds is not active on streptomycin, nor is there any apparent relationship with streptomycin phosphotransferase.

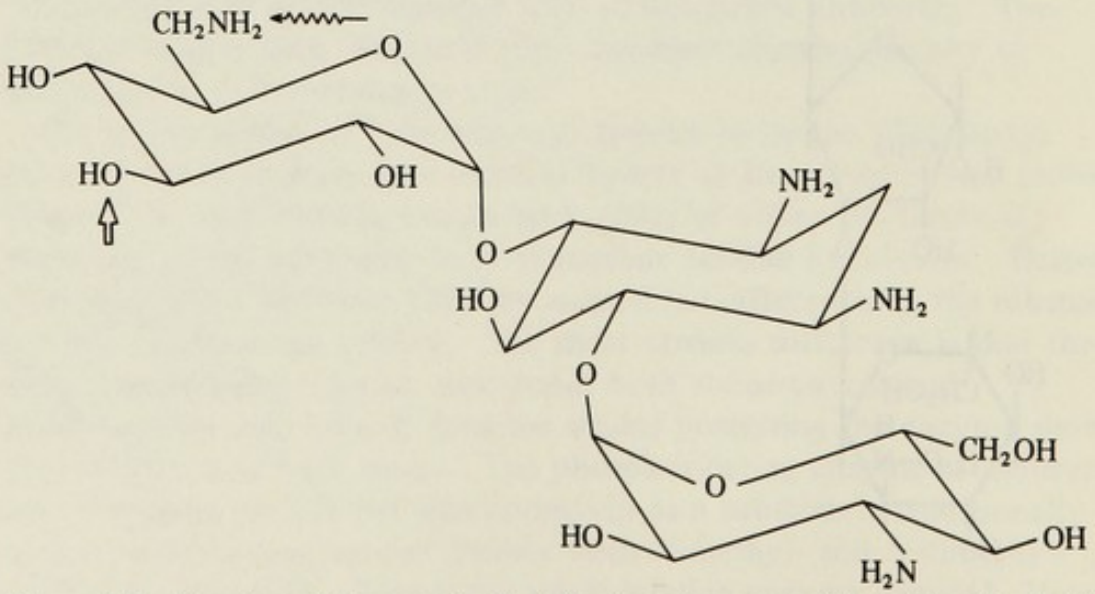

Figure 9.4. Structure of kanamycin A. The jagged arrow points to the site that is enzymatically modified by *N*-acetylation. The other arrow points to the site enzymatically modified by *O*-phosphorylation.

Likewise the kanamycin acetyltransferase bears no relationship to the R-factor-mediated enzyme that acetylates chloramphenicol. The enzymes are elaborated by completely separate genes, and both kinds of kanamycin-inactivating enzymes are not found simultaneously in the same R-factor genome.

The phosphorylated derivatives of kanamycin and related compounds are all totally inactive as antibiotics. Similarly *N*-acetylkanamycin A is no longer capable of inhibiting bacterial growth. However, *N*-acetylkanamycin B, *N*-acetylgentamicin C_{1a}, and *N*-acetylneomycin B are still antibiotics, albeit weaker than their unacetylated parent compounds. Indeed, although gentamicin C_{1a} is one of the best substrates for the acetylation reaction, the R-factor strains are still almost completely sensitive to the *N*-acetylated antibiotic, indicating that this enzymatic modification alone is not sufficient to eliminate its antibiotic activity. The retention of antibiotic activity by the *N*-acetyl derivatives of neomycin B, kanamycin B, and gentamicin C_{1a} has been interpreted in structural terms to mean that the presence of an amino group in either the 2 or the 6 position of the hexose moiety attached to the deoxystreptamine of these aminoglycosides is an important element in determining antibiotic properties. Such structure–activity studies demonstrate the usefulness of the R-factor-mediated enzymes in determining the nature of antibiotic structures. By the same token one can see that these findings lay the groundwork for the synthesis of antibiotics that can overcome (at least for a time) R-factor-mediated resistance. For example, blocking the 6-amino group of kanamycin may be an effective way to prevent acetylation, and the proper substitution on the 3-hydroxyl group in the same sugar ring of kanamycin could prevent its inactivation by phosphorylation. In a practical vein H. Umezawa and his coworkers have attempted to prepare antibiotics active against organisms which have the phosphorylation enzyme. The synthesis of tetra-*N*-phenylalkylkanamycins did in fact provide seven

Table 9.4. Modification of aminoglycosides by kanamycin phosphotransferase (KP) and kanamycin acetyltransferase (KA). (Modified from Davies *et al.*, 1971.)

Antibiotic	Relative effectiveness as substrate to:	
	KA	KP
Kanamycin A	1·00	1·00
Kanamycin B	0·66	0·89
Neomycin B	0·61	1·11
Paramomycin	0·00	1·40
Gentamicin A	0·00	0·98
Gentamicin C_1	0·00	0·00
Gentamicin C_{1a}	1·76	0·03
Streptomycin	0·00	0·00

derivatives which showed considerable activity against R-factor-mediated kanamycin-resistant *E. coli in vitro* and against resistant strains of *Staph. aureus* and *Ps. aeruginosa* as well. A semisynthetic kanamycin derivative, BBK-8, has found recent use and is sensitive only to kanamycin acetyltransferase. It may be of future practical interest that aminoglycosides which are not substrates for enzymatic modification may nonetheless be powerful inhibitors of the enzymes. For example, gentamicin C_{1a} is acetylated but retains antibiotic activity and at the same time is a potent inhibitor of the phosphotransferase which inactivates gentamicin A. Conversely gentamicin A is a potent inhibitor of the acetyltransferase activity. Benveniste and Davies point out that in clinical use the gentamicin complex would seem to have a distinct advantage over the use of isolated components of the mixture.

9.3.3 Additional mechanisms of R-factor-mediated gentamicin resistance

The principal components of the gentamicin complex used clinically are gentamicin C_1, C_{1a}, and C_2. As already noted, C_{1a} is acetylated but retains antibiotic activity, C_2 is acetylated poorly and C_1 not at all. Moreover the only members of the gentamicin complex phosphorylated are gentamicin A and B. Thus the gentamicin complex has already proved valuable for treatment of R-factor-bearing strains which possess the phosphorylating or acetylating enzyme and, in fact, the clinical use of this antibiotic (and its advertisement in medical journals) has increased dramatically in the past few years. In 1971 Christopher Martin and his associates described a sudden outbreak of serious infections, including pyelonephritis and bacteraemia, caused by gentamicin-resistant strains of *Klebsiella pneumoniae*. These strains were also resistant to streptomycin, kanamycin, ampicillin, tetracycline, sulphonamides, and chloramphenicol, but generally retained sensitivity to cephalothin and the polymyxins. The epidemiological findings were consistent with the view that the resistant *Klebsiella* were being transmitted from patient to patient via the hands of hospital personnel attending patients with indwelling catheters. When these strains were tested for their ability to transfer antibiotic resistance it was found that selection for transfer of gentamicin resistance disclosed a simultaneous transfer of resistance to streptomycin, kanamycin, chloramphenicol, tetracycline, sulphonamides, and ampicillin, that is to say an R-factor was indeed present.

The R-factor associated with this strain possessed streptomycin phosphotransferase activity but this enzyme was ineffective against the gentamicin complex. Moreover *O*-phosphorylation or *N*-acetylation of gentamicin was not observed in extracts of strains bearing this R-factor. Benveniste and Davies found, however, that inactivation of gentamicin by these cell extracts had an absolute requirement for Mg^{2+} ions and ATP. Because of this they reasoned that the modification of gentamicin occurred by adenylylation, as had been previously shown for streptomycin.

This expectation was borne out and, indeed, several other aminoglycosides were also substrates for this enzyme, as shown in table 9.5. It can be seen that this adenylylating enzyme, which modifies gentamicin and kanamycin, is different from the enzyme which adenylylates streptomycin, since neither streptomycin nor spectinomycin is a substrate. Conversely gentamicin is not a substrate for the enzyme that adenylylates streptomycin and spectinomycin. The site of inactivation by this new adenylylating enzyme is thought to be the 2-hydroxyl group of the garosamine ring of the gentamicin complex.

The original *K. pneumoniae* strain isolated from the nosocomial outbreak possessed kanamycin phosphotransferase, streptomycin phosphotransferase, and the new adenylylating enzyme that inactivated kanamycin and gentamicin. The strain was therefore resistant to all aminoglycoside antibiotics in current clinical use.

It is interesting to see that there are three separate mechanisms for the inactivation of the kanamycin and the gentamicin complex: *O*-phosphorylation, *N*-acetylation and *O*-adenylylation. The number of reported distinctive R-mediated aminoglycoside inactivating enzymes has continued to grow. Most recently, for example, two additional inactivating enzymes of the gentamicin complex have been described which are called gentamicin acetyltransferase I and gentamicin acetyltransferase II. Tables 9.6 and 9.7 summarize the mode of action and substrate specificity of these and all other aminoglycoside-modifying enzymes. Fortunately R-mediated gentamicin resistance still remains relatively uncommon, so that gentamicin still remains a major 'drug-of-choice' in combating nosocomial infection caused by gram negative bacteria. Nevertheless the clinician can only view the potential R-mediated armamentarium against the aminoglycoside antibiotics with awe and some trepidation. On a more happy note, the adenylylating enzyme has proved of value in assaying the gentamicin level in the serum of patients receiving this antibiotic.

Table 9.5. The enzymatic adenylylation of aminoglycosides by gentamicin adenylyltransferase. (Modified from Benveniste and Davies, 1971.)

Antibiotic	Relative activity
Gentamicin A	1·00
Gentamicin C_1	0·78
Gentamicin C_{1a}	0·25
Gentamicin C_2	0·28
Kanamycin A	0·65
Neomycin B	<0·01
Streptomycin	<0·01
Spectinomycin	<0·01

Table 9.6. Aminoglycoside-modifying enzymes. (Modified from Benveniste and Davies, 1973.)

Enzyme	Modification of antibiotic
Streptomycin-spectinomycin adenylyltransferase (SAdT)	Hydroxyl group of a D-*threo*methylamino alcohol moiety is adenylylated
Streptomycin phosphotransferase (SPT)	3-Hydroxyl group of *N*-methyl-L-glucosamine is phosphorylated
Kanamycin-neomycin phosphotransferase (NPT)	3-Hydroxyl group of an aminohexose is phosphorylated
Kanamycin acetyltransferase (KAcT)	6-Amino group of an aminohexose is acetylated
Gentamicin adenylyltransferase (GAdT)	2-Hydroxyl group of an aminohexose of both kanamycin and gentamicin is adenylylated
Gentamicin acetyltransferase I (GAcTI)	3-Amino group of 2-deoxystreptamine is acetylated
Gentamicin acetyltransferase II (GAcTII)	2-Amino group of an aminohexose is acetylated

Table 9.7. The enzymatic inactivation of aminoglycoside antibiotics (data from Benveniste and Davies, 1973).

Antibiotic	Inactivating enzyme[a]						
	SPT	SAdT	NPT	KAcT	GAdT	GAcTI	GAcTII
Kanamycin A	−	−	+	+	+	−	−
Kanamycin B	−	−	+	(+)	+	(+)[b]	−
Kanamycin C	−	−	+	−	+	−	−
Neomycin B	−	−	+	(+)	−	−	−
Paromomycin	−	−	+	−	−	−	−
Gentamicin C_{1a}	−	−	−	(+)	+	+	+
Gentamicin C_2	−	−	−	(+)	+	+	+
Gentamicin C_1	−	−	−	−	+	+	+
Gentamicin A	−	−	+	−	+	−	(+)[b]
Tobramycin	−	−	−	(+)	+	+	+
Streptomycin	+	+	−	−	−	−	−
Spectinomycin	−	+	−	−	−	−	−
BBK-8[c]	−	−	−	+	−	−	−

[a] The abbreviations for the enzymes are given in table 9.6. + means an enzymatic modification inactivates the antibiotic, (+) that it is only partially inactivated, and − that it is not a substrate.

[b] These two antibiotics are modified by the enzyme but are poor substrates, and strains are essentially sensitive to them.

[c] BBK-8 is a semisynthetic kanamycin derivative.

9.4 The mechanism of R-factor-mediated tetracycline and sulphonamide resistance

Tetracycline resistance is probably the most common R-factor-mediated drug resistance found in Nature. Despite this fact the mechanism of R-factor-mediated resistance to this drug has received relatively little attention. It appears that the resistance of R^+ strains is due to their inability to concentrate tetracycline. Whereas drug-sensitive bacteria actively accumulate tetracycline, leading to inhibition of ribosome function, R^+ strains possess a constitutive low-level resistance to the drug, which prevents it from reaching the ribosome target. The decreased uptake of the drug, and concomitantly the level of resistance, is appreciably increased in the presence of tetracycline, suggesting that the inhibitor of tetracycline uptake is inducible. One might expect therefore that constitutive mutants of R-Tc, resistant to high levels of drug, should exist and indeed this was shown in 1971 by Franklin and Cook. The specific mechanism whereby tetracycline is inhibited from entering the cell is not known; studies of radioactivity-labelled tetracycline provide no evidence for enzymatic inactivation or modification.

The mechanism of sulphonamide resistance mediated by R-factors has likewise received little attention. A recent brief report by Unowsky and Krainski suggests that, as with tetracycline, permeability is a mechanism for R-factor-mediated resistance to sulphonamides. This conclusion was drawn from the observations that [^{35}S]sulphisoxazole is neither destroyed nor modified by R^+ strains. Moreover R^+ strains incorporated only 10–50% as much labelled drug as sensitive strains. Increasing the drug concentration by threefold caused a ninefold increase in incorporation by sensitive strains, whereas incorporation by R-Su strains remained unchanged. These observations were essentially the same with both fi^+ and fi^- factors.

R-factors also mediate 'resistance' to a number of heavy metals, colicins, and ultraviolet irradiation. Anne Summers and Simon Silver reported in 1972 that R-factor-mediated mercury resistance seems to be associated with the conversion of Hg^{2+} ion into metallic mercury. The volatilizing activity is inducible and has other properties of enzymatic catalysis. There are, as yet, no clearcut experiments shedding light on the mechanisms underlying the other resistance properties cited.

9.5 The origin of resistance determinants

Shortly after the discovery of R-factors it seemed logical to presume that these sex factors acquired drug-resistance genes from the chromosomes of their hosts by a mechanism of 'gene pick-up' analogous to the acquisition of *gal*$^+$ genes by phage λ or *lac*$^+$ by F. The evidence for a chromosomal origin of resistance determinants is weak, however, since there has been no evidence for the allelic counterparts of many of the resistance determinants of R-factors on the chromosomes of *E. coli*, *Salmonella*, and *Shigella*.

In the main the data on the mechanisms of drug resistance lead to the interesting conclusion that the basis of drug resistance carried by R-factors is quite different from that which has been characterized for chromosomal resistance.

For example, reactions involving adenylylation of any class of substrates are relatively rare in Nature. When this does occur, as in amino acid activation, the adenylylated molecule is generally found as an intermediate which is subsequently broken down to another product. Alternatively one finds that enzyme activation occurs through covalent binding of AMP, as seen for muscle phosphorylase and glutamine synthetase. In any case the enzymatic adenylylation of antibiotics or sugars had not been reported before the discovery of the adenylylating enzymes found on R-factor chromosomes. By the same token the O type β-lactamase of R-factors appears to be unique, and the TEM type enzyme does not have an allelic counterpart in *E. coli* K-12. As noted earlier, enzymes virtually identical with R-factor TEM type β-lactamase have been identified in nontransmissible ampicillin-resistant clinical isolates, but it is not definitively established whether the cellular location of these determinants is chromosomal or extrachromosomal. In *Pseudomonas aeruginosa,* for example, a 'naturally resistant strain' was described that possessed a TEM β-lactamase and kanamycin phosphotransferase essentially identical with R-factor enzymes. When Richard Sykes and Mark Richmond examined similar *Pseudomonas* strains for genetic transfer in 1970, they discovered that Ap, Tc, and Km were transmissible and thus carried an fi^- R-factor of a type (P) not usually found in *E. coli* and other Enterobacteriaceae.

The isolation of this type P compatibility-group plasmid has additionally given new insight into the possible origin of the TEM type β-lactamase. Datta and her associates showed that the TEM type Ap gene of this P plasmid could be translocated to an I plasmid *and* to the chromosome of *E. coli* K-12. Robert Hedges and Alan Jacob have recently extended this observation to show that the Ap genes could not only be translocated onto various other replicons, but that plasmids which acquired this resistance showed a small increase (about 2×10^6 daltons) in molecular weight. Moreover they showed that once a plasmid gained the Ap gene, it could, in turn, translocate the gene to other replicons. Hedges and Jacob suggest therefore that the ubiquity of the structural gene for the TEM β-lactamase in R-factors probably reflects both its association with a DNA fragment, which can be readily translocated from one replicon to another, as well as the high molecular efficiency of the TEM type β-lactamase genes themselves. The implications of this work, that the genetic segment which includes the TEM type β-lactamase genes might be essentially identical in all R-factors regardless of the compatibility group, geographical source, molecular size, and G + C content, have been confirmed by Frederick Heffron working in my laboratory. Heffron employed a small (5×10^6 daltons) naturally occurring replicon, RSF1030,

which encodes for a TEM β-lactamase. As shown in table 9.8, DNA–DNA duplex studies between RSF1030 and R-Ap$^+$ factors from a variety of sources showed a significant level of nucleotide sequences in common in those cases where the R-factor under study elaborated a TEM type β-lactamase. R-factors which were Ap$^+$ by virtue of an O type β-lactamase failed to form DNA–DNA duplexes with RSF1030. That the sequences being measured were largely restricted to a stretch of DNA containing Ap genes was shown by a comparison of duplex formation between RSF1030 and two homogenic plasmids, S-a and S-a-1, which differed only in that S-a-1 carried a 2×10^6 daltons insertion containing the genetic information for the biosynthesis of a TEM type β-lactamase. Also, in those cases where highly related plasmids of the same compatibility group, differing only in the type of β-lactamase, were examined the RSF1030-plasmid DNA reacted only with the plasmid carrying the TEM type β-lactamase. These data show, therefore, that a sequence of DNA which includes the genetic information for TEM type β-lactamase is likely to be of a common ancestral source in most R-factors. The translocation of this DNA

Table 9.8. DNA–DNA duplex studies between RSF1030 and R-factors carrying TEM and O β-lactamases. (Data from F. Heffron, S. Falkow, R. W. Hedges, A. Jacob, and N. Datta, *J. Bacteriol.*, in press 1975.)

R-factor	Compatibility class	10^{-6} × Mol.wt. (daltons)	Type of β-lactamase	Origin of R-factor	Percent relative homology with RSF1030[a]
S-a	W	25	None	*E. coli*, Japan	<1
S-a-1[b]	W	27	TEM	*E. coli*, Japan	36
R388	W	21	None	*E. coli*, UK	<1
R7K	W	22	TEM	*P. rettgeri*, Greece	30
R906	P	?	O	*Ps. aeruginosa*	<1
RP4	P	34	TEM	*Ps. aeruginosa*	41
222	FII	72	None	*Shig. flexneri*, Japan	<1
R1	FII	65	TEM	*Salm. paratyphi*, UK	45
R455	FI	?	O	*P. morganii*	<1
R55	C	?	O	*K. pneumoniae*, France	<1
R746	C	?	TEM	*Providencia*, sp., Canada	31
R6K	X	45	TEM	*E. coli*, Greece	46
R16	O	48	O	*E. coli*, Mexico	<1

[a] [^{3}H]thymine-labelled RSF1030 DNA was duplexed with DNA isolated from each of the indicated R-factors. The data are presented as the percent nucleotide-sequence relationship relative to the homologous reaction, which was taken as 100.

[b] S-a-1 is homogenic with S-a except that it contains an Ap$^+$ translocation. Electron-microscope heteroduplex analysis shows that S-a-1 differs from S-a in a single contiguous region by about $2{\cdot}1 \times 10^6$ daltons.

fragment has been shown by Hedges and Jacob to be independent of the *rec* genes of the bacterial host and that the DNA segment *per se* is probably not capable of independent replication. While the precise genetic mechanism of this translocation phenomenon is not presently known it does bear some resemblance to the IS1 and IS2 insertion segments described in an earlier chapter (chapter 8). Of course, these data do not tell us anything about the actual origin of the TEM type β-lactamase gene, but they do provide a rather interesting view into the mechanism by which these genes may become widely distributed both on plasmids and on host chromosomes.

The best evidence for an allelic counterpart of an R-factor enzyme on the bacterial chromosome has been the observations by William Shaw. He found that a few strains of *E. coli, Serratia marcescens* and, particularly, *Proteus mirabilis*, which were phenotypically sensitive to chloramphenicol, nonetheless possessed low activities of a constitutive chloramphenicol-acetylating enzyme. In *Proteus mirabilis* the enzyme produced by phenotypically sensitive strains and spontaneous Cm^r mutants show a high degree of gross homology with the chloramphenicol acetyltransferase produced by R-factor-bearing strains (table 9.9). The sole difference noted to date is that the enzyme isolated from the Cm^s wild-type strain has a significantly lower affinity (higher K_m) for chloramphenicol. The mutation to Cm^r is accompanied by an increased affinity for chloramphenicol and a concomitant higher degree of activity on chloramphenicol. The affinity of the mutant enzyme still does not equal that found on R-factor chromosomes, however.

At the best, one could make the argument that the resistance genes were derived from several different bacterial species in single genetic events. The finding, however, that the resistance determinants of fi^+ R-factors predominantly reside on a small apparently homogeneous replicon possessing an average base composition of 56% G + C is not very consistent with this hypothesis. One could go on with pros and cons, but there is really no definitive evidence bearing on the question of whether the resistance markers originated as plasmid genes or chromosomal genes.

Table 9.9. Properties of chloramphenicol acetyltransferase from wild-type and mutant strains of *Proteus mirabilis*. (Data from W. V. Shaw, 1971.)

Enzyme source	Enzyme activity	Enzyme synthesis	Michaelis constant (K_m) for chloramphenicol (μM)	Molecular size	Weight subunit
E. coli R^+ Cm	High	Constitutive	6·8	80000	20000
P. mirabilis R^+ Cm	High	Constitutive	6·8	80000	20000
P. mirabilis R^- Cm^s	Low	Constitutive	98·0	80000	?
P. mirabilis R^- Cm	High	Constitutive	18·0	80000	20000

The major point is that one need not assume that the plasmid genes have been extracted from the chromosome—or if they have, that it is a very recent event in the evolutionary sense.

9.5.1 R-factors before 'the antibiotic era'

The latter interpretation infers that the present-day examples of R-factors represent a continuation of a 'primitive' condition. Of course, there is no doubt that there is a relationship between the prevalence of R-factors in man and his domestic animals and the use of antibiotics. Yet did R-factors predate our present 'antibiotic era' and are antibiotics a *sine qua non* for the existence of R-factors? The best evidence for an early existence of R-factors was the discovery by David Smith of an R-Tc–Sm factor in a strain of *E. coli* freeze-dried in 1946—before the clinical introduction of these drugs.

Indirect evidence of a similar nature came from the observation by Smith, and from my laboratory, that R-factors mediating resistance to semisynthetic penicillins and several aminoglycoside antibiotics were present in enteric bacteria isolated years before these agents were available. For example, our studies revealed that strains of *E. coli, Shig. flexneri,* and *Salm. typhimurium* isolated in Mexico in 1956 contained R-factors mediating O type β-lactamase ampicillin resistance. The finding, that a β-lactamase with a high degree of affinity for semisynthetic penicillins was already present in 1956, suggested that it was previously evolved to fit a naturally occurring substrate. Similarly the R-factor in the 1946 lyophil was streptomycin resistant by virtue of a streptomycin phosphotransferase that cannot be distinguished from present-day examples. Although the data are not overwhelming, it does seem fair to conclude that R-factors are not just a recent phenomenon and that selective forces were operative in Nature before 'the antibiotic era' to confer sufficient evolutionary advantage for the association of a transfer factor with drug-resistance genes. Watanabe suggested in 1966 that if R-factors have been evolving in natural populations of microorganisms, then it would be most useful to examine 'antibiotic virgin' populations to determine their prevalence and characteristics under such conditions. Such a study was conducted by I. Maré among Kalahari bushmen and wild animals in 'drug-free' communities of Rhodesia. No R-factors could be detected among the fifty-seven strains of drug-resistant enteric bacteria examined. Two recent independent studies in the South Pacific were successful, however. In one of these, Gardner, Smith and their associates examined twenty-one stool specimens and nineteen soil specimens taken from a community in the Solomon Islands. The inhabitants of the island had remained virtually untouched by modern 'civilization'. R-factors, R-Tc–Sm, were recovered from two of the forty specimens processed; both elaborated streptomycin phosphotransferase. One was present in an *E. coli* strain resident in the bowel of a native from the innermost bush country. The other was from an

'alcaligenes-like' species of the soil! The isolation of an R-factor from a soil organism is important for several reasons. Thus far, studies on the epidemiology of R-factors have naturally centred primarily on factors affecting human and animal colonization. The ability of an R-factor to exist and be maintained in a soil organism suggests that soil may be an important cryptic reservoir of R-factors. Moreover, considering the fact that soil is the principal ecological niche for antibiotic-producing organisms, this finding would be consistent with the view that natural selection by the small amounts of natural antibiotics produced in the soil may have an important bearing on the evolution of R-factors. Despite this enticing report, I am unaware of any study which has examined the flora of soil to assess the prevalence of R-bearing organisms.

The second successful study of an 'antibiotic-virgin' community was undertaken by Charles Davis and Jeraldine Anandan. These workers examined the enteric flora of 128 people who inhabited an isolated village in North Borneo. Of 1017 bacterial isolates, 50 were multiply resistant and six strains (12%) from four different individuals could transmit their resistance by conjugation. Unfortunately the other multiply resistant isolates were not tested to see if their resistance complements could be mobilized. Two strains were R-Su-Sm-Cm and three were R-Tc-Su-Sm-Ap. These R-factors were transferred invariably as a single genetic unit. However, one strain, R-Su-Sm-Tc-Cm; Ap showed a segregation of resistance at transfer; some progeny received only Ap whereas others received Ap together with combinations of Tc, Sm, Su, and Cm. This latter example may represent a strain harbouring two R-factor types or may be an example of a dissociated unit *à la* Anderson.

These studies[8] of isolated communities reveal therefore that R-factors can be found under natural conditions, without the selective force of deliberately administered antimicrobial drugs. The prevalence is, however, low—although, in truth, no lower than that seen in the community-at-large in some surveys of urban residents in the USA.

9.5.2 Natural selection for drug resistance

If one begins to think about the ecological factors responsible for the natural selection of bacteria resistant to antibiotics in a pre-antibiotic era, it is natural, as mentioned above, to take note that drugs to which R-factors mediate resistance are commercially produced by microorganisms found normally in the soil. One can presume that this was the rationale which led Gardner *et al.* in their search for R-factor-bearing strains in the soil of the Solomon Islands. The most straightforward explanation therefore is that antibiotics elaborated in the soil have a role in the

[8] One may well wonder how the inhabitants of these isolated regions reacted when suddenly faced with the invasion of strangers intent on placing cotton swabs in their rectums and coveting their faeces!

selection of resistant bacteria. The nature of the drug-inactivating enzymes themselves lends some support to the notion that they were specifically evolved as detoxifying enzymes directed against antibiotics and related molecules. For example, chloramphenicol acetyltransferase has an affinity only for those chloramphenicol analogues which are biologically active. This is true, as well, for the chromosomal chloramphenicol acetyltransferase found in cells of *Proteus mirabilis*. Yet these *Proteus* strains also produce significant amounts of a protein that cross-reacts with chloramphenicol acetyltransferase but has very low catalytic activity as measured by chloramphenicol acetylation. Shaw designates this cross-reacting protein 'x-acetyltransferase' and infers that its principal specificity may not be directed towards chloramphenicol at all. Thus, as an alternative to evolving specifically as antibiotic resistance genes *per se*, one can envisage that initially the products specified by R-factor resistance genes may have been involved more generally in the formation of biosynthetic intermediates or in transport or membrane processes. Smith, Harwood and Rubin point out a useful comparison in this respect with regard to an acetylating enzyme of *E. coli*, thiogalactoside transacetylase. This enzyme may aid in the active transport of galactosides into the cell, where the sugar is acetylated and eventually metabolized or, if non-metabolizable, excreted as an acetylated product. On the one hand, the enzyme may play a role in metabolism; on the other hand, since galactosides are often toxic to bacterial growth, the enzyme may serve, as Zabin proposes, to inactivate toxic galactosides. Thus it can be argued teleologically that enzymes, initially present to metabolize or detoxify general classes of compounds, have been selected for highly specific substrates over the course of evolution. The entire question of the origin and nature of the drug-resistance determinants may become more amenable to experimental attack when (and if) it becomes possible to isolate more strains carrying the drug-sensitive alleles present on R-factors.

Possibly the most intriguing hypothesis on the origin of the drug-resistance genes and the enzymes that they specify has been advanced by Raoul Benveniste and Julian Davies. These workers initiated a search in the actinomycetes for aminoglycoside-modifying enzymes like those that have been characterized in strains carrying R-factors. The actinomycetes are a group of organisms considered to be intermediate between bacteria and fungi, but their cellular dimensions, cytology, and genetic properties place them among the bacteria. One of the best appreciated properties of the actinomycetes is the extent to which they produce antibiotics; most of the aminoglycoside antibiotics are produced by them. Benveniste and Davies found that *Streptomyces kanamyceticus* (which produces kanamycins) contained an enzyme that acetylated the 6′-amino groups of kanamycin A and B, gentamicin C_{1a}, and neomycin. This was the same reaction as that catalyzed by R^+ *E. coli* which elaborate kanamycin acetyltransferase. In a similar vein *Streptomyces spectabilis* (a producer

of spectinomycin) was found to produce an enzyme that acetylated the 2′-amino groups of the hexose ring of gentamicin C_{1a}, which was identical to the reaction mediated by R^+ bacteria which produce gentamicin acetyltransferase II. The metabolic role of the aminoglycoside-modifying enzymes in actinomycetes is not known. They may be required for the synthesis of precursors of the finished antibiotic, for detoxification of antibiotics as is the case for R^+ bacteria, for transport of antibiotics in or out of the cell, or they may have nothing to do with antibiotic synthesis and may play a role in other biosynthetic processes. The discovery of aminoglycoside-modifying enzymes in actinomycetes catalyzing the same reactions as those found in clinical isolates of other bacteria suggests to Benveniste and Davies an origin for resistance determinants. Of course, they can only speculate as to the mechanism of the possible genetic transfer of genetic information from actinomycetes to other bacteria. Presumably genetic transfer by transduction, conjugation, or transformation may have occurred through a chain of related organisms even though the initial gene donor and final recipient may be totally unrelated bacterial species. It will be interesting to see whether this stimulating hypothesis gains any further experimental support. It somehow seems fitting that the very organisms that now provide us with antibiotics, may also be the origin of the genes that other bacteria employ to detoxify them!

For all of this speculation and skimpy experimental evidence, it nonetheless seems an inescapable conclusion that, whatever their origin, R-factors, as we now know them, were present in free-living bacteria before the widespread use of antibiotics by man. And one presumes that these R-factors conveyed some degree of selective advantage to their hosts. How much and what kind of selective advantage, one can only guess, particularly since we can roughly estimate that less than half of the theoretical complement of R-factor genes has been identified functionally. But, judging from the prevalence of R-factor-bearing bacteria in 'pre-antibiotic' populations, the selective advantage was not overwhelmingly profitable. Our own work (F. J. Skerman and S. Falkow, unpublished observations) with a 'virgin' population in Australia, for example, disclosed only a single composite R-factor in over 300 strains of *E. coli* isolated from man and his domestic animals. Yet 38% of these strains harboured a sex factor of some sort, which was capable of mobilizing a nontransmissible drug-resistance plasmid. In fact a fair proportion of the strains (about 7%) were multiply drug-resistant and contained nontransmissible plasmids. It seems fair to suppose, then, that obligate enteropathogens in the pre-antibiotic era would have had little chance to acquire, or to profit from, R-factors. Obviously the introduction of therapeutic antibiotics has now changed all this. The occasional free-living or facultatively pathogenic bacterial species bearing composite or dissociated R-factors have been able

to emerge in many circumstances as the predominant members of the microbial population. Given the small reservoir of R-factors that had accumulated in the 'pre-antibiotic' world, one can perhaps better appreciate how it was that R-factors emerged in so relatively short a time after the introduction of therapeutic agents.

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The transmission of R-factors *in vivo* and the public health implications of R-factors for man and his domestic animals

10.1 R-factor transfer *in vivo*

Whatever the origin of R-factors, the evidence cited in the previous chapters suggests they have been with us in some form for quite some time. Given an R-factor residing within an enteric organism, the immediate thought might be that R-factor transfer could occur quite readily to other enterobacterial strains. This conclusion would seem justified by the laboratory observation that R-factor transfer from one strain to another can usually be demonstrated without difficulty, even when it occurs at a low rate. The gut of a mammal is a far cry from a test tube, however, and from a practical point of view it is important to ask to what extent transfer occurs *in vivo*.

The available evidence indicates that transfer does not occur on so grand a scale *in vivo* as it does *in vitro*. To be sure there are a number of studies which have established that the transfer of R-factors can occur within the alimentary tract of man and animals. Most often the demonstration *in vivo* has been achieved by modifying the normal flora of the alimentary tract by first feeding with antibiotics before instilling prospective donor and recipient strains. Other workers have employed germ-free mice or newly hatched chicks, which possess a nearly sterile alimentary tract. All of these studies revealed that antibiotic resistance was transferred from one strain of *E. coli* to another or from *E. coli* to enteropathogens. Yet transfer was still of a relatively low order of magnitude even with the experimental modifications of the host's alimentary tract that were employed.

The transfer of antibiotic resistance in a host with a normal gut flora is even less pronounced. H. Williams Smith employed a human volunteer (himself) in which the predominant resident *E. coli* flora consisted of a serologically well-defined nalidixic acid-resistant mutant. Over a two year period various other *E. coli*, harbouring R-factors, were taken by mouth in the form of 24 h broth cultures. The flora were examined for the occurrence of nalidixic-acid-resistant residents that had acquired the donor R-factor. Some of the results are summarized in table 10.1. There is no doubt that transfer did indeed take place. Nonetheless there is no evidence of either an epidemic spread of R-factors among the resident *E. coli* or, for that matter, of persistence in the gut of those comparatively few resident organisms that had acquired R-factors. Of course, this series of experiments was performed in only one individual, but similar studies in animals also reveal that the detection of transfer *in vivo* is generally of a low order in the absence of antibiotic selection.

This latter point is extremely important, as demonstrated in some unpublished observations from my own laboratory. In these experiments,

Table 10.1. Transfer of antibiotic resistance from animal and human *E. coli* strains to the resident *E. coli* in the alimentary tract. The resident *E. coli* was a nalidixic acid-resistant mutant comprising >20% of the total *E. coli* flora. Donor strains were nalidixic acid-sensitive, and taken orally in the form of 24 h broth cultures. Faecal samples were smeared over the surface of a differential medium containing nalidixic acid and discs containing various antibiotics were applied. (Modified from H. Williams Smith, 1969.)

Source of donor strain	Pattern of resistance transfer *in vivo*	Dose of donor organism ingested	Number of days on which the donor strain formed <20% or >20% of the *E. coli* faecal flora		Resistance transfer to resident strain		
					Number of days detected	Number of colonies of resident strain per selection plate	Pattern transferred
			<20%	>20%			
Pig-2	Su-Sm-Tc-Km-Ap	10^9 (once)	3	2	2	1	Su-Ap-Km
		10^9 (once on six other occasions)	2-5	2-3	0	0	-
Ox-B63	Su-Sm-Tc-Cm	10^9 (once)	3	0	4	1-150	Su-Sm-Tc-Cm and Su-Sm-Cm
		10^9 (once on five other occasions)	2-4	0-1	0	0	-
Ox-B119	Su-Sm-Tc-Km	10^9 (once)	3	1	7	5-20	Su-Km
		10^9 (once on three other occasions)	1-3	0-1	0	0	-
		10^9 (daily for seven days)	11	2	18	5-10000	Sm-Km
Human-H-13	Su-Sm-Tc-Ap	10^6 (daily for seven days)	0	0	0	0	-
Human-1RH14	Su-Sm-Tc-Cm	10^9 (once)	10	0	0	0	-
		10^9 (once)	22	16	2	1-2	Tc
		10^9 (once)	35	25	2	1-2	Tc

a well-marked *E. coli* carrying a de-repressed R-factor was implanted as the predominant *E. coli* strain within the gut of a group of laboratory mice. As is well known, such de-repressed strains display a transfer frequency of $>10^{-1}$ per donor cell as compared with 10^{-3}–10^{-5} for most R-factor isolates. In fact when this prospective donor was tested *in vitro* with normal *E. coli* isolates from the mice, high-frequency R-factor transfer was almost invariably observed. Nonetheless, after the implantation of this donor within the alimentary tract of the mice, there was demonstrable evidence of R-factor transfer to other enteric residents in only one of ten animals over a two week period. The converted strains were few and their persistence, at best, was variable. Since the total number of coliforms within the gut of these experimental animals exceeded $10^7/0{\cdot}5$ g of faeces, of which about 20% were the donor strain, one might reasonably have expected that there was ample opportunity for mating. Also, the detection level was of the order of one resistant cell in 10^6. The animals were then fed with a single dose of streptomycin. Within seventy two hours, nine of the ten animals were shedding organisms that had received the de-repressed R-factor! The plasmid was predominantly observed in *E. coli* and *Enterobacter* but was also observed on one occasion each in *Serratia* and *Proteus*. These findings simply reaffirm earlier findings of others, the only difference being that the donor R-factor was deliberately chosen so that virtually any other strains that came with it should theoretically have been infected. In a 'normal' gut of a 'conventional mouse', R-factor transfer must have been of a low order since the selective use of antibiotics was necessary to detect converted strains.

Is there a clinical counterpart to this? In this connection it may be instructive to consider the findings of Aserkoff and Bennett, in the aftermath of an outbreak of *Salmonella* food poisoning. Roughly 1900 persons contracted *Salm. typhimurium* gastroenteritis after consuming turkey meat at a large outing. The *Salmonella* isolated from the contaminated meat was antibiotic-sensitive. A total of 272 patients were examined by faecal culture some twelve days after this point-source epidemic. As luck would have it, 87 patients had received no treatment, 97 had been treated with chloramphenicol, 48 with ampicillin, and the remainder with various other drugs. Not a single resistant *Salmonella* strain was isolated from the untreated patients. Eighteen resistant isolates were found in treated patients, however, and three-quarters of these strains carried an R-factor. Since the initial strain was drug-sensitive, it seems probable that the subsequent isolation of R-factor-bearing strains was a reflection of transfer *in vivo* from the enteric flora of the patients. Thus both in laboratory and in clinical situations, R-factor transfer within a normal alimentary tract appears to be of a low magnitude. Often it can be brought to a detectable level only upon the application of the selective force of antibiotic feeding. Is it that R-factor transfer occurs at an

extremely low frequency before feeding with the drug and that the drug selects for the ascendance of these rare strains? Or is it also that transfer does not occur at high frequency until the animal receives the drug; in other words, does the drug not only select for pre-existing resistant organisms but also favour transfer *in vivo*?

10.2 The best defence against R-factor transfer?

Shortly after leaving a sterile prenatal existence, man is subjected to a massive invasion by microorganisms, which quickly leads to the development of a reasonably stable gut flora. Although the representative kinds and the numbers of microorganisms present in the gut might lead one to believe that this is a chaotic process, recent evidence suggests this is a finely honed evolutionary product of long-standing relationships. This is never so clearly apparent as when something disturbs the normal equilibrium, and organisms of the well-populated large bowel progress into the small bowel and stomach, which normally contain only a few indigenous organisms. Then, as in the blind-loop syndrome, the normal flora can hamper digestion, compete for nutritional factors and intoxicate the host with microbial metabolic products. The full import of the normal intestinal flora of man remains a mystery[9]. In animals other than man there is clearly an important energy contribution from faecal microbial fermentation, but this is probably not a major property of man's flora. However, one major property of the intestinal flora of both man and animal is that they apparently can act as part of the host defence system and effectively interfere with the establishment of pathogenic bacteria in the intestine.

The normal flora not only acts to prevent the establishment of pathogenic organisms, such as *Salmonella* and *Shigella*, but equally to the establishment of nonpathogenic enteric species as well. The ingestion of just 'any' *E. coli* does not mean that it will be able to colonize the gut. This is far from the case. An ingested organism must be able to tolerate the acid of the stomach together with the low pH (about 6·1) of the intestines, the inhibitory activity of volatile fatty acids, a low oxidation–reduction potential (E_h about −200 mV) as well as a number of 'antagonistic' products produced by bowel inhabitants. Even at best the doubling time of an organism such as *E. coli* is five to twelve hours in the gut as compared with its nominal thirty minutes in broth.

Returning to R-factor transfer *in vivo*, however, one can see that a strain must be a reasonably good colonizer of the alimentary tract to be successful in carrying out eventual R-factor transfer. Even if an R^+ strain

[9] Haenal speculated that in the development of *Homo sapiens* it was necessary to reduce the frequency of emptying the bowel in the fight for survival. In return, man must carry the burden of billions of microbes in his large bowel, and a concomitant potential hazard to his health. Interestingly enough, during bio-isolation, for example, during space flights, the flora of man shows considerable simplification.

possesses enough genetic plasticity to colonize the bowel, it is not easy to see how there could be much R-factor transfer. The low E_h of the bowel is a reflection in part of the activity of aerobic organisms and, in fact, the predominant microorganisms of the 200–300 billion microbes per gram of faeces are strictly anaerobic bacteria. Studies on the physiology of conjugation of F-factors and of R-factors show that optimum conjugation is inhibited by similar anaerobic conditions and conditions of pH and fatty acid concentration. It is probably reasonable to conclude therefore that the normal bowel flora and their metabolic activity is largely responsible for the diminished transfer of R-factors that has been observed *in vivo*. By the same token, it is not so difficult to understand why the prevalence of R-factors from aboriginal populations and from normal urban populations still remains low in most parts of the world. The best defence against R-factor transfer therefore may be a healthy gut and a normal intestinal flora. The same mechanisms that operate to interfere with the multiplication of *Salmonella* in the intestine, low E_h and volatile fatty acids produced by normal gut flora, likewise seem to function to prohibit the epidemic spread of R-factors between members of the microflora.

Elimination of the normal flora from an animal might be expected to have a profound effect on the susceptibility of a host to infection and to R-factor-mediated transfer *in vivo* as well. In fact, the oral administration of streptomycin to mice increases their susceptibility to *Salm. enteriditis* infection from an IP_{50} value of 10^6 per mouse to less than 10 bacteria. Moreover it is a general practice in many laboratories to render animals susceptible to infection with *Shigella,* enteropathogenic *E. coli, Salmonella,* and even *Vibrio cholerae* by the oral administration of antibiotics. On a more practical level it has been established that the administration of an antibiotic may potentiate acute enteritis in a *Salmonella* carrier. Of course, the most-feared side effects of antibiotic-induced modification of bowel flora in clinical practice are enteric superinfections, of which staphylococcal entericolitis is the best known. Such sweeping changes in host susceptibility are more likely to be observed after administration of broad-spectrum drugs such as the tetracyclines, ampicillin, and some of the amino-glycosides. As noted above, it is much easier to detect the transmission of R-factors *in vivo* after drug administration under both clinical and laboratory circumstances. Guy Meynell has shown in mice that concomitant with the elimination of a large segment of the intestinal flora by a single dose of streptomycin, the faecal contents showed an average rise of 0·5 pH unit, and the mean E_h increased from −200 mV to +200 mV. Moreover the concentration of inhibitory volatile acids fell from an effective normality of 0·3 to 0·02. In other words there is a shift from conditions quite unsuitable to conjugation to conditions that are theoretically rather satisfactory. If these conditions are crudely

duplicated in the test tube, one sees about a 100-fold or greater increase in R-factor transmission. Such findings must be extended, and certainly it is dangerous to generalize. For example, R-factor transmission occurs at a quite high level in an avian host with a normal alimentary tract in the absence of antibiotics. Even in this contrary example, however, antibiotic administration usually increased the rate of transfer *in vivo*. It seems that antibiotic administration may be a double-edged sword, in that it can select not only for pre-existing drug-resistant cells, but also lead to conditions which favour their dissemination.

10.3 Public health implications of R-factors

Obviously the physician is often in a dilemma over whether or not to use antibiotics. It has been emphasized in academic medicine and by clinical microbiologists that ideally antibiotics should not be used prophylactically. Yet many clinicians feel strongly that their patients benefit from such use. All medical students are taught that antibiotics should be prescribed only after a diagnosis has been established and the sensitivity of the offending organism has been determined. This ideal is often not met, either by choice or because clinical circumstances dictate otherwise. Many physicians feel that antibiotic therapy is of little value in uncomplicated cases of *Salmonella* gastroenteritis and other forms of infectious diarrhoea, since these infections are largely self-limiting. But this attitude is by no means universally accepted. These are but a few examples of the continuing discussion about antibiotic usage, in which there is a spectrum of opinion. Maxwell Finland wrote in 1951 that certain people would give antibiotics to a mother before the baby was born, to the baby after it was born, and find an excuse for giving antibiotics until the individual died and was interred. Then, he suggests, they would inject antibiotics to make sure that deterioration of the body was slowed down or prevented. In general, however, the concern among physicians about the possible overgrowth of resistant organisms has led to a growing conservative view towards chemotherapy, so that antibiotics are now being used against infection more like rifles than shotguns.

The answers to the search for a rational use of antibiotics can only come from clinicians on the basis of controlled studies in which the welfare of the patient must be the important parameter. R-factors must, of course, be considered in these investigations since they and their analogues in the staphylococci (see chapter 12) constitute the largest reservoir of drug-resistance genes in two groups of organisms very commonly encountered in infection. It cannot be overemphasized, however, that despite the preponderance of articles in the literature on drug resistance, at present most enteric strains isolated from infection are relatively drug-sensitive and do not contain R-factors. The importance of R-factors to the public health is not simply a matter of whether antibiotic usage encourages the selection of resistant bacteria more difficult to treat

clinically, or even whether it encourages the transmission of resistance *in vivo*. Rather the questions are: does the presence of R-factors affect the epidemiology of infection; does the presence of R-factors and the general policies in the use of antibiotics adversely affect the community; and what are the possible long-term effects of selection for bacteria carrying resistance plasmids? "From a practical standpoint", says Pierce Gardner and David Smith, "the R-factor story has a clear moral for the physician: he must take cognizance of the public-health aspects of antibiotic usage and consider the effect of antibiotics on the communal ecology as well as on the individual and his illness".

10.3.1 Nosocomial infection

Hospital-acquired (nosocomial) infection has been discussed in a general way in chapter 4. A large proportion of these infections are directly related to the character of the patient population and to the type of therapy to which the patients are necessarily subjected. As noted earlier, nosocomial infections are increasingly associated with nonfastidious bacterial species, particularly Gram-negative rods, which are multiply antibiotic-resistant. The problem of nosocomial infection and the role that R-factors play in such infections can probably be best appreciated by examining a relatively small epidemiological unit, where the selective pressures of antibiotics may exert all its effects and the number of variables are more limited. The well-documented prospective study of a hospital in Denver, Colorado by Richard Selden and his associates seems worth considering in some detail as an illustrative example.

It was noted by bacteriologists at the Denver hospital during the summer of 1967 that some of the *Klebsiella pneumoniae* isolates from infected patients were resistant to an unusual number of antibiotics. Within several months the prevalence of this multiply resistant (Su, Sm, Tc, Cm, Km, Ap) *Klebsiella* had increased, particularly among patients in an intensive therapy unit. Most of the strain belonged to a single serotype, 30. Over the next year the organism was recovered from an average of 2% of all patients admitted to acute care wards. The organism was isolated from a variety of nosocomial infections, the commonest being the urinary tract, the next the respiratory tract, and then surgical wounds. To identify the reservoir of the multiply resistant *Klebsiella* in the hospital, bacteriological surveys of patients, hospital personnel, and the inanimate environment were carried out. Roughly 25% of the patients cultured on the acute care wards after residence for three or more days were intestinal carriers of the multiply resistant *Klebsiella*. In contrast the examination of a control group from a psychiatric ward within the hospital failed to reveal a single carrier. Among the hospital personnel only about 2% were intestinal carriers of the strain. Pharyngeal carriage was rare in all groups.

Since the endemic (epidemic?) *Klebsiella* strain was clearly associated with intestinal colonization of patients, patients on identical wards with

and without *Klebsiella* were compared. Several factors were significantly associated with intestinal colonization: urinary catheterization, inhalation therapy, and antibiotic therapy. The incidence of isolation of the strain from inanimate objects throughout the hospital is shown in table 10.2, where it can be seen that isolates were most commonly encountered from patient care areas. Clearly then a particular strain of *Klebsiella* type 30 had been selected within this hospital. The strain possessed several important attributes: it could survive in the inanimate environment; it was multiply resistant (because of an R-factor); and it was a successful colonizer of the intestinal tract subsequent to antibiotic therapy or certain hospital procedures that may have served as methods of inoculation. Although these studies clearly implicated intestinal colonization as the major reservoir of the strain within the hospital environment, Selden and his associates also examined, prospectively, the significance of *Klebsiella* intestinal carriage, regardless of antibiotic-resistance pattern, in the eventual appearance of overt infection.

Briefly, this study was conducted by examining all patients admitted on Mondays and Thursdays from October, 1968 through February, 1969 to general medicine, urology, ear–nose and throat, and general surgery wards. Cultures were taken within twenty-four hours of admission and twice weekly thereafter until the patient was discharged or developed a clinically significant *Klebsiella* infection. Ward procedures, surgery, antibiotic therapy, as well as clinical and laboratory findings, were duly recorded. A total of 31 out of 162 patients included in this study acquired a *Klebsiella* infection after admission. The commonest site of infection was the urinary tract (24 out of 31), and there were three respiratory infections and four surgical-wound infections. Seventeen of these 31 patients (about 55%) had been gastrointestinal carriers of the infecting strain before its first isolation from the infection. Eleven infected patients (about 35%) were not intestinal carriers, and the remaining three patients were carriers of *Klebsiella* serotypes other than the one isolated from their infection. The infection rate of *Klebsiella* nosocomial infection was highest for those patients who acquired this organism in their intestine after admission to the hospital. In contrast, only 3 out of 30 patients who were intestinal

Table 10.2. Isolation of multiply resistant *Klebsiella* from the hospital environment. (Adapted from Selden *et al.*, 1971.)

	Floor	Sink drain	Horizontal surface	Inhalation equipment	Germicide	Total
Patient rooms	4/25	5/12	3/38	4/30	0/4	16/109
Storage areas	1/4	1/3	1/14		0/4	3/25
Operating room	-	0/3	0/6	0/5	0/1	0/15
Nurses' station		0/1	0/6		0/6	0/13

carriers on admission subsequently developed an infection with the same serotype. The infection rate for those who were not intestinal carriers was similarly low (table 10.3).

Table 10.4 shows the serotype and antibiotic-resistance pattern of the *Klebsiella* strains isolated from the sites of infection. Of the eight serotypes encountered, the 'hospital' multiply resistant strain, serotype 30, accounted for about 35% of the nosocomial infections. It should be noted that the vast majority of strains were resistant to five or more antibiotics. In this context an interesting finding was that the initial *Klebsiella* isolate from 5 of the 31 infected patients was either sensitive to all antibiotics tested or resistant only to ampicillin. In 4 of these 5 patients, as shown in table 10.5, the same serotype subsequently isolated from the gastrointestinal tract, after interim antibiotic therapy, showed additional resistance traits. In each patient the antibiotic-resistance pattern of the *Klebsiella* isolated from the infection site was the same as the resistant intestinal isolate. Two of these strains were shown definitively to now possess a transmissible R-factor. Although it certainly appears that these strains acquired their R-factors by transmission *in vivo*, within the patient's gut, one cannot rule out the possibility that these resistant strains were initially present and later emerged under the

Table 10.3. Relationship of nosocomial infection to previous intestinal colonization. (Adapted from Selden *et al.*, 1971.)

Intestinal colonization	Number of patients	Number showing nosocomial infection	Number infected by same serotype as carried	Infection rate (%)
On admission	30	5	3	17
Acquired	31	15	14	48
None	101	11	0	11
Totals	162	31	17	19

Table 10.4. Antibiotic-resistance pattern of *Klebsiella* serotypes isolated from 31 nosocomial infections. (Adapted from Selden *et al.*, 1971.)

Antibiotic-resistance pattern						*Klebsiella* serotype								Total
Su	Sm	Tc	Cm	Km	Ap	2	16	20	21	30	31	47	63	
−	−	−	−	−	+	2			1					3
−	−	+	−	+	+						1		1	2
+	+	+	+	−	−		1	1				1	2	5
+	−	+	−	+	+				2					2
+	+	+	+	+	+				1	11		2	5	19
Totals						2	1	1	4	11	1	3	8	31

selective pressure of antibiotic therapy. Indeed, not unexpectedly, one of the significant factors associated with the acquired intestinal carriage of *Klebsiella* was antibiotic therapy (table 10.6).

It is to be emphasized that the experience in Denver is not unique. One could have chosen literally dozens of published accounts, which are qualitatively similar, concerning *E. coli, Serratia marcescens, Pseudomonas aeruginosa,* and other bacterial species in hospitals in England, Japan, Sweden, Germany, and throughout the world. All of these studies agree that Gram-negative rods are presently the leading agents found in nosocomial disease. The significant role that antibiotic resistance has played in establishing this microbial population in the hospital environment is obvious. The role that R-factors must play is equally obvious. In the light of previous discussion it is interesting to see that the transmission of R-factors *in vivo* seems of less importance than the colonization of patients by specific strains already harbouring an R-factor. The implied

Table 10.5. Acquisition of additional resistance determinants by *Klebsiella* during hospitalization. (From Selden *et al.*, 1971.)

Serotype	Drug-resistance pattern		Interval[a] (days)	Site of infection	Interim antibiotic therapy
	On admission	Infection isolate			
16	Ap	Tc-Sm-Cm-Ap	13	Urinary tract	Ampicillin and sulphonamide
20	Ap	Su-Sm-Tc-Cm-Ap	4	Urinary tract	Tetracycline
21	Sensitive	Su-Tc-Ap-Km	6	Surgical wound	Sulphonamide
31	Ap	Tc-Km-Ap	4	Urinary tract	Ampicillin

[a] Interval in days between admission and the development of overt infection.

Table 10.6. Antibiotic therapy and the acquisition of *Klebsiella* in the intestinal tract. Twenty-four patients who acquired intestinal *Klebsiella* after admission to the hospital were compared with 24 patients who remained negative for *Klebsiella* in all sites throughout hospitalization. Each patient in the control group was matched by ward, month and year, and duration of hospitalization. (Adapted from Selden *et al.*, 1971.)

Antibiotic therapy	Patients acquiring *Klebsiella*		Matched controls	
	Number	%	Number	%
One or more antibiotics	18	75	5	21
Cephalothin	5	21	0	0
Sulphonamides	7	29	1	4
Ampicillin	12	50	4	17

importance of R-factors and their mechanisms of transmission should not, however, lead one to ignore the bacteria that are involved in the disease processes. The R-factor is but a single component of total genetic apparatus of the colonizing strain, and almost certainly is not the primary reason for the 'success' of the organism. Such microorganisms are rarely the primary causative agent of a patient's disease. Rather they are robust bacteria (in the physiological sense) which can cause overt disease if they are inoculated by some hospital procedure into an antibiotic-induced vacuum present in a susceptible patient. Often these organisms are characterized as microbial opportunists. As has been pointed out, however, opportunism is a real property of every parasite and is dependent upon inherent pathogenic properties as well as the susceptibility of the host. Certainly it must be more than just opportunism or an R-factor that led to the ascendancy of the type 30 serotype of *Klebsiella* in the Denver hospital. It is in just this vein that Maxwell Finland warns us to keep a clear distinction between 'colonization' and 'disease'. He points out that if, during the course of chemotherapy, a primary causative organism (say a pneumococcus) disappears and another organism (say an *E. coli*) appears and even multiplies, so long as there is no evidence of change in the infectious process, such as a return of fever or inflammatory response, then one may be justified in considering this as colonization rather than disease. Mere presence does not necessarily correlate with disease in the patient, except in the sense that statistically the rate of subsequent infection is significantly higher in colonized antibiotic-treated patients. And it may be recalled (chapter 4) that patients with nosocomial infection tend to be the very young and the very old who have severe debilitating disease, and/or whose normal defence mechanisms have been breached by instruments, radiation, antibiotics or cytotoxic medication. Thus there are three general concomitants to nosocomial infection. First, there must be a susceptible ecological niche available for colonization. Second, there must be a bacterial vacuum or lowered host-defence mechanism, and third, the secondary invader must be available—and genetically competent.

Given the correct set of circumstances, bacterial strains are introduced into the hospital and then may cause an infection in an individual patient or, exceptionally, may circulate more or less permanently in the hospital as an epidemic or endemic strain. The question of how such 'successful' strains circulate, or in the specific example used above how *Klebsiella* serotype 30 was introduced into the intestinal tract of different patients, is one of the complexities of the epidemiology of nosocomial infection. In 'epidemic' situations the reservoir of the nosocomial pathogen is generally restricted to certain focal points and often can be traced to specific factors such as inhalation equipment or contaminated solutions. With regard to endemic nosocomial infection, however, the reservoir is more diffuse. Thus, in the Denver example, the onset and pattern of

multiply resistant *Klebsiella* infection may have been epidemic but, with the passage of time, was evolving into an endemic pattern. An important parameter in both epidemic and endemic nosocomial infection patterns appears to be patient-to-patient spread by hospital personnel. Indeed several studies show that relatively simple hygienic measures may significantly reduce colonization of patients with resistant organisms. The reason for such reemphasis on technique is underscored by the demonstration by Theodore Salzman and his fellow workers that over 20% of hospital employees harboured antibiotic-resistant coliforms on their hands. In the Denver experience two of eight nurses on the intensive therapy unit had *Klebsiella* type 30 on their hands. Despite the probable significant role of hand contamination in the colonization of patients, in no study have the hospital personnel been found to be intestinal carriers in significant numbers. Presumably, as suggested by Salzman, this absence of intestinal carriage is related to the obvious fact that the hospital personnel are not receiving antibiotics. Thus the high correlation between intestinal carriage and antibiotic treatment is probably completely analogous to the rendering of animals more susceptible to infection with a pathogen by feeding with antibiotic (see above).

The control of nosocomial infection depends upon reemphasis of aseptic technique and the search for common sources of contamination such as catheters, respirators, soap dispensers, etc. Where facilities permit, it has also been suggested that patients with open infections due to resistant organisms should be isolated. These relatively simple practices are extremely important and can be most effective deterrents. But (at the risk of being tiresome) the major selective force favouring the emergence and dissemination of multiply resistant bacteria of nosocomial infection is antibiotic usage. Carefully imposed restrictions in the use of antimicrobials for unnecessary topical and prophylactic use usually coincide with a sharp decrease in the emergence and interpatient transmission of resistant bacteria. Even with stringent regulations it is important to maintain a vigilant surveillance system to detect changes in antibiotic-sensitivity patterns among clinical isolates, which may herald a renewed nosocomial outbreak. Such vigilance will often serve to demonstrate the two mechanisms by which an R-factor may be distributed in nature: the spread of R-factors from cell to cell and the spread of a predominant host species carrying an R-factor. The availability of R-factor compatibility typing often gives an insight to the epidemiological basis for a nosocomial outbreak and its control. Datta and Hedges report, for example, that sudden outbreaks of nosocomial infection are sometimes associated with the demonstration of a plasmid belonging to a compatibility group seldom or never seen before in a particular institution. Thus the appearance of carbenicillin-resistant *Pseudomonas aeruginosa* in a burns unit, described by Roe and Lowbury, probably reflected both the use of the antibiotic

together with the epidemic spread of a P plasmid which could infect pseudomonads. Similarly the sudden appearance of one unusual biotype of *Klebsiella* sp. in three London hospitals was probably the result of a selective advantage conferred on a single strain because it carried a W plasmid which conferred resistance to the new drug, trimethoprim. It cannot be expected that many institutions will be able to do R-factor compatibility typing. However, Dr. H. Ericsson gives a simple rule of guidance: "the pattern of antibiotic resistance or sensitivity of organisms in the hospital, or indeed in any environment, is the negative imprint of the use of antibiotics in that environment". It is also, in large part, an imprint of the R-factor gene pool.

10.3.2 R-factors in the community-at-large

In chapter 4 the results of surveys were presented showing that enteric bacteria harbouring R-factors are reasonably common in 'healthy' individuals in the population-at-large and have been increasing in recent years. Moreover R-factor-bearing strains can complicate treatment in the physician's office but not at all to such a degree as seen in the hospital. The public health implications of R-factors in the community, however, may be of more profound interest from the standpoint of the long-term patterns of infectious disease. We know that some enormous changes have occurred in the aetiology of infection within the hospital; we further know that, on a far smaller scale, similar changes may be occurring within the community. The important question is whether the same problem of infection by indigenous flora is destined to replace other infectious disease outside the hospital or whether it will remain largely limited to the most susceptible members of the population, who would have inevitably ended up in a hospital anyway. Preliminary data suggest that there has been an increase in the number of Gram-negative infections coming into the hospital from the community. Moreover in some hospitals there appears to be an increased occurrence of *primary* Gram-negative bacillary pneumonia. But we do not have definitive data on these points yet and it seems absolutely imperative that we start to obtain good baseline data from large populations. All of this is speculative, but it does reflect the unhappy fact that we know relatively little about our 'normal' flora, the mechanisms of host resistance, production of disease, and microbial 'invasiveness'.

Thus the full import of R-factors within bacteria inhabiting the community population and the role they may play within this population in the future remains unknown. Additionally we remain almost totally ignorant about the source of these R-factor-bearing strains. We only know they exist in significant numbers. One hopes again that baseline and prospective studies will delineate the reservoir of these resistant species and indicate whether or not there is a significantly greater likelihood that colonized individuals in the community will later suffer infections from

these organisms rather than noncolonized counterparts. Even very young infants possess strains carrying R-factors.

Since most individuals in today's society begin life within a hospital, one may suppose that colonization could begin in earnest within the first few hours of life, when the gut is still empty and most susceptible to implantation. This simply is meant to point out that, of course, the hospital environment does not exist as a distinct entity from the community and there must be some degree of flow of microbes from the hsopital population to the community (and vice versa). Indeed I already know of one (so far unreported) instance in which a particular multiply resistant *Salmonella* strain, endemic in a hospital for about a year, suddenly spread to the community with particularly severe effects. Likewise Damato, Eitzman and Baer have shown that 45% of infants whose intestinal tracts were colonized by R-Km factors during their hospital stay were still excreting these organisms twelve months later and that 33% of the infants' family group also harboured the R-factor in their rectal flora. Notwithstanding the significant specialized microbiological pool within the hospital, the occurrence of R-factors in aboriginal populations and in the flora of infants born at home without any known intervening antibiotic therapy points to other less obvious selective pressures, which remain to be elucidated.

10.3.3 R-factors in animals

There is a striking parallel between the finding of an emerging drug-resistant human faecal flora and that observed in man's domestic animals. Since domestic animals have been given antibiotics in much the same way as man to control infectious disease, it is not surprising that R-factors have been found in veterinary medicine. In man, severe enteric disease outside of hospitals is now relatively uncommon in technologically developed countries. H. Williams Smith points out that the very opposite is often true in veterinary medicine. Animals kept by human societies are intensively reared to yield the greatest amount of produce (protein) in the least possible space. Smith points out that such conditions are ideal for the outbreak of disease caused by enteric bacteria, and he cites as an example the fact that in Britain (and elsewhere) *E. coli* is generally considered the most important single cause of mortality in swine. The drugs employed to treat animals are essentially identical with those used in man, so that one expects a similar ascendence of R-factor strains and complications in the treatment of serious veterinary infections. Aside from the significance to veterinary medicine, the emergence of resistant bacterial populations in animals has human public health implications, the most obvious being that the major source of *Salmonella* infection for man is meat and meat products. One would also like to know whether other bacterial species bearing R-factors reach humans through the food chain.

If one were simply to consider the emergence of resistant strains in animals associated with therapeutic use of antibiotics, I suppose that the situation would largely mimic that already described for man. The entire problem of bacterial drug resistance in animals is incredibly complicated by the fact that most classes of animal grown for food are fed continuously on diets containing small supplements of antibiotics, which have as their purpose the stimulation of animal growth. This has become a common farm practice, particularly in the USA and in Britain, since the 'fifties. From the outset of this practice there was concern about the public health implications of the addition of small amounts of antibiotics to animal feed. But it was argued that the economic gain overshadowed any minimal health risk since there was a ready supply of new antibiotics and relatively few enteric bacteria of animal origin that can colonize the human gut. Indeed this view is still strongly held by many investigators. Nonetheless the changes in patterns of antibiotic resistance in animal *Salmonella* and *E. coli* during the past decade leave little doubt that there has been an enormous ecological change, which mirrors the use of antibiotic-supplemented feed and the therapeutic use of antibiotics in animals. It is not a completely uniform picture, variations occurring with bacterial species and, to some extent, animal species. For the *Salmonella* which cause clinical salmonellosis in animals, P. A. M. Guinee has found that, in the Netherlands, *Salm. dublin* and *Salm. cholerasuis*, which cause systemic infection, have shown a relatively small rise in resistance from 1956 to 1962. Resistance of *Salm. dublin*, for example, rose from 1·4% resistance to tetracycline in 1959 to only 4·17% in 1962. The experience in the Netherlands, USA, and Britain with *Salm. typhimurium*, which causes gastroenteritis but also asymptomatically colonizes the animal gut, is entirely different. In England, for example, Anderson showed that the incidence of antibiotic-resistant strains of *Salm. typhimurium* increased from 3·3% in 1962 to 61% in 1965, with strains often resistant to as many as five drugs. As might be expected the majority of these isolates owed their resistance to R-factors. Both common and enteropathogenic species of *E. coli* isolated from animals have also become predominantly resistant. This resistance is directly attributable to the employment of antibiotic-supplemented feeds. It is evidenced by many observations that the alimentary tracts of animals which were not fed with antibiotics remain relatively free of drug-resistant strains, whereas animals continuously fed with small amounts of antibiotics almost uniformly contain drug-resistant *E. coli* (table 10.7). The resistance is usually transmissible, but often only singly resistant, depending upon the drug employed (usually tetracycline). Studies by Smith have shown that probably the majority of the pig population in Britain has organisms that are tetracycline-resistant. An additional finding of particular significance is that the tetracycline-resistant *E. coli* present in the swine population appear to be able to retain their dominant position for quite long periods when their

environment becomes antibiotic-free. Lest one conclude that the effect of using such diets is restricted to the enteric bacteria, it should be noted that intestinal lactobacilli and streptococci likewise rapidly become resistant to the included antibiotic. Moreover there is a sharp increase in incidence of antibiotic-resistant *Staphylococcus aureus* on the skin and external nares of pigs and fowl fed on diets containing penicillin and tetracycline. This increased incidence is reflected as well on the skin and external nares of the human attendants of the animals.

The patterns of drug resistance in animals are still changing. Although R-factor resistance has been predominantly solely towards the drug employed in the diet, the tendency during the past few years has been towards a multiply resistant pattern. Obviously the use of a single drug is perfectly suitable for the selection of a multiply resistant R-factor. Because of the large reservoir of drug-resistant strains in animals, it has become increasingly common to find resistant, often multiply resistant, *E. coli* in animals on an antibiotic-free diet or even in newborn animals (table 10.8). The problem is not just confined to our warm-blooded domestic animals. Watanabe *et al.* showed in 1972 that antibiotics and other chemotherapeutic agents used for treating infections in cultured fish are associated with a dramatic rise in R-factor-mediated drug-resistant Gram-negative organisms in the water and in the intestines of the fish.

What are the hazards to human health of a predominantly antibiotic-resistant flora in domestic animals? This question has been the subject of an emotional debate, as much economic and political as it is scientific. To the minds of many investigators the selection of any large reservoir of multiply antibiotic-resistant bacteria constitutes a major hazard to the

Table 10.7. An example of the effect of feeding with tetracycline on the incidence of resistant *E. coli.* Data from the Netherlands (adapted from Guinee, 1971).

	Feeding of drug	Number with resistant *E. coli*/number tested	Percentage of resistant *E. coli* in flora
Newborn calves	None	1/20	<1
Fattening calves	Tetracycline (50 p.p.m.)	75/75	10-100
Breeding calves	None	0/20	-

Table 10.8. Increasing prevalence of antibiotic-resistant bacteria in animals not given drugs in their diets. Data from the Netherlands (adapted from Guinee, 1971).

Animal	Number with resistant *E. coli*/number tested	
	1961	1971
Adult cattle	0/32	19/26
Horses	0/6	16/39

health of both humans and animals. But the major debate has revolved about whether the resistant bacteria are capable of entering the human body and causing disease problems which were not present before the development of resistance. The potential hazard to man seems rather obvious, if judged solely on the basis that: 1, antibiotics, even in the small amounts used to promote animal growth, render animals (including man) more susceptible to colonization by *Salmonella* sp., and has probably enlarged the reservoir of these organisms; 2, *Salm. typhimurium* in animals often carry R-factors; 3, meat and meat products are the main direct and indirect source of *Salmonella* infection in man, and 4, a high percentage of all human isolates of *Salm. typhimurium* carry R-factors, and the resistance pattern is correlated very highly with that seen in animals. The proof that the hazard was not only potential but real came from the epidemiological studies of E. S. Anderson and his associates on phage type 29 of *Salm. typhimurium*. These studies clearly documented that human disease and death resulted from multiply resistant *Salmonella*, which had acquired this resistance through the use of antibiotics in calves. Although even the most zealous proponents of antibiotic-supplemented diets concede that this practice selects for R-factor-bearing bacteria and has some hypothetical potential hazard, they do not consider Anderson's findings to be sufficient proof. Dr. Thomas H. Jukes, the discoverer of the growth-promoting effects of antibiotics in animal feeding, points out that the outbreak studied by Anderson appears to have been due to "shockingly poor sanitation", a wide dispersal of infected animals, and poor therapeutic use of antibiotics rather than use of antibiotic-containing foods. Indeed, in the continuing debate, the argument is usually raised that, outside of Anderson's findings, no other 'hard' epidemiological data exist relating resistant strains of animal origin to human disease. In the USA it is argued that, if human disease problems have increased concomitantly with feeding of antibiotics, it is not a significant problem since a dramatic change in the frequency has not occurred.

Aside from the *Salmonella* problem, several investigators have attempted to determine whether there is a connection between the animal reservoir of resistant *E. coli* and its increasing prevalence in man. In England E. M. Cooke, R. A. Shooter and fellow workers found one hospital environment in which new strains of *E. coli* were constantly entering the patients' gut flora through the hospital food. It is conceded, however, that the situation was perhaps exceptional because of unhygienic practices. Nonetheless, although many serotypes of *E. coli* belong primarily to animals and do not establish themselves in humans (and vice versa), some serotypes survive well in both animal and humans. Studies throughout the world have clearly shown that contamination of carcasses with intestinal microorganisms during the slaughtering process cannot be prevented. In this way meat and meat products, contaminated with resistant *E. coli* (and *Salmonella*), reach the human consumer (table 10.9). Guinee showed that

meat-eaters are no more likely to harbour R-factor resistant organisms than vegetarians (see the survey in chapter 3), but obviously one cannot conclude from this finding that meat and meat products do not play a role in the colonization of humans with resistant strains. Obviously, if animals do contribute significantly to either the bacterial or the plasmid reservoir of human bacterial strains, then one should expect that farm workers or families with livestock might show a different pattern from their urban counterparts. In fact the total numbers of individuals with resistant *E. coli* and the percentage of strains harbouring R-factors do not appear to be significantly different between rural and urban inhabitants. What does appear to be different, however, is that farm workers show a quantitatively higher proportion of resistant cells in their total enterobacterial flora than do urban residents (table 10.10). Moreover, Ellen Moorhouse has obtained evidence to show that whereas the incidence of R-factors is not significantly different between rural infants and urban infants, the proportion of infants excreting *E. coli* carrying R-factors conferring resistance to three or more antibiotics is more common in rural infants whose families had contact with livestock (table 10.11). Thus there do appear to be some ecological differences in

Table 10.9. Isolation of resistant *E. coli* in meat products. Data from the Republic of Ireland (Moorhouse *et al.*, 1969).

Specimen	Number	Number with resistant *E. coli*	Number with R$^+$ *E. coli*
Shop sausages[a]			
Skins	21	8	4
Meat	21	6	3
Pigs' faecal swabs	12	11	10
Carcas meat	3	3	2
Factory sausages			
Skins (uncooked)	6	6	4
Meat (uncooked)	6	4	3
Skins (cooked)	6	0	-
Meat (cooked)	6	4	3

[a] Twenty-one different shops, which sold sausages produced on the premises.

Table 10.10. Percentages of resistant cells in the enterobacterial flora of urban residents and farm workers. Data from Germany (from Wiedemann and Knothe, 1971).

	Number of individuals			
Percentage of resistant enterobacteria	$<10^{-4}$	10^{-4}-10^{-2}	10^{-2}-1	over 1
Urban residents	21	34	32	1
Farm workers	7	25	34	33

the quantitative numbers of resistant bacteria and the complexity of resistance seen in rural as compared with urban populations. The data do not, of course, definitively establish that the rural population has shown this shift in their *E. coli* flora because of livestock—but it certainly seems a reasonable hypothesis and suggests this is one route by which the strains enter the population or, barring colonization, how new R-factors may enter the human plasmid reservoir.

The concern about feeding animals with antibiotics generated enough concern to the governments of Great Britain and the USA to cause the establishment of 'blue ribbon' panels to study the question in depth and provide appropriate recommendations. After months of testimony, in which the committees were asked to balance the benefits of feeding against the risks, recommendations relative to the use of antibiotics have been made by both groups. The Swann Committee report from Great Britain grouped antibiotics into 'feed' and 'therapeutic' categories, and suggests that antibiotics used as feed supplements should not include those drugs used for the therapeutic treatment of disease in man or animals. Such therapeutic drugs, they recommend, should be reserved for limited use in animals only on prescription by veterinarians. The USA has recommended that tetracyclines, streptomycin, sulphonamides, and penicillin be prohibited from their present use as growth promoters by 31st December 1973[10]. Further, the USA report recommends that tetracyclines, streptomycin, neomycin, spectinomycin, penicillins, and the sulphonamides be reserved for therapy, and should be available to farmers only on a veterinarian's prescription, for use at therapeutic doses for short-term treatments only. Chloramphenicol, the semisynthetic

Table 10.11. Resistance patterns of *E. coli* isolated from urban and rural infants. Data from Republic of Ireland (modified from Moorhouse, 1971).

	Urban		Rural	
	Distribution of total resistance (%)	Distribution of R-factors (%)	Distribution of total resistance (%)	Distribution of R-factors (%)
Resistance to:				
One antibiotic	33·8	27·0	25·9	12·3
Two antibiotics	35·3	38·1	18·6	14·7
Three antibiotics	14·1	15·9	33·4	43·9
Four antibiotics	11·2	12·7	18·6	24·4
Five or more antibiotics	4·6	6·3	3·5	4·7
	100	100	100	100

[10] This deadline has been extended to April 1975 to provide for the submission of data by interested parties (mostly pharmaceutical houses) to show that antibiotic-supplemented feeds are not potentially detrimental to the health of animals or man.

penicillins, gentamicin, and kanamycin were also specifically prohibited from use in animal feeds. If these kinds of recommendations are implemented politically, they should help to stem the rise of drug-resistant organisms which would not be amenable to present chemotherapeutic agents and those developed in future. Nonetheless the present reservoir of tetracycline-resistant plasmids in animal *E. coli* strains would not disappear very quickly even if the suggestions were to be implemented tomorrow. The R-factors appear to be relatively stable in the bacterial populations and, as Smith has observed, can almost be looked upon as a normal component of the genetic apparatus of *E. coli* residents in certain animals. One may reasonably suspect that as other substances are substituted for penicillin and the tetracyclines as growth-promoting additives of animal diets, new plasmid genes may appear. Since, of course, the feeding with a single agent selects for all genes carried on a plasmid, the problem may still remain most difficult to manage. For example, spectinomycin was released for therapeutic use in human and animal infections only a relatively short time ago. Yet selection for Sm^r R-factors has resulted in a 'preexisting' reservoir of spectinomycin-resistance genes. The drug bacitracin would qualify as a feed additive, since it has growth-stimulating activity in animals and it is not used extensively therapeutically. Yet were it to be employed extensively in animal feeds, would selection for plasmid genes specifying bacitracin resistance also result in coselection for genes (say for penicillin resistance) which may have a limiting effect on animal and human antibiotic therapy? From a bacteriological standpoint the addition of drugs to animal feeds may prove to be indefensible in the long run. Yet this may be too pessimistic a view. A number of drugs, such as nalidixic acid, the furan derivatives, and the polymyxins, enjoy fairly wide use therapeutically in man and animals, but resistance is still rare and, when present, almost never found on R-factors. No one really knows why this is the case but, at any rate, it does show that antibiotic classes do exist that might conceivably serve both the farmer and the physician. Moreover one drug, flavomycin (flavophospholipol), now being used as a feed additive in many countries (although not to my knowledge in the USA or Great Britain), actually is far more effective against bacteria carrying all classes of sex factors than against impotent strains. Apparently the presence of sex pili is responsible for the increased sensitivity, since cells carrying de-repressed sex factors are even more sensitive than those which show repressed pili synthesis. Thus flavomycin seems to satisfy the requirement of the Swann and US committees—for the time being anyway.

The data presented in this chapter from hospitalized patients, the population-at-large, and domestic animals all show that there has been a profound selection for plasmid-containing bacteria in the intestinal flora. The full ecological implications of this man-made increased incidence of transfer factors in enteric organisms (quite apart from any drug-resistance

genes already carried) remains to be seen. Has man hastened an evolutionary tendency for the dissemination of transfer factors in Nature, or could it be that we have reversed an evolutionary trend? All we know is that there has been a dramatic change from what was the steady state a decade ago.

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11

Plasmids which contribute to the pathogenicity of enteric organisms

11.1 Plasmids and enteric disease

Even in very comprehensive epidemiological surveys, the well-characterized enteric pathogens, *Salmonellae* and *Shigellae*, as well as known viral and protozoan agents, account for less than half of the cases of infectious diarrhoea. In the past ten years there has been more and more compelling evidence to show that *E. coli* and other members of the 'normal' gut flora are causally related to a significant proportion of diarrhoeal disease seen in man and his domestic animals. These *E. coli* strains provoke diarrhoea by either of two general processes: a dysentery-like mechanism in which the organism invades the intestinal mucosa and elicits an acute inflammatory response; or a cholera-like mechanism in which the organism multiplies solely within the lumen of the bowel and exerts its effect by producing an enterotoxin that stimulates the secretion of water and electrolytes.

Veterinary scientists have provided the most detailed information on the causal relationship of *E. coli* to diarrhoeal disease through their studies on 'scouring' in calves and 'oedema' disease in piglets. For example, one characteristic common to enteropathogenic strains of *E. coli* of porcine origin is their ability to produce enterotoxin. A large proportion of these toxigenic strains also produce an α-haemolysin and possess a common surface antigen K88*ab* or *ac* (referred to hereafter as K88 for simplicity). Our perspectives of the biology of these toxigenic *E. coli* were enormously increased in the mid-1960's by the findings of Ørskov and Ørskov, and by Smith and Halls, that K88, enterotoxin, and haemolysin production were often governed by transmissible plasmids designated K88, Ent, and Hly, respectively. H. Williams Smith and Margaret Linggood further showed that transmission of K88 and Ent to normal resident *E. coli* from healthy pigs sometimes 'converted' them into a form which experimentally produced a disease in piglets, this being indistinguishable from that produced by naturally occurring enteropathogenic *E. coli*. Thus, in *some but not all* strains the acquisition of these two plasmids was enough of an addition to a strain's genetic potential to tip the balance from that of a nonpathogenic commensal of the normal flora, to that of a strain now capable of producing overt disease. In parallel with the studies in domestic animals, enterotoxin production by *E. coli* isolated from human adults and children have also been shown to be plasmid-mediated. The implications of these findings have led to a concerted effort by several laboratories to define better the genetic and molecular nature of Ent, K88, Hly, and analogous plasmids, as well as the mechanisms by which these plasmids modify a bacterial cell so as to permit them to colonize and cause disease in an animal host.

11.2 The *E. coli* enterotoxins, the K88 antigen, the α-haemolysin and their detection

It is easy to employ antibiotic discs on a plate of nutrient medium to determine the drug-resistance traits carried by a strain, or to streak a plate of differential medium to determine the sugar fermentation properties of an isolate. However, no bacteriological medium exists at present which serves to distinguish a toxigenic *E. coli* strain from a nontoxigenic strain. It is therefore necessary to design the genetic analysis of Ent, K88, Hly, and analogous plasmids, around the technical limitations of their detection.

11.2.1 The *E. coli* enterotoxins

The enterotoxins produced by *E. coli* are found in cell-free supernatant fluid after the growth of cells in broth (that is to say, they are exotoxins). They are conveniently divided into two classes (table 11.1). One class, a heat stable toxin (ST), is of low molecular weight and is apparently nonantigenic. The response of a sensitive bowel to this toxin is characterized by an immediate accumulation of fluid in the bowel lumen, but the duration of its action is short-lived. The second class of *E. coli* enterotoxin is inactivated by heating at 65°C for ten minutes and is called labile toxin (LT). The LT toxin appears to be a high-molecular-weight protein and can stimulate neutralizing antibodies. The response of the bowel to LT is usually delayed but the accumulation of fluid in the bowel lumen is sustained for a long period of time. Neither the ST nor the LT toxin has been purified to any great degree despite concerted efforts by several groups; hence the concept of high and low doses is relative, and generally refers to a volume of culture supernatant fluid or the amount of lyophilized culture filtrate required to elicit a response in a test animal under a given set of experimental conditions.

The mode of action of the ST toxin has remained largely enigmatic, but during the past few years a good deal has been learned about the LT toxin. One of the most important findings has been that the *E. coli* LT toxin bears a partial antigenic identity with the well-characterized

Table 11.1. Properties of heat stable (ST) and heat labile (LT) *E. coli* enterotoxins.

	ST	LT
Resistance to 65°C	+	−
Resistance to acid	+	−
Size	1000–10000	82000 (protein)
Effect on gut secretion	Rapid onset, short duration	Slow onset, prolonged duration
Stimulation of adenyl cyclase	−	+
Stimulation of neutralizing antibody	−	+

enterotoxin of *Vibrio cholerae*. The effects of cholera toxin on the epithelial cells of the small bowel have been linked to the stimulation of the adenyl-cyclase–cyclic 3′–5′-adenosine monophosphate (cyclic-AMP) system which leads to the secretion of water and electrolytes into the bowel lumen. It now seems apparent that the effects of the *E. coli* LT enterotoxin are also mediated through this same mechanism. The effects of a toxigenic *E. coli* infection in man are generally not so severe as that seen in patients suffering from cholera. Perhaps this is a reflection that smaller amounts of toxin are produced by *E. coli* or that the part of the *E. coli* toxin molecule that is unrelated to cholera mitigates its effects to some degree.

The adult-rabbit ligated-ileal-loop method has been the recognized standard technique for the assay of both ST and LT enterotoxins. Basically this method involves exposing surgically the small bowel of a rabbit and isolating small segments along its length with surgical thread. Segments are then injected intraluminally with culture supernatant fluid or with whole cells; some ligated segments are uninoculated or injected with the supernatant fluid of known toxigenic and nontoxigenic strains to serve as controls. The bowel is then replaced and the abdomen closed. Sometime later the animal is sacrificed and the small bowel excised. The volume of fluid in each ligated segment is measured and the lengths of the segments are determined; the volume per length ratios (ml/cm) are then employed to roughly quantitate the response. Ratios of 0·1 or less are seen in negative controls. The ST toxin usually gives a maximal response within six to eight hours with a ratio of about 0·4–0·6, while the LT toxin generally gives maximal ratios of about 1·0 to 2·0 in eighteen to twenty-four hours. The assay can be used to measure the effects of neutralizing antibody or inhibitors etc. Unfortunately the method works best with the LT toxin and may be negative with some preparations, particularly of ST. Moreover the sensitivity of the small bowel to both enterotoxins decreases as one moves from the anterior small bowel towards the ileo–caecal valve. This reflects the fact that both toxins affect only small-bowel epithelium and that the toxigenic diarrhoeas are basically diseases of the small bowel. The *E. coli* ST toxin is often assayed by the simple alternative of injecting culture supernatant fluid intragastrically into the milk-filled stomach of a one to three day old infant mouse (plate 9). The most reproducible results for any toxin are obtained if one can prepare ligated segments in the same animal from which the toxigenic strains were isolated. Fortunately in the past year the assay of the LT toxin has been considerably simplified as a result of the observation in several laboratories that tissue culture monolayers of mouse adrenal cells or chinese hamster ovary cells react to LT (and cholera toxin) by undergoing morphological changes and increasing their steroid production (plate 10). These changes are correlated with the effects of toxin on the adenyl-cyclase–cyclic-AMP systems of the cells.

The tissue culture method is sensitive and holds great promise as a means for screening cultures in epidemiological surveys, evaluating the effects of toxin inhibitors, and assessing the antibody content of sera. The drawback is that it cannot detect *E. coli* ST toxin. Presumably the enteric effects of the ST toxin are mediated by mechanisms not directly involving the adenyl-cyclase–cyclic-AMP systems.

Suffice it to say that, from the standpoint of a clinical laboratory, the tests for *E. coli* enterotoxin are cumbersome. From the standpoint of the microbial geneticist who may be required to score hundreds of colonies from a mating, these tests are a nightmare. It is also important to keep in mind that these laboratory assays of ST and LT are tests for toxigenicity and not necessarily for the entero-pathogenicity of the strain that elaborates the toxin. It will become apparent that this distinction is quite important.

11.2.2 The K88 antigen and α-haemolysin

K88 is a surface antigen of *E. coli* and is classified as a thermolabile L type. Most K-specific substances have been found to be acidic polysaccharides. K88 is the first *E. coli* K antigen shown to be a protein. It is a large filamentous protein ($s^0_{20w} = 36 \cdot 7S$) and contains all the common amino acids except cysteine–cystine. Transfer of the K88 plasmid to a cell line which is nonflagellated and does not contain Type 1 pili (see chapter 2) has revealed that the K88 antigen is manifested on the surface of the bacterial cell as a 'fur of fine filaments'. These filaments have a measured length of 0·1 to 1·5 μm and a diameter of 70 to 110 Å. They differ from common Type 1 pili in their amino acid composition (that is to say, Type 1 pilin protein contains cysteine–cystine, but no tryptophan or methionine). Moreover the K88 filaments are somewhat thinner and more flexible than Type 1 pili. The fine 'fur' of K88 are not the sex pili associated with a transfer factor, since the two can be readily distinguished with donor-specific bacteriophages. The filaments of K88 confer an interesting property of adhesiveness upon the host cell, so that they can 'stick' to red blood cells and to epithelial cells. As will be shown below this adhesiveness seems important in the pathogenesis of diarrhoeal disease.

The detection of cells carrying the K88 determinant is reasonably straightforward. In some instances (depending upon the strain) $K88^+$ clones can be distinguished from $K88^-$ clones by their darker yellow colour on agar plates containing bromthymol blue and a fermentable carbohydrate. A totally reliable method is to prepare monospecific K88 antiserum which can be employed in a slide agglutination test. Of course, this is not a substitute for a sensitive method of genetic selection but one can screen a reasonable number of colonies for K88 with this method. K88 antigen appears to be associated solely with

strains of porcine origin. Another K antigen, also plasmid-mediated, has been associated with toxigenic strains from lambs and calves. Very little is known about this K antigen, called K99, although its contribution to pathogenicity appears to be analogous to that of K88 (see below). K99 can also be reliably detected by slide agglutination tests with appropriate absorbed antisera.

The precise biochemical nature of the α-haemolysin carried by Hly plasmids is not known. Obviously, this phenotype is easily detected on nutrient medium containing washed red blood cells. Indeed, compared with the detection of K88 and Ent, Hly detection is quite sensitive since it is possible to see a single haemolytic colony among several thousand nonhaemolytic sister clones.

11.3 The genetic transfer of Ent, K88, and Hly and their incidence in normal and toxigenic *E. coli*

In the absence of any direct sensitive genetic selection method, the study of the transfer of Ent, K88, and Hly has to rely upon methods which simply maximize the probability of plasmid transfer between a toxigenic cell line and a suitable recipient cell line. Of these, the most direct method is to mix a nalidixic-acid-sensitive toxigenic culture with a nalidixic-acid-resistant *E. coli* (*E. coli* K-12 F^- sublines are adequate) and pass the mixture serially two or three times in nutrient broth. The mixture can finally be plated on a blood–agar medium containing nalidixic acid, which eliminates the donor cells but permits the recipient cells to grow normally. Any recipient clones receiving Hly can be immediately distinguished by means of zones of haemolysis surrounding them, and individual Hly^+ and Hly^- clones can also be picked for K88 slide agglutination tests, and for testing in an animal or tissue culture system for Ent. Another alternative is to use the resistance-mobilization test (see chapter 5), which determines whether a strain has any transfer factor capable of mobilizing a nontransmissible R-plasmid. The rationale here is that if Ent, Hly, or K88 are present as transmissible plasmids in a toxigenic strain, they will mobilize the R-plasmid into a suitable drug-sensitive recipient cell. In this way one can at least concentrate on recipient cells known to have mated, and determine whether the recipient cells which have received the nontransmissible R-plasmid have also received Ent, K88, or Hly from the toxigenic donor strain. Clearly, no matter what method is employed, Ent, K88, and Hly transfer will be demonstrated only when they are directly or indirectly transmitted at a relatively high rate.

Table 11.2 summarizes the results of a survey of plasmid incidence in toxigenic and nontoxigenic *E. coli* isolates from man, pigs, and calves, as determined by the resistance-mobilization test. It can be seen that some 35% of *E. coli* isolated from asymptomatic individuals were

found to carry a detectable transfer factor capable of mobilizing a nontransmissible drug-resistance plasmid. In striking contrast, well over 90% of toxigenic *E. coli* possessed a transfer factor of some sort capable of R-determinant mobilization. The recipient clones which received the nontransmissible R-plasmid were further screened to determine whether they also received a Col, Hly, K88, or Ent determinant from the donor culture. Although all of the toxigenic strains were selected precisely because they elaborated an enterotoxin, only 21% of the toxigenic strains could be shown to harbour an Ent plasmid. The K88 determinant was transmissible from 30 of 60 strains that showed this property. Both normal and toxigenic *E. coli* were found to possess transmissible Hly determinants, although the incidence of transmissible Hly was considerably higher (24 of 43) from toxigenic haemolytic strains than was observed for haemolytic normal strains (8 of 31). In contrast, the incidence of transmissible Col factors in normal and toxigenic *E. coli* was roughly the same.

Obviously toxigenic *E. coli* as a group have engaged in conjugation more often than 'normal' *E. coli*. While many toxigenic *E. coli* have demonstrable Hly, Ent, and K88 plasmids, most of the transfer factors

Table 11.2. Incidence of plasmids in toxigenic *E. coli*, and in *E. coli* isolated from healthy individuals. *E. coli* from asymptomatic individuals and toxigenic *E. coli* were tested for the production of colicin (Col); haemolysin (Hly); K88 or K99 antigens (K), and the production of enterotoxin (Ent). Each strain was then tested in the resistance-mobilization test to determine if they carried a transfer factor of any kind. For each mating that gave a positive resistance-mobilization test, about 100 recipient clones were tested to see if they had also received Col, Hly, K, or Ent. [Data from Gyles, So and Falkow (1974) and unpublished observations by the author.]

Source	Number of strains	Properties of strains:				Number containing transfer factor of any kind	Number containing transmissible			
		Col	Hly	K	Ent		Col	Hly	K	Ent
E. coli of the normal flora										
Human	43	3	8	0	0	12	3	0	-	-
Bovine	62	20	12	0	0	24	12	4	-	-
Porcine	37	32	31	0	0	14	6	4	-	-
Total	142	55	51	0	0	50 (35·2%)	22	8	-	-
Toxigenic E. coli										
Human	24	3	10	?	24	18	4	2	?	4
Bovine	4	0	0	4	4	4	0	0	4[a]	4
Porcine	72	10	42	56	72	69	9	22	26[b]	13
Total	100	13	52	60	100	91 (91%)	13	24	30	21

[a] K99; [b] K88.

that were detected in these strains by the resistance-mobilization test could not be associated with any *known* function. The significance of this finding is not known. It is possible that this higher incidence of transfer factors is associated with attributes other than Ent, Hly, and K88 which contribute to the pathogenicity of these organisms. In my mind a simpler alternative is that strains which are toxigenic were simply better genetic recipients than were the 'run-of-the-mill' *E. coli*. Perhaps this property itself is a useful attribute for a successful enteric pathogen?

One of the prominent features of toxigenic strains is that they often carry a variety of transmissible determinants. For example, 17 of the toxigenic strains employed in the survey of table 11.2 were ultimately found to carry Ent together with Hly or K88. Indeed several of these strains harboured a transmissible Col, Hly, K88, and Ent plasmid simultaneously. In instances such as these, it can almost always be shown that each of the transmissible determinants resides on a distinct entity that segregates in a genetic cross. Table 11.3 shows one example of the genetic segregation of determinants among recipient cells mated with a wild-type donor *E. coli* with Ent, Hly, K88, Col, and an R-factor. Thus K88, Ent, and Hly generally reside on distinct plasmid species that appear to be mutually compatible with each other.

The genetic analysis of Ent$^+$ recipients has consistently revealed a feature that has led to considerable confusion and speculation about the structural genes for toxin biosynthesis. The observed differences between the ST and LT enterotoxins with respect to size, immunogenicity, and kinetics of fluid accumulation suggested that the two *E. coli* enterotoxins were quite distinct entities. Many toxigenic *E. coli* of porcine and bovine origin produce only ST and, as expected, recipients receiving an Ent plasmid from such a strain also produce only the ST toxin. Yet a number of porcine strains, as well as most human toxigenic strains, produce both ST and LT. In matings in which the donor is both ST$^+$ and LT$^+$, all Ent$^+$ recipients likewise produce both ST and LT. The determinants cannot be separated no matter how many Ent$^+$ recipients

Table 11.3. The distribution of determinants in an *E. coli* recipient grown in mixed culture with a donor *E. coli* with transmissible Ent, Hly, R, Col, and K88. [Data from Smith and Linggood (1970).]

Number of colonies with pattern	Col$^+$	R$^+$	Ent$^+$	Hly$^+$	K88$^+$
34	+	+	+	+	−
17	+	+	+	−	−
16	+	+	−	+	−
18	+	+	−	−	−
4	+	−	−	−	−
3	−	+	−	−	−
1	+	+	−	+	+

one examines. The ST toxin produced by these strains is indistinguishable from the toxin produced by strains carrying a plasmid which determines only ST, although, of course, the properties of ST that can be compared are varied and few in number. In point of fact no toxigenic *E. coli* strain has been reported that solely produces LT; there is always some fraction of ST toxin present. These findings have led to the speculation that the two toxins are related in some fashion, that the Ent ST plasmid is an immediate precursor of Ent St + LT plasmids, or that LT toxin synthesis requires the cooperative action of two distinct plasmid genes for expression. The genetic nature of Ent plasmids became even more complicated by the description of both fi^+ and fi^- types. Obviously it becomes important to deal with Ent, K88, and other plasmids associated with virulent strains in terms of their precise molecular nature rather than simply as transmissible genetic elements.

11.4 The molecular nature of Ent, K88, and Hly plasmids

11.4.1 Hly and K88

There is relatively little information available concerning the molecular nature of either the K88 or Hly plasmids. Werner Goebel and his associates have examined plasmids controlling synthesis of α-haemolysin in wild-type strains of *E. coli*. Their findings to some extent typify the kinds of difficulties that one encounters in analyzing the plasmid content of wild-type strains. Out of eight naturally isolated α-haemolytic *E. coli* strains, four contained three distinct covalently-closed circular DNA molecules corresponding to molecular weights of 65×10^6, 41×10^6, and 32×10^6 daltons. Three of the strains contained only a single plasmid corresponding to 41×10^6 daltons, and one strain carried two plasmids corresponding to 41×10^6 and 32×10^6 daltons. The Hly plasmids in two of the three strains carrying only the 41×10^6 dalton plasmid were nontransmissible. Unexpectedly it was found that the independently transmissible 41×10^6 and 32×10^6 dalton plasmids could *both* encode for haemolysin. Moreover in some cases it appears that the separate 41×10^6 and 32×10^6 dalton Hly plasmids can recombine to form a 73×10^6 dalton single unit of transmission. The 65×10^6 dalton plasmid does not have haemolytic activity and can, at present, be simply classified as a transfer factor. These data are obviously difficult to interpret. The independently transmissible 41×10^6 and 32×10^6 dalton Hly plasmids must be compatible, since they can coexist in the same strain. Yet they appear to recombine, indicating some degree of homology between them. Goebel did not determine whether the Hly plasmids he isolated were fi^+ or fi^-. Earlier studies have indicated that Hly strains are F-like and, in fact, Goebel's findings that both the 41×10^6 and 32×10^6 dalton plasmids can integratively suppress a *dna* A mutant of *E. coli* suggest that they are F-like (chapter 8). Miguel Ortiz (unpublished observations), working in my laboratory, has examined a nontransmissible 41×10^6

dalton Hly plasmid. This plasmid could be readily mobilized by a variety of R-plasmids from different compatibility groups. DNA–DNA duplex studies with this plasmid indicate that it is not significantly related to the DNA plasmids belonging to the FI, FII, I, N, W, P, or X compatibility groups. On the other hand, this plasmid shows from 20% to 65% nucleotide-sequence similarity with all other Hly plasmids that were tested, including some of those studied by Goebel and his associates. Hly plasmids appear, therefore, to have some common core of genetic information.

The information on the molecular nature of K88 is also limited. Genetic experiments by the Ørskovs suggested that K88 transfer was a Class 1 transfer system comprising an fi^+ transfer factor and a nontransmissible plasmid which carried the structural genes for the K88 antigen determinant itself. Later studies by A. L. Bak and associates in large part confirm this impression and suggest that a 10×10^6 dalton nontransmissible determinant encodes for K88. It was also clear, however, that K88 could be found as part of a composite molecule about 50×10^6 daltons in size, which could be transferred as a single unit. This composite structure appeared to dissociate, in *E. coli*, into an fi^+ 40×10^6 dalton transfer factor and a 10×10^6 dalton molecule, the latter presumably encoding for K88. Experiments in my own laboratory indicate that plasmids encoding for K88 are both transmissible (fi^+) and nontransmissible and most often associated with a single genetic element of from 40×10^6–50×10^6 daltons. Insofar as I am aware, there have been no DNA–DNA duplex studies performed with K88.

11.4.2 The Ent plasmids

A number of Ent plasmids encoding for ST and ST + LT have been characterized by Carlton Gyles, Magdalene So and me. Table 11.4 summarizes these findings. The Ent plasmids associated with both ST + LT biosynthesis form a reasonably homogeneous group. All are found as a single covalently-closed circular species of about 60×10^6 daltons in size containing 0·50 mol fraction G + C, regardless of whether they were isolated from domestic animals or man. These plasmids, which were transferred into *E. coli* K-12, specify the same levels of ST and LT enterotoxin as found in the original toxigenic isolates. The association of a single plasmid-DNA species with both ST and LT suggests either that two plasmid loci are involved or that the smaller ST toxin molecule is either an incomplete form or a breakdown product of the larger LT moiety. The Ent plasmids of porcine origin which encode for only ST have proved to be considerably more heterogeneous. These Ent ST plasmids range in size from 21×10^6 daltons to 80×10^6 daltons, and show a mol fraction G + C content of 0·41 to 0·50.

The apparent homogeneity of Ent ST + LT plasmids has been confirmed by DNA–DNA duplex studies (see table 11.5). Ent ST + LT plasmids of

Table 11.4. The molecular nature of Ent, K88, K99, and Hly plasmids found in toxigenic *E. coli* strains. [Data from So, Crandall, Crosa and Falkow (1974).]

Plasmid	Phenotype	Source	10^6 x Mol.wt.	Mol fraction G + C
P307	Ent (ST + LT)	Porcine	60	0·50
P25	Ent (ST + LT)	Porcine	60	0·50
P155	Ent (ST + LT)	Porcine	58	0·50
P130	Ent (ST + LT)	Porcine	61	0·50
P75	Ent (ST + LT)	Porcine	55	0·50
SF119	Ent (ST + LT)	Human	60	0·50
H10407	Ent (LT + LT)	Human	60[a]	-
P16	Ent (ST)	Porcine	25	-
P95	Ent (ST)	Porcine	21	0·41
P215	Ent (ST)	Porcine	21	-
P57	Ent (ST)	Porcine	52[a]	-
P3	Ent (ST)	Porcine	42[a]	-
P116	Ent (ST)	Porcine	40[a]	-
P2176	Ent (ST)	Porcine	80	-
B41	Ent (ST)	Bovine	65	0·50
K88-1	K88 antigen	Porcine	47	0·50
K88-2	K88 antigen	Porcine	43[a]	-
K99	K99 antigen	Bovine	47[a]	-
Hly-1	α-haemolysin	Porcine	41	0·48
Hly-2	α-haemolysin	Human	71	0·48

[a] In these instances the particular plasmid was 'assigned' because its presence was invariably associated with the phenotypic trait. It was not technically possible, however, to isolate a clone harbouring only this molecular species. In the other cases, the plasmid was unequivocally isolated as a single molecular entity whose presence was associated with the stated phenotype.

Table 11.5. Polynucleotide sequence relationships between an Ent ST + LT plasmid and other Ent plasmids. [Data from So, Crandall, Crosa and Falkow (1974).]

Plasmid	Origin	Toxin type	Percentage of sequences in common
P307	Porcine	ST + LT	100[a]
P130	Porcine	ST + LT	87
P155	Porcine	ST + LT	88
SF119	Human	ST + LT	55
P95	Porcine	ST	41
P16	Porcine	ST	1
Vibrio cholerae[b]	Human	LT	1

[a] In every case the degree of DNA-DNA duplex formation was calculated relative to P307, as this was the reference labelled DNA.
[b] The *Vibrio cholerae* strain used here was toxigenic and contained three distinct plasmids, but it is not known if toxin biosynthesis in *Vibrio* is plasmid-mediated.

porcine origin share about 85% of their sequences in common, and even an Ent ST + LT plasmid of human origin is significantly related to those derived from animals. Ent ST + LT plasmid DNA, however, does not share a significant proportion of sequences in common with any Ent ST plasmid that has been tested. Because of the partial immunological cross reactivity of *E. coli* LT toxin and cholera toxin, DNA–DNA duplex studies were also performed using *Vibrio cholerae* DNA. While *Vibrio cholerae* strains generally harbour one or more plasmids that could conceivably be concerned with toxin biosynthesis, it is clear that *E. coli* ST + LT plasmid DNA shares no discernible sequences in common with the DNA extracted from the *Vibrio cholerae*. The high degree of polynucleotide similarity among the Ent ST + LT plasmids suggested that they were likely representatives of a common compatibility class (chapter 8). Table 11.6a shows that the DNA of a representative Ent ST + LT plasmid was significantly related to the DNA both of FI and FII R-plasmids but not to any other representative R-compatibility class. In a reciprocal experiment (table 11.6b), labelled FII R-factor DNA was shown to share 45% of its sequences with all Ent ST + LT plasmids of porcine origin, and, what is more, 75% of its sequences with an Ent ST + LT plasmid of human origin! Obviously there is nothing particularly unique about Ent ST + LT plasmids, at least with respect to their transfer and/or replicative fractions. They appear to belong to the F-compatibility complex (that is, FI–FIV, see chapter 8). The groundwork that has already been established for the detailed structural mapping of F- and R-factors can be obviously exploited to define better the precise functional similarities between the Ent plasmids and other F-like plasmids. However, even this preliminary look at the molecular nature of Ent is sufficient to show that they are but a variation on a common biological theme encountered with the bacterial plasmids. For example, the difference between the R-factor R1 and the Ent ST + LT plasmid (SF119) is rather minimal and reflects that basically the same transfer factor has in one instance acquired drug-resistance genes and, in another, genes determining enterotoxin biosynthesis in response to different selective pressures.

DNA–DNA duplex studies have not been quite so helpful in sorting out the origins of the porcine Ent ST plasmids. Thus far the Ent ST plasmids tested have not been found to be related to each other, to Ent ST + LT plasmids, or to any known plasmid-compatibility class. Fortunately this is not always true, for preliminary studies with Ent ST plasmids of bovine origin indicate that they are a homogeneous class of plasmids but, at this time, little more is known about them. The ST toxin is a perplexing biochemical and genetic problem. The clinical observation that toxigenic isolates produce either ST or ST + LT suggested to many investigators that Ent ST plasmids were immediate ancestors or, alternatively, a derivative of Ent ST + LT plasmids. Thus far there are

absolutely no data to support this view. Of course, by analogy to the drug-resistance determinants of R-factors, the actual structural genes determining toxin biosynthesis undoubtedly occupy but a small fraction of the plasmid genome, so that any heterogeneity in a plasmid size or gross molecular organization need not reflect any significant degree of heterogeneity in toxin structure. Hence it is possible that the ST toxin genes are identical (or nearly identical) on all Ent plasmids; the DNA–DNA duplex studies simply show that the genetic vehicles which carry these genes are not directly related in anyway. In a similar vein the *E. coli* LT toxin and cholera toxin genes may have a common ancestor, but the DNA–DNA duplex studies certainly show that the *E. coli* Ent

Table 11.6. Polynucleotide-sequence relationships between Ent ST + LT plasmids and plasmids of known compatibility type. [Data from So, Crandall, Crosa and Falkow (1974).]

(a) Duplex formation between P307 and R-plasmids of various compatibility groups[a]

Plasmid	Compatibility class	Percentage of sequences in common with P307
P307	?	100
R144	Iα	5
R1*drd*	FII	45
F	FI	21
N-3	N	<1
S-a	W	<1
RP4	P	<1
R6K	X	<1

(b) Duplex formation between a representative FII plasmid and Ent plasmids[b]

Plasmid	Compatibility class	Percentage of sequences in common with R1*drd*19
R1*drd*19	FII	100
P307	?	46
P155	?	41
SF119	?	75
P95	?	7

[a] ^{3}H-labelled P307 plasmid DNA was reassociated with *E. coli* DNA containing each of the indicated representative plasmids of different compatibility classes. The degree of duplex formation is expressed relative to the homologous P307 reaction (actual binding 88%) corrected for the *E. coli* F^- contribution (6·5%).

[b] ^{3}H-labelled R1*drd*19 plasmid DNA was reassociated with *E. coli* DNA containing each of the indicated Ent plasmids. The degree of duplex formation is expressed relative to the homologous R1*drd*19 reaction (actual binding 92%) corrected for the *E. coli* F^- contribution (13·2%).

plasmids were not directly derived from *Vibrio* and vice versa. The recent advances in 'plasmid dissection', using restriction endonucleases (chapter 7) and fine-structure heteroduplex mapping (chapter 8), should permit a better focus on the nature and precise relationships between the toxin genes. Moreover it can be anticipated that the differences in specificity of K88 for porcine tissue, and K99 for bovine tissue (see below), will also be rewarding in terms of defining host–parasite inter-relationships.

Although the data are by no means comprehensive, the sum total of the genetic and molecular findings with K88, Hly, and Ent are reassuring in the sense that they have been found to be previously known plasmid types wearing different phenotypic garb. Indeed they run the same gamut of genetic behaviour seen so often with the R-factors; they form Class 1 and Class 2 transfer systems (chapter 5), form dissociable composite genetic elements (chapter 5 and 6), and may be either transmissible or non-transmissible. Perhaps one of the most striking features is that Hly, K88, and Ent ST + LT all appear to be F-like or else are associated with F-like plasmids. Since one often finds them all together in the same cell, they must be mutually compatible. Nevertheless, although F-like plasmids are the most common plasmid type present in *E. coli*, it seems unusual that other common *E. coli* plasmid types, N, or I, for example, have not yet been found associated with any of these determinants. This may not be a significant point but I find it curious, since the same Ent, K88, or Hly plasmid types recur so often together in different *E. coli* serotypes around the world. It almost seems that by bearing very similar transfer genes, the coinheritance of Ent, Hly, and K88 is more likely assured.

11.5 The significance of Ent, K88, and Hly in the pathogenesis of *E. coli* diarrhoea

The observation that some normal *E. coli* strains could be 'converted' into a form pathogenic for piglets by the acquisition of Ent, K88, and Hly plasmids showed definitively the important role that they must play in the pathogenesis of diarrhoea in pigs. This finding was re-enforced by the consistent discovery of the Ent, K88, Hly genetic constellation among independent *E. coli* serotypes around the world. The implication was that together these plasmids played some cooperative role in enteropathogenicity. But what part did each plasmid play? The role of Ent seemed clear and was assumed to be the most important factor. Yet a number of workers had noted that a cell which receives only an Ent plasmid is not converted into an 'instant' pathogen when fed to an animal. H. Williams Smith and Margaret Linggood set out to determine the part played by each plasmid determinant. By means of plasmid transmission and by 'curing' with acridine orange, they prepared a series of strains that differed only in regard to the particular combination of Ent, Hly, and K88 plasmids they possessed. These strains were then directly

employed in infection experiments. Their work, which probably ranks as the finest example of the application of microbial genetics to the study of bacterial pathogenesis, revealed that the critical factor in determining enteropathogenicity was K88. Table 11.7a, for example, shows the effect of feeding piglets an Ent$^+$ *E. coli* with different combinations of Hly and K88. The data clearly show that Hly has no apparent affect on the pathogenicity of the strain. If K88 was absent, however, the strain was totally innocuous. The experiment was repeated but this time various combinations of Ent and K88 were employed in the infecting strain. As shown in table 11.7b, the Ent plasmid alone did not permit the strain to elicit a diarrhoeal response in piglets. The same strain carrying just the K88 plasmid induced a very mild diarrhoea in a few animals but most remained well. The combination of the Ent plasmid *plus* the K88 plasmid in an infecting strain was associated with a high incidence of severe diarrhoea. Thus, while K88 and Ent were not particularly noteworthy by themselves, together they permitted an organism to be fully enteropathogenic. We may well ask ourselves why should enterotoxin biosynthesis alone not be sufficient? After all, sterile culture filtrates prepared from strains containing only an Ent plasmid can duplicate the diarrhoeal disease syndrome when directly instilled into the small intestine of an animal. The critical role played by K88 became apparent by examining the concentration of infecting organisms in different parts of the alimentary tract (table 11.8). To begin with it is important to understand that the anterior small bowel of pigs (also of people and most other animals) is maintained relatively free of aerobic organisms (see chapter 10). Thus, if one feeds an animal an *E. coli* culture, the organisms that successfully reach the small bowel are ordinarily rapidly cleared into the large bowel. This is

Table 11.7. The role of Ent, K88, and Hly in the pathogenesis of diarrhoea. [Data from Smith and Linggood (1971).]

Plasmid in strain	Number of piglets fed	Number of piglets that developed diarrhoea
(a) The effect of giving day old piglets forms of an Ent$^+$ *E. coli* possessing different combinations of Hly and K88 plasmids		
Hly$^+$ K88$^+$	10	9
Hly$^-$ K88$^+$	10	9
Hly$^+$ K88$^-$	13	0
(b) The effect of giving piglets forms of *E. coli* possessing different combinations of K88 and Ent plasmids		
K88$^+$ Ent$^+$	25	20
K88$^-$ Ent$^+$	11	0
K88$^+$ Ent$^-$	20	6
K88$^-$ Ent$^-$	8	0

precisely what happens when a piglet is fed an Ent$^-$ K88$^-$ strain or an Ent$^+$ K88$^-$ strain. In contrast, a K88$^+$ Ent$^-$ strain or a K88$^+$ Ent$^+$ strain fed to piglets is found in high concentrations at all levels of the small bowel. The data clearly show, therefore, that the possession of K88 permits an organism to proliferate in the small intestine. Organisms possessing just Ent have no distinctive advantage in colonizing the small bowel. The failure of these Ent$^+$ K88$^-$ organisms to produce disease is probably due to the low concentration of toxin that can be produced as the organism is just 'passing through'. While the Ent$^+$ K88$^-$ strain may be present in the large intestine in high numbers, and may even be maintained there for days and weeks, the toxin is not effective because it is specific for the anterior small-bowel epithelium. A K88$^+$ Ent$^-$ strain reaches very high concentrations indeed in the anterior small intestine, and it is probably fair to assume that the occasional mild diarrhoea seen with such strains is a host reaction to such large numbers of bacteria. The very high numbers of K88$^+$ cells are not maintained indefinitely in the small bowel, but obviously an organism which can selectively multiply in this region has a decided advantage over its fellows (table 11.9). Thus it becomes clear why the combination of K88 and Ent can be literally deadly; organisms carrying this plasmid combination can not only multiply selectively in the anterior small bowel but, in addition, the enterotoxin that is synthesized can immediately bathe its specific target tissue.

The experiments of Smith and Linggood are extraordinarily clear-cut and permit us to focus quite specifically upon the role of the Ent and K88 plasmids in the pathogenesis of one type of porcine diarrhoea. It is important to keep in mind, however, that not all strains of toxigenic *E. coli* that are enteropathogenic for pigs, possess K88. To some extent this is related to the peculiar distinction between Ent ST and Ent ST + LT plasmids noted earlier. That is, the Ent plasmids found in nature along with K88 are almost invariably the Ent ST + LT type. Yet, most Ent ST strains isolated from sick piglets are generally K88$^-$ (even though one can transfer K88 to such strains in the laboratory. Presumably

Table 11.8. The concentration of infecting organisms in different parts of the alimentary tract of piglets given *E. coli* possessing different combinations of K88 and Ent. [Data from Smith and Linggood (1971).]

Organ	Log_{10} number of infecting organisms per gram of tissue in a piglet given an *E. coli* that was:			
	Ent$^-$ K88$^-$	Ent$^+$ K88$^+$	Ent$^-$ K88$^+$	Ent$^+$ K88$^-$
Stomach	5·3	5·7	4·0	4·8
Proximal small bowel	6·0	9·5	8·3	4·4
Distal small bowel	7·4	9·2	10·2	6·0
Colon	8·4	9·5	9·7	6·9

these Ent ST K88⁻ strains isolated from nature either contain a determinant (plasmid or chromosomal) that functions in an analogous manner to K88, or perhaps the nature of the enigmatic ST toxin itself plays a determining role. And what about Hly? This plasmid is found in combination both with Ent ST and Ent ST + LT. While Hly can be observed to increase the virulence of *E. coli* for mice on intraperitoneal injection, there is no indication that it plays any part in the pathogenesis of diarrhoea. Why then do such a high proportion of toxigenic *E. coli* possess Hly? All of the selective forces at play still remain very much a mystery.

The data of Smith and Linggood are interpreted to mean that the K88 antigen permits organisms to adhere to the epithelium of the small intestine. This is a reasonable assumption consistent with the observations made with purified K88 protein. This is a very specific host–parasite interrelationship, however. The transmission of K88 to *Salmonellae*, for example, is neutral or even detrimental to the strain with regard to its pathogenicity for pigs. Moreover a K88⁺ form of *E. coli* does not have any particular selective advantage in the colonization of the small bowel of animals other than pigs. This also seems to be the case for strains isolated from calves, where a combination of an Ent plasmid and K99 function together very much like K88 and Ent in pigs. The K99 antigen is specific for the colonization of lambs and calves but not of pigs. This rather exquisite specificity is also found to some degree in the enterotoxins. That is, the enterotoxin determined by an Ent plasmid of bovine origin may not function very well in the bowel of a piglet, even in concert with K88 antigen.

Table 11.9. The concentration of *E. coli* organisms in different parts of the alimentary tract of piglets given mixtures of a K88⁻ Ent⁻ nalidixic-acid-resistant mutant (Nx^r) and a K88⁺ Ent⁻ streptomycin-resistant mutant (Sm^r) *E. coli* strain. [Data from Smith and Linggood (1971).]

Ratio of Nx^r to Sm^r organisms[a]	Organ	Log_{10} number of infecting organisms per gram of tissue	
		Nx^r K88⁻ Ent⁻	Sm^r K88⁺ Ent⁻
10:1	Stomach	5·5	3·4
	Proximal small bowel	4·3	7·2
	Distal small bowel	3·7	7·3
	Colon	8·6	6·9
100:1	Stomach	6·7	3·4
	Proximal small bowel	6·0	7·3
	Distal small bowel	6·4	7·3
	Colon	8·9	7·2

[a] Each piglet was given 1·0 ml of a broth culture of a Nx^r mutant of the K88⁻ Ent⁻ *E. coli* at the same time as 0·1 or 0·01 ml of a broth culture of a Sm^r K88⁺ Ent⁻ form of the same strain.

Obviously there is still a great deal more to be learned about the specificity and mode of action of both Ent and the K antigens, and one should not be tricked into thinking that the very clear-cut results obtained by Smith and Linggood totally explain the pathogenesis of porcine *E. coli* diarrhoea or its epidemiology. The results with K88 and Ent do point to a significant new feature of pathogenesis of course. A plasmid-mediated substance that can modify a bacterial cell so that it can preferentially colonize a particular animal host is surely now worth considering in all enteric infections. Moreover, since toxigenic diarrhoeas are similar in many respects irrespective of whether they occur in piglets, calves, lambs, or humans, it is logical to assume that many of the conclusions drawn from these studies on animals may be equally applicable to human babies.

11.6 Some final comments concerning plasmids and bacterial pathogenesis

The recognition that plasmids may directly contribute to the pathogenicity of a bacterial cell is still very much a recent observation. To the clinician and epidemiologist, these findings have confirmed suspicions held for many years that 'something' in certain *E. coli* strains conferred the specific property of enteropathogenicity. Plasmids appear to be that 'something'. It is important that there be no misunderstanding about the precise role that these plasmids play. For example, *E. coli* often invade the bladder and cause cystitis; this infection occurs because of the entry of an organism with a certain potential pathogenicity into an otherwise sterile environment. Under these circumstances toxigenic strains are not isolated any more frequently from the bladder than any other *E. coli*— indeed, they are quite uncommon in such infections. The plasmids described in this chapter do not encompass the total pathogenic potential of *E. coli*, but rather are confined to a very specific kind of host–parasite relationship that is concerned with the colonization of the small bowel of a specific or limited number of hosts. The critical factor in one type of porcine diarrhoea is the possession of the K88 antigen, which confers a degree of survival value on an organism so that it can colonize the small bowel, a region that ordinarily contains very few bacteria. In many ways the role of K88 is analogous to the survival value of an R-factor to a strain that finds itself in an antibiotic-containing environment.

The possession of Ent and Hly plasmids did not appear to have any obvious survival value in porcine strains with regard to colonization of the small bowel. It would be a mistake, I think, to consider that their presence does not contribute in some way to the survival of the strain within the host. In this vein, we (L. P. Williams and S. Falkow, unpublished observations) have had the opportunity to monitor calves fed *E. coli* K-12 with and without a single Ent ST plasmid of bovine origin. While the calves totally cleared an *E. coli* F^- strain from their

intestinal tract within three days, the same *E. coli* K-12 subline, carrying the Ent plasmid, was maintained and excreted by these animals at detectable levels for about two weeks. There was no sign of clinical disease. In fact calves, after 96 hours of age, are relatively resistant to *E. coli* diarrhoea, but this does not mean that organisms containing Ent or K99 are without selective advantage with respect to other *E. coli* in these animals. I suspect, therefore, that while Ent alone may not appear to have survival value when studied in a short-term experimental infection, when it is considered from the standpoint of long-term intestinal carriage it may be of considerable significance. The point is that we should not overlook the fact that, in the basic biological sense, the plasmids that we associate with enteropathogenicity were probably individually selected on the basis of some degree of survival value that they conferred upon their bacterial host. Individually the plasmids aided colonization without causing overt disease. The combination of the additive survival value of Ent and K88 (and, although not apparent yet, probably Hly as well) in some cases can result in the development of a strain that is not only very well adapted to its animal host but can now cause overt disease. Possibly one way of describing the evolution of the strains associated with outbreaks of toxigenic diarrhoea may be that they have simply accumulated too much of a good thing.

In the concluding sentence of their paper on the specific role of Ent and K88 in the pathogenesis of diarrhoea, Smith and Linggood have remarked that "since the transfer factors of K88, Ent, Hly, and antibiotic-resistance determinants can be common to all these plasmids, it is interesting to speculate whether 'new' enteropathogenic strains of *E. coli* will emerge at a more rapid rate in the present antibiotic era than hitherto". It may be that this will be a prophetic remark and not necessarily restricted to *E. coli*. There are now several well-defined outbreaks of Salmonellosis that appear to be correlated with the acquisition of an R-factor. For example, LeMinor has documented a sudden severe outbreak of *Salmonella vienna* in France. From 1956–1970 this serotype appeared only in sporadic cases of Salmonellosis and, with one exception, these isolates were antibiotic-sensitive. Since August 1970, there have been numerous outbreaks of this serotype and the great majority have carried an R-factor which confers a resistance pattern Su-Sm-Ap-Tc-Cm-Km. LeMinor concludes that somehow the acquisition of the R-factor is associated with the abrupt multifocal epidemics that have appeared throughout France. Similar epidemiologic observations concerning the acquisition of an R-factor by the typhoid bacillus which altered the endemic pattern of typhoid fever in Mexico, led Gangarosa and his associates to speculate that there might be a plasmid which determines virulence acquired at the same time as the R-factor. This is, at present, just speculation based more on clinical and epidemiological evidence than any genetic evidence. Certainly

multiresistance may hinder clinical therapy in individual cases, but the epidemic spread of these strains cannot be laid simply to antibiotic selection. That the R-factor itself, seen in the epidemics described so far, is associated with increased virulence is unlikely. Certainly a plasmid may acquire a genetic determinant for both drug resistance and a K antigen or Ent as evidenced by our recent isolation of a plasmid carrying K99 and Tc. What may be more likely *in vivo*, however, is that multiplasmid exchanges will occur and be preserved under antibiotic selection. The analogy here is the resistance-mobilization test employed experimentally to detect Ent, K88, and Hly. It is quite clear that antibiotic selection gives some selection for Ent etc. by preserving those cells that have mated, whether they have received just drug-resistance determinants or the whole plasmid complement of a toxigenic donor. As noted in chapter 10, antibiotic selection has increased the probability of successful *in vivo* conjugation and provided for selection of plasmid-containing bacteria in the intestinal flora—certainly this must be having some effect on the distribution of those plasmids which can contribute to pathogenicity. Whether antibiotic selection will, as Smith and Linggood speculate, hasten the emergence of enteropathogens, remains to be seen. It is not speculation to say that further research will identify plasmids other than Ent, K88, Hly, and K99 that can modify the pathogenicity of bacterial cells.

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12

The staphylococcal plasmids—and some final comments

One of the first suspicions that drug resistance might have its foundation in a mechanism other than simple mutation and selection came with the observation by Barber and others in the early 1950s that staphylococci could occasionally lose their resistance to penicillin (chapter 1). A number of investigators stimulated by this observation subsequently demonstrated that penicillinase production in *Staphylococcus aureus* showed extra-chromosomal inheritance and was situated as part of a plasmid. Such plasmids are not restricted only to penicillin resistance; in addition, plasmid-linked resistance to erythromycin, tetracycline, chloramphenicol, and kanamycin as well as to toxic cations, arsenate and arsenite, have also been identified (table 12.1).

Katherine Dornbusch reported in 1971 that methicillin resistance, staphylococcal enterotoxin B, and β-haemolysin in a clinical staphylococcal isolate may be plasmid-linked. Other workers have suggested that the ability to make pigment, coagulase, haemolytic toxin, and fibrinolysin are plasmid-linked in some strains. Possibly other plasmid-linked resistances and determinants will be identified in the future. Richard Novick and Daniel Bouanchaud already think it reasonable to suggest that most, if not all, clinically significant resistance in the staphylococci will show plasmid inheritance. The staphylococci are notorious for their resistance to antibiotics, and nosocomial staphylococal infection literally strikes fear in the heart of an attending physician. One can see that there is a striking parallel between the R-factors and other plasmids of enteric bacteria and the plasmids of the staphylococci.

It seems worthwhile at the end of a discussion of the plasmids of Gram-negative rods briefly to consider this analogous group of plasmids in a Gram-positive organism as a means of understanding the phylogenetic status of these hereditary elements. This discussion of the staphylococcal elements will be at best sketchy and the interested reader is referred to the reviews of Mark Richmond, Richard Novick, and R. W. Lacey for details.

Table 12.1. Genes known to be or suspected of being plasmid-linked in *Staph. aureus.*

Antibiotics
Penicillin (Pn), erythromycin (Em), tetracycline (Tc), chloramphenicol (Cm), kanamycin (Km), methicillin (Me), and fusidic acid (Fu)
Cations (resistance, unless noted otherwise)
Antimony (Sb), cadmium (Cd), lead (Pb), mercury (Hg), and bismuth (Bi and Bi ES) (ES indicating epistatic sensitivity)
Anions (resistance)
Arsenate (Asa) and arsenite (Asi)
Other properties suspected of being plasmid-linked in some strains
Pigment, coagulase, haemolytic toxin, fibrinolysin, and enterotoxin B

12.1 Genetic properties of staphylococcal plasmids

Staph. aureus does not mate; indeed conjugation as seen in Gram-negative organisms is very rare in Gram-positive species. The staphylococcal plasmids are therefore not autotransmissible. So far the only method generally available for transferring staphylococcal plasmid markers from cell to cell is phage-mediated transduction. One can either propagate a staphylococcal phage (usually one of the international typing series) in a donor strain, or induce prophage in the donor with ultraviolet light. Transductional analysis has provided a good deal of insight into the genetic structure of the plasmids specifying penicillinase production. At the practical level, transduction provides an explanation for the spread of clinical antibiotic resistance in *Staph. aureus*. Novick and Morse mixedly infected mice with paired plasmid-positive and plasmid-negative strains with well-defined distinguishing characteristics. Abscesses were established in the mouse kidneys and it was demonstrated that interstrain plasmid transfer occurred *in vivo* quite readily when the plasmid-positive strain was lysogenic for a transducing phage. The conversion from plasmid-negative to plasmid-positive occurred at a level of 10^{-6}–10^{-4} per donor organism. This proportion could be increased about 10000-fold by the parenteral administration of an appropriate antibiotic. Plasmid transfer in the natural environment of staphylococci probably occurs therefore by low-level spontaneous transduction.

The plasmids of *Staph. aureus* have been characterized genetically in a number of ways. The best studied are the plasmids specifying penicillin resistance ('penicillinase' plasmids), which, in addition to Pn, possess a variety of marker patterns including Em, and resistance to inorganic cations and anions. Plasmids have been identified in some clinical isolates of staphylococci that appeared to be genetically homologous to the 'penicillinase' plasmids but, in fact, lacked the Pn gene. This prompted the inclusion of cadmium resistance (Cd) as a genetic marker, because either this marker or Pn is invariably present in this class of plasmids. To simplify matters further virtually all of the Pn–Cd plasmids studied thus far belong to one of two known incompatibility classes, 1 and 2—really quite analogous to the compatibility classes described earlier for R-factors. No two members of the same class can stably coexist in the same cell. Members of one class are always compatible with those of the other. Incompatibility specificity has been correlated with other genetic traits by Novick. Thus all members of incompatibility class 2 exhibit epistatic bismuth (Bi) sensitivity whereas class 1 plasmids show bismuth resistance or indifference. Three out of eight class 2 plasmids studied do not carry Pn, but all but one of 18 members of class 1 examined were Pn^+. A rather considerable number of marker variations in individual plasmids have been described but the primary differentiation into the two incompatibility types permits one to consider that such variations in markers represent deletion or accretion of additional genes by the two separate plasmid lines.

As with R-factors, the genetic map of a Pn–Cd plasmid appears to be distinguisable into carried determinants and 'essential' genes governing replication, plasmid maintenance, and plasmid apportionment. One assumes that the incompatibility is a reflection of the essential regions. Plasmids conferring resistance to tetracycline or chloramphenicol are compatible with each other and with both class 1 and 2 Pn–Cd plasmids. The Tc and Cm plasmids therefore presumably represent distinct classes. They do not carry resistance to any other known antibiotics or to inorganic ions, resistances which are so commonly carried by the Pn–Cd group.

The genetic analysis of resistance to erythromycin has been enigmatic so far. There are at least two known phenotypic types of staphylococcal resistance to erythromycin. One type is constitutive and linked to Pn in a series of plasmids studied by Mitsuhashi and his associates in Japan. The other type of erythromycin resistance is inducible and, when induced, confers cross-resistance to a large number of macrolide antibiotics that are not themselves inducers. The inducible type of erythromycin resistance is not linked to Pn and there is conflicting evidence as to whether this gene is borne on a separate plasmid or is chromosomal in nature.

12.2 Plasmid autonomy and the molecular nature of staphylococcal plasmids

All the classes of staphylococcal plasmids, like R-factors, are genetically quite stable despite their nonessentiality to the cell. An inquiry into the maintenance mechanism employed by the staphylococcal plasmids to ensure genetic continuity is extraordinarily similar to that seen in R-factors and other plasmids of enteric species (see chapter 7). For the Pn–Cd plasmids there are at least three genetically distinct maintenance genes in the host chromosome. The genes *seg* A and *seg* B are responsible for maintenance of class 1 and class 2 plasmids respectively, and a third gene, *seg* B, is involved in the maintenance of any plasmid type. The plasmid itself determines at least two additional genes concerned with plasmid replication and plasmid apportionment to a daughter bacterial cell. Novick and Bouanchaud have examined the kinetics of segregation by a thermosensitive replication-defective Pn–Cd plasmid under non-permissive conditions. The kinetics of plasmid loss indicates that this plasmid is present at two or three copies per cell—like most R-factors in *E. coli*. Similar experiments done with a Tc plasmid suggested, however, that this plasmid is present as 30 or more copies per cell—like the nontransmissible Tc plasmids or the Col E1 plasmid of enteric species described earlier.

The examination of the DNA of the staphylococcal plasmids reveals, not unexpectedly, that they may be isolated as covalently-closed ring molecules from the host cell. There appear to be two distinct types of molecular species involved (table 12.2). The Pn–Cd plasmids are relatively large, about 20×10^6 daltons, and present in a limited number

of copies per cell. The replication of this plasmid type, like sex factors, is likely to be analogous to the host chromosome and keyed to cell division. The presumption is that the apportionment of replicas of this plasmid type is somehow regulated by attachment, at some time, to a cellular structural component. The other molecular type, exemplified by the staphylococcal Cm and Tc plasmids, is much smaller, about 3×10^6 daltons, and is present in a large number of copies per cell. The replication of this type of plasmid, like that of Col E1, or the nontransmissible tetracycline-resistance determinant of R-factors, is presumably random in nature and probably not keyed to the cell-division cycle. It may be possible that these smaller plasmids, by analogy with their enteric analogues, will replicate in the absence of net host protein synthesis.

Table 12.2. Molecular nature of staphylococcal plasmids.

Plasmid	Compatibility class	Approximate number of copies per cell	$10^{-6} \times$ Mol.wt. (daltons)
Pn-Cd 258	1	2-3	18
Pn-Cd 147	2	2-3	21·8
Tc 169	Compatible with 1, 2, Cm-22·1	30	2·7
Cm 22·1	Compatible with 1, 2 and Tc 169	30	3·1

12.3 Biochemical basis of drug resistance in staphylococci

At this time the mechanism of resistance determined by most genes on the staphylococcal plasmids remains unknown. It has been suggested that resistance to the mercury and cadmium cations probably reflects a change in membrane properties, which excludes these ions from the cell interior. However, it is likely that resistance to mercury is associated with an enzyme which converts the mercury into a volatile form, as reported for the R-factors (R. P. Novick, personal communication; see also chapter 9). The biochemical basis of resistance to tetracycline and erythromycin is unknown.

The biochemical basis of resistance to penicillin and chloramphenicol in the plasmid-bearing strains of staphylococci has received considerable attention, however. Penicillin resistance is invariably associated with a high activity of β-lactamase. Unlike all classes of enteric β-lactamase, the staphylococcal enzyme is inducible. There is no immunological cross-reaction between enzymes from the Gram-positive and Gram-negative species, and the only common property shared, other than performing the same function within the architecture of the enzyme molecule, is that they are sensitive to iodine/potassium iodide. The chloramphenicol

acetyltransferase in R^+ enteric bacteria has its counterpart in plasmid-bearing staphylococci. The information currently available suggests that the enteric and staphylococcal enzymes have grossly similar properties with respect to substrate specificity, pH optimum and molecular weight (80000; subunits of 20000). The enzymes are readily distinguished immunologically, however, and possess different affinities for chloramphenicol as well as divergent electrophoretic properties. As with β-lactamase, the chloramphenicol-acetylating enzyme of the staphylococci is inducible, in contrast to the constitutive nature of R^+ Cm enteric bacteria.

12.4 Some final remarks

The purpose of this very rapid look at the staphylococcal plasmids has been to point out that multiple drug resistance in both enteric bacteria and staphylococci takes place through similar biochemical mechanisms and is mediated largely by extrachromosomal elements. This gross similarity in natural history has several important implications. Although it has not yet been definitively shown, it seems highly unlikely that the genetic material of R-factors and the staphylococcal plasmids will exhibit any significant degree of relationship. I presume that they are products of completely distinct lines of parallel evolution in totally unrelated bacterial species (table 12.3). One can, of course, make the assertion that, somewhere back in the (oft-cited) primaeval ooze, the grandfather of all bacteria possessed plasmids and that in the subsequent major division of Gram-positive and Gram-negative species, the ancestral plasmid(s) were retained, etc. Perhaps. But the point is that the importance of plasmids to the life history and genetic organization of bacteria cannot be underestimated. It seems fascinating that, at the molecular and replicative level, two clearly distinct plasmid classes are observed. The preliminary

Table 12.3. Parallel evolution of staphylococcal plasmids and R-factors.

Staphylococcal plasmids	R-factors
Nontransmissible	Transmissible or nontransmissible
Two molecular classes:	Two molecular classes:
20×10^6; 1-3 copies/cell	35×10^6-70×10^6; 1-3 copies/cell
1×10^6-3×10^6; multiple copies/cell	5×10^6-12×10^6; multiple copies/cell
Predominant cause of resistance clinically: Carries resistance to penicillin, chloramphenicol, tetracycline, mercury, lead, cadmium, bismuth, antimony, arsenate	Predominant cause of resistance clinically: Carries resistance to ampicillin, chloramphenicol, tetracycline, sulphonamides, streptomycin, kanamycin, gentamicin, mercury, etc.
Mechanism of penicillin resistance: β-lactamase (inducible)	Mechanism of penicillin resistance: β-lactamase (constitutive)
Mechanism of chloramphenicol resistance: chloramphenicol acetyltransferase (inducible)	Mechanism of chloramphenicol resistance: chloramphenicol acetyltransferase (constitutive)

evidence reveals remarkable similarity in relative molecular size and replicative behaviour of the plasmids found in these evidently divergent bacterial species. All in all it would seem that this may well represent a biological generalization in the nature of the extrachromosomal genetic structure and replicative properties, which may be common throughout all bacterial genera. In this same general respect, the larger molecular plasmid classes probably share with chromosomes a basically similar mechanism of replication. They represent therefore ideal model systems, experimentally and conceptually, for outlining basic replication, replication control, and the distribution of hereditary replicas. The genetic and molecular analysis of plasmids, coupled with the study of the structural components of plasmid distribution, seems to be particularly useful. One would have considerable difficulty performing a mutational analysis of the genetic control and structural basis of bacterial chromosome distribution since interference would be presumably lethal. However, conditionally dependent mutations involving plasmid distribution are easy to isolate and much more amenable both to genetic and molecular analysis. The analysis of these mechanisms in plasmids should provide specifically a better understanding of the replica-distribution process in bacteria, and may even provide a certain degree of insight into the mitotic mechanisms of more highly evolved organisms.

Many of the studies on plasmids can lead to a rather jaded view of their role in the life of bacteria. Most of this volume has dealt with resistance to antibiotics, with a generous sprinkling of haemolysins and enterotoxins. Of necessity I have largely restricted the discussion to the Gram-negative enteric species and the staphylococci. Plasmid-mediated drug resistance appears to be widespread in other bacterial genera, however, as evidenced by the recent demonstration of plasmids, some transmissible, in *Staphylococcus faecalis*, which encode for Em and for Em–Sm–Tc–Km (*en bloc*), as well as for Ap in *Hemophilus influenzae* (L. Elwell, D. Siebert, and S. Falkow, unpublished observations). And, of course, one is reminded that toxin production in other bacterial genera are associated with prophages or other plasmids. William Hayes' remark that we may well be indicting the bacteria in general for the sins of their viruses (plasmids) seems well taken indeed. I do not wish to underestimate the importance of plasmids to our understanding of pathogenesis, but the question really is why do properties such as drug resistance and toxin production so often appear to be associated with extrachromosomal elements? That is, of course, a human's view. The effect of plasmid products on human tissue and manufactured pharmaceutical products may be purely incidental to its primary function as a phage or plasmid gene and in its selective advantage to the host. In most ways we are still quite ignorant of the biochemical mechanisms of several biologically active compounds elaborated by bacteria. In this respect the recent finding that the *E. coli* enterotoxin

is a stimulator of adenyl cyclase may have as much significance in our understanding of the Ent plasmid as a biological entity, as it has to the pathogenesis and control of disease.

Aside from the public health considerations of plasmids (which we may just be really beginning to appreciate), it is interesting to think about their role in the more numerous groups of microorganisms in Nature that do not cause disease in man or his animals. Every introductory textbook in Microbiology points out the essentiality of bacteria to life on this planet, and it is the rare scientist who is not fascinated by the amazing variety of specializations that the bacteria exhibit. It is in the free-living groups of organisms that our ignorance of bacterial plasmids is most obvious. More examples, such as the recent demonstrations of plasmid-mediated control of lactose fermentation in *Streptococcus lactis*, regulation of octane and camphor degradation in *pseudomonads*, and control of sporulation in *Bacillus pumilus* may well provide us with a firmer grasp of the role of these extrachromosomal hereditary determinants in the evolution of bacteria.

Finally there is no doubt that one can employ the concepts uncovered for bacterial plasmids as a framework within which the processes of more highly evolved organisms can be profitably compared. The autonomy and extrachromosomal inheritance of plasmids, for example, can serve as a useful model for understanding the autonomy and extrachromosomal inheritance of mitochondria, chloroplasts, and the other extrachromosomal organelles of higher organisms. In addition to these visible organelles may there not also be direct plasmid-like analogues in higher organisms? Are there perhaps RNA plasmids? Or do we already regard certain plasmids of mammalian cells as RNA oncogenic viruses? Obviously the prophage model has already been of inestimable value in the continuing analysis of certain tumour viruses. By the same token, other tumour viruses may adopt the plasmid-like pattern of autonomous quiescent intracellular existence in their process of cellular transformation. The plasticity of the immune response has already spawned 'episome' theories and the plasmid concept has been invoked in developmental biology. The most profitable aspects of the study of bacterial plasmids may yet prove to be their use as a model and intellectual springboard for understanding the processes in higher organisms.

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