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Transmissible Multiple Drug Resistance in Enterobacteriaceae

The structure, replication, and mode of action of drug-resistance episomes are discussed.

J. E. Davies and R. Rownd

Ever since the introduction of antibiotics as a means of controlling infectious disease, bacterial strains have emerged that are resistant to a variety of chemotherapeutic agents. The examination of the mechanism of resistance in resistant strains which have been isolated from nature or derived in the laboratory has revealed a variety of ways in which microorganisms can survive in the presence of antibiotics. According to the tenets of microbial genetics, mutant strains should acquire resistance to only one antibiotic at a time, and organisms resistant to several drugs should arise in nature only by the accumulation of successive mutations. It is therefore quite extraordinary that there has been a dramatic increase in the frequency of occurrence of Enterobacteriaceae with multiple drug resistance in essentially all countries in which the problem has been examined.

In general, the multiple resistance of these microorganisms does not seem to have arisen in a series of discrete steps; but rather, resistance to all of the drugs appears to have been acquired simultaneously. Genetic analysis has revealed that multiple drug resistance is specified by an extrachromosomal element, which is referred to as a drug-resistance factor or R factor. Over the past decade, R factors have been studied extensively throughout the world because of their theoretical and practical consequences. We will review a number of their properties, particularly those that are related to the biochemical mechanism of multiple drug resistance and to the ways in which the level of drug resistance of host bacteria may be varied by the replication and the dissociation and reassociation of the components of R factors.

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Epidemiology

The first R factors were detected in Japan in the 1950's during an outbreak of bacillary dysentery (*1–3*). Since that time, extensive epidemiological studies that were carried out in Japan, England (*4, 5*), the Netherlands (*6*), Switzerland (*7*), and the United States (*8*) have shown that in numerous clinical situaitons, R factors are the agents responsible for resistance to antibiotics in Enterobacteriaceae. The occurrence of R+ strains is not simply restricted to hospital outbreaks; R factors are present in domestic animals (*5, 9*) and fish (*10*), particularly when antibiotics are used as routine diet additives. Recently, R+ *Escherichia coli* strains derived from urban sewage (*11*) were found in river water.

Resistances to at least ten antibiotics are specified by R factors (Table 1), and it seems likely that additional resistance determinants will be found in the future. As far as we are aware, no R factor that has been isolated from nature harbors all of these resistance determinants simultaneously, but examination of the patterns of resistance for all ten antibiotics has probably not been carried out with all R factor isolates. There appears to be no obvious pattern of development of antibiotic resistance, although the number of resistance markers can increase as a result of exposure of the organism to additional antibiotics (*4, 5, 7, 12*).

From such studies it can be concluded that new resistance markers can be acquired by existing R factors in nature and replicated, transferred, and expressed as part of the composite structure thus formed. Smith (*13*) and Gardner *et al.* (*14*) have shown that enteric bacteria that carried R factors were present before the advent of widespread antibiotic therapy. More extensive studies with proven antibiotic-virgin subjects should be carried out to establish any correlation between the administration of new antibiotics and the appearance of transferable resistance markers.

Numerous classifications of R factors are possible, and different R factor isolates have characteristic groupings of resistance markers (Table 2). The R factors are often given different designations by different groups of workers; for example, NR1, R_{100}, and 222 are one and the same (*15*). This could lead to some confusion; many of the R factors that were isolated in different parts of the world and differently named may in fact be identical. It would be desirable (although difficult) to have nomenclature standardized so that biochemical studies of R factors from different laboratories could be compared accurately.

Table 1. Resistance markers found on different R factors.

Resistance to:	Name of marker	References
Ampicillin	AP	(*51*)
Chloramphenicol	CM	(*59, 60*)
Kanamycin	KM	(*66, 71–75*)
Neomycin	NM	(*66, 71–75*)
Streptomycin	SM	(*66, 68*)
Spectinomycin	SP	(*68, 70*)
Gentamicin	GM	(*76, 77*)
Sulfonamides	SU	(*1*)
Tetracycline	TC	(*47, 48*)
Colicinogenic factors		(*45*)
Mercury		(*44*)
Nickel and cobalt		(*44*)
Viruses		(*56, 58*)
Ultraviolet light		(*46*)

Genetic Nature of R factors

Several groups of Japanese geneticists have shown that R factors are extrachromosomal genetic elements that are capable of autonomous replication in host cells; that is, they replicate independently of the host chromosome (*1–3, 16, 17*). Most of the initial studies were on the R factor NR1, which had originally been isolated from nature in a strain of *Shigella* by Nakaya *et al.* (*16*). In general, most of the initial results obtained with NR1 appear to be characteristic of other R factors, which have not been studied in as detailed or systematic a manner. The R factors can be transferred by bacterial conjugation to essentially all members of the Enterobacteriaceae (*1, 3, 5, 16*). The transduction of multiple drug resistance by appropriate temperate phages has been observed in *E. coli* (*16*), *Shigella flexneri* (*16*), several *Salmonella* species (*1, 2, 18, 19*), and *Proteus mirabilis* (*20*).

The transfer of multiple drug resistance, either by bacterial conjugation or transduction, takes place independently of the transfer of host chromosomal genes. Additional evidence that R factors are extrachromosomal agents is the elimination of R factors from host cells by treatment with acridine orange or other intercalating dyes (*1*). Studies (to be described below) on the molecular nature and the replication of R factors in various hosts have also shown that these elements exist as independent units of replication (replicons) in host cells.

On the basis of transduction data and the patterns of spontaneous segregation of drug resistance genes, Watanabe (*1, 2*) has suggested that R factors are composed of two genetically distinguishable units: a transfer factor (RTF) and a unit which harbors drug resistance genes (r-determinant). The RTF is believed to mediate the autonomous replication of R factors and to promote their transfer from one bacterium to another by conjugation. The r-determinants harbor genes which specify resistance to a wide range of common antibiotics.

Whether r-determinants themselves are capable of autonomous replication in host cells has been a matter of controversy. The orginal findings were interpreted to mean that r-determinants are unable to replicate unless they are attached to the RTF or are integrated into the host chromosome (*2, 3, 21, 22*). However, more recent genetic (*5*) and physical evidence (*23–26*) suggests that in some host strains both the RTF and r-determinants are independent replicons whose duplication is regulated by different control mechanisms.

Drug-sensitive enteric strains that harbor only RTF's and not r-determinants (*3, 5, 27, 28*) have also been isolated from nature. Similarly, strains that are resistant to several drugs but are unable to transfer multiple drug resistance have been isolated (*5*). These strains can transfer their r-determinants, however, if they are infected with an RTF. Since the transfer of multiple drug resistance occurs independently of host chromosomal genes, it appears that the r-determinants of these strains exist as independent replicons.

Stability of Association between RTF's and r-Determinants

The stability of the association between RTF's and the r-determinants for various antibiotics appears to vary for different R factors, and the stability of this association for a given R factor appears to depend upon the host. For NR1, the association between the RTF and the determinants of antibiotic resistance is quite stable in *E. coli*. However, there are occasional segregants that have lost resistance to tetracycline (marker TC) or to all or various re-

stricted combinations of the antibiotics chloramphenicol, streptomycin, and the sulfonamides (markers CM, SM, and SA). These segregants occur at low frequency, either spontaneously or during transfer by conjugation or transduction (*12*, *29*). The RTF is usually not lost by cells that harbor any r-determinants, since the drug-resistance genes are still transferable by conjugation.

The behavior of the same R factor in *Salmonella* is quite different. After transfer to *Salmonella* by conjugation, the genes CM, SM, and SA are segregated simultaneously so frequently that it is possible to do clonal analysis on the segregation (*21*). After repeated subculturing of the cells, only TC is retained by the population. The majority of the tetracycline-resistant segregants retain the ability to transfer the marker, so the RTF is not segregated by the cells.

During transduction of NR1 in *Salmonella typhimurium* by phage P22, segregation of TC from the block that contains CM, SM, and SA is almost always observed (*1*, *18*). In essentially all cases both segregants of NR1 have lost the ability to transfer their drug resistance and thus, at least phenotypically, have lost the RTF. As will be discussed below, physical studies on the DNA of NR1 in *P. mirabilis* have suggested that the RTF and r-determinants of R factors dissociate and reassociate in this genus to provide a novel mechanism by which the number of copies of drug-resistance genes per cell can be regulated.

Successive Intergeneric Transfers

One of the most interesting genetic studies on the association and dissociation of a transfer factor and the determinants of resistance to several antibiotics was carried out by Anderson and Lewis (*5*, *27*, *30*). They studied successive intergeneric transfer of ampicillin resistance (AP), streptomycin and sulfonamide resistance (S), and TC from a multiply resistant strain of *S. typhimurium* to a drug-sensitive strain of *E. coli* and back again.

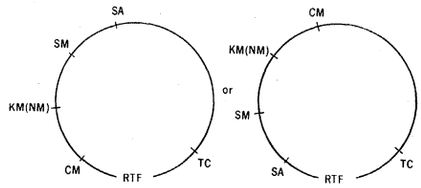

Fig. 1. Two possible linkage maps for R factors. Markers are defined in Table 1 [from Watanabe (*2*)].

In initial matings, AP and S were transferred independently to the *E. coli* recipient strain in most instances, each marker having a transfer frequency of about 10^{-2} per donor cell. Traits AP and S were transferred simultaneously at a frequency of 10^{-3}. The TC marker was initially transferred at a very low frequency (10^{-6}). However, in subsequent matings the transfer frequency for TC was greatly increased to about 5×10^{-1}, which was the transfer frequency for the transfer factor itself (here called Δ). Moreover, in these subsequent matings transfer of TC was always accompanied by transfer of Δ. Anderson and Lewis suggested that the original low frequency for transfer of TC was due to a rare pickup of the chromosomal gene TC by Δ. Once formed, the TC-Δ complex was quite stable, and TC and Δ were always transferred together.

The transfer frequencies for AP, S, and AP-S in further matings were the same as in initial matings (10^{-2}, 10^{-2}, and 10^{-3}, respectively). With appropriate genetic techniques it could be shown that the transfer of Δ alone occurred at a frequency of 5×10^{-1}. In long (overnight) mating experiments, most cells that received resistance determinants were capable of transferring resistance. In short (interrupted) matings, some recipient cells received AP or S but were incapable of transferring these resistances. Thus, there was independent transfer of AP, S, and Δ. Since the frequency for simultaneous transfer of AP and S was ten times higher than that expected for random transfer events, it appears that these two independent determinants frequently associate with the same structure, presumably Δ, in a reversible manner.

Circular linkage maps (Fig. 1), which are based on transduction data, have been proposed for two R factors that are stable units in *E. coli* (*1*, *2*). Although these two R factors have similar genetic maps all R factors might not fall into the same pattern, since different permutations of resistance genes and the RTF are likely to exist.

Transmissibility

Several episomal elements in Enterobacteriaceae are capable of autonomous replication and transfer; these episomes include F factors, colicin factors, and R factors (*31*). Each (collectively termed sex factors) possesses several genes that are responsible for the transfer and other characteristic properties of the episome. Interstrain transfer (from *Shigella* to *Shigella* or to *Escherichia*) of multiple drug resistance was first demonstrated by Ochiai *et al.* (*32*) and Akiba *et al.* (*33*), and sex factor activity was found in R+ strains shortly thereafter (*16*, *17*). Genetic transfer that is mediated by R factors (and other episomes) involves conjugation, since it requires cell-to-cell contact and the presence of surface structures called pili and can be interrupted mechanically. The detailed mechanism of DNA transfer is not known (*34*).

Specific pili have been recognized on the surface of bacteria that carry F

Table 2. Patterns of resistance markers on some typical R factors. Markers that are not identified in Table 1 are HG, mercury resistance; PM, paromomycin resistance. Chloramphenicol resistance (*4*), combined streptomycin and spectinomycin resistance (*70*), and mercury resistance (*106*) are often, but not exclusively, associated with the R(f) type (ND, not determined).

R factor	Resistance marker										R factor type	References
	AM	CM	HG	KM	NM	PM	SA	SM	SP	TC		
NR1* (R_{100}, 222)	—	+	+	—	—	—	+	+	+	+	R(f)	(*16*, *17*, *40*)
NR3*	—	—	ND	—	—	—	+	+	—	+	R(i)	(*66*, *72*)
NR79*	—	+	+	+	—	—	+	+	+	+	R(f)	(*66*, *72*)
NR84*	+	+	+	—	—	—	+	+	+	+	R(f)	(*66*, *72*)
R_6	—	+	ND	+	—	—	+	+	ND	+	R(f)	(*65*)
R_d	—	+	ND	+	+	+	+	+	ND	+	R(f)	(*7*)
JR_{36}	+	—	ND	+	+	+	+	+	—	+	R(i)	(*66*, *75*)

* Isolated and characterized in the laboratory of R. Nakaya (*16*, *88*).

factors, colicin factors, or R factors; and R factor piliation and its relation to surface structures associated with other episomes have been studied extensively by Meynell, Meynell, and Datta (*35, 36*). They found that one group of R factors [R(f)] produce pili that are similar to those for F factors and that act as receptors for F-specific phages (f1 and f2), while another group of R factors [R(i)] produce pili similar to those found on bacteria that carry the colicin-I factor.

Many episomes can be transferred between most Gram-negative species. In general, R factors are transferred at a much lower frequency than are F factors (*16, 37*). However, when certain types of R factors are present in cells that harbor an F factor, the transfer frequency for the F factor is considerably diminished. When no other episomes are present, F factors produce sex pili in virtually all cells. When certain R factors are present, alone or with an F factor, only a minority of cells produce sex pili. This is due to the reduced ability of R+ strains to produce pili.

According to Meynell *et al.* (*36*), these observations are the result of the production of a repressor of pilus formation in certain R+ strains; a series of repressor mutants that exhibited high transfer frequencies were found to possess an increased number of surface pili. The R factors that inhibit sex-pilus formation and fertility in F-factor strains have usually been called fi+, while those R factors that lack this ability have been termed fi− (*38*). Considerations of the pilus type on these two R factor classes have led to the suggestion that fi+ be renamed R(f) and fi− be renamed R(i) (*39*). In all other respects, transfer and subsequent expression of R factors is assumed to occur in the same way as for other episomes. The RTF therefore carries all of the genetic information necessary for conjugation and transfer and like other sex factors can incorporate chromosomal genes at low frequency (*40*).

The R factors differ from other episomes in their response to dyes such as acridine orange and ethidium bromide, which inhibit the transfer of sex factors and bring about curing (promotion of segregation of an episomal element). The transfer of R factors can be inhibited by acridine dyes, but R factors are extraordinarily inert to curing by acridine orange (*41*) or ethidium bromide (*42*). Whereas an *E. coli* strain that carries an F-lac episome can be

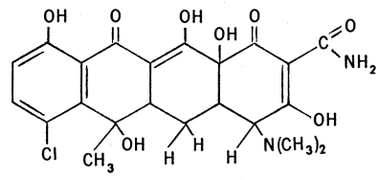

Fig. 2. The structure of tetracycline.

100 percent cured of the episome by a brief exposure to ethidium bromide, a typical R factor strain is quite refractory to such treatment. This dissimilarity has not been satisfactorily explained. However, it must indicate a fundamental difference in molecular structure, replication, or segregation of the two types of episomes; and it emphasizes the need for more information on these subjects.

When comparisons are made between R factors and other episomes or between different R factors, it is important to realize that the host bacterium can markedly influence the properties of the R factor. This influence is usually seen as quantitative differences in antibiotic resistance when the episome is transferred from one strain to another (*29, 43*). In addition, certain R factors can prevent another from being established in the host cell. These are termed homologous; a heterologous R factor can be accepted. The mechanism of this type of exclusion is not known, but two processes might be responsible. One is exclusion of the episome and involves the cell surface while the other is the failure of the R factor to become established once it has entered (*1, 39*). Novick (*39*) proposes that the terms entry exclusion and incompatibility be used to describe these processes in place of the current terms superinfection immunity and mutual exclusion. Superinfection immunity suggests a close analogy to bacteriophage systems, which is not justified.

Mechanisms of Resistance

Apart from scientific curiosity, there are many reasons why one would like to know how R factors determine resistance to antibiotics. Knowledge of the molecular mechanisms would allow accurate predictions of the usefulness of new antibiotics against different R+ strains, the synthesis of antibacterial agents or antibiotic derivatives to which R factors cannot confer resistance, and the design of inhibitors of resistance conferred by R factors. In addition, knowledge of resistance mechanisms may give clues to the origin of r-determinants and will be useful in interpreting studies on the genetics, molecular structure, and properties of R factors.

The resistance characters on R factors are listed in Table 1; different R factors carry different combinations. A resistance character does not necessarily define a gene, since a single enzyme can be responsible for resistance to two or more antibiotics. The mechanisms of resistance are known for about half of these characters. For the others—resistance to heavy metals (mercury, cobalt, nickel) (*44*), sulfonamides (*1*), colicinogenic agents (*45*), and ultraviolet irradiation (*46*)—there are speculations but no clear experiments to suggest mechanisms responsible for phenotypes; perhaps this article will stimulate inquiry into these problems.

Tetracycline

Resistance to the tetracycline antibiotics (Fig. 2) in R+ strains has been studied (*47, 48*). The R− bacterial strains that are sensitive to tetracycline actively accumulate the drug, which then inhibits ribosome function. The resistance of R+ strains is due to their inability to concentrate tetracycline. It appears that R− strains possess a mechanism that mediates the entry of the drug (*49*). In R+ strains, the constitutive, low resistance is amplified in the presence of tetracycline; this suggests that R+ strains possess an inducible inhibitor of drug uptake. Most evidence for this mechanism comes from studies of the uptake of radioactive tetracycline in R+ and R− strains; there is no evidence for inactivation of the drug or alteration of its target site on the ribosome (*48*). This model predicts that constitutive mutants that are resistant to a high concentration of tetracycline should exist. Such a mutant has been reported recently by Franklin and Cook (*50*). Tetracycline resistance is probably the most common naturally occurring R factor resistance, perhaps because the gene that determines this trait is closely linked to the RTF in most R factors (*1, 3*).

Penicillinases

The mechanism of resistance to penicillin (Fig. 3) in R+ strains (*51*), similar to that in other strains, is the production of a penicillinase (β-lactamase),

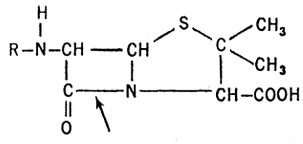

Fig. 3. The structure of penicillin. The arrow indicates the point of hydrolysis of the β-lactam ring by penicillinases. A semisynthetic β-lactam antibiotic, ampicillin (AP), has been most commonly used in R factor studies since it is more active than penicillin.

of which many different types have been isolated. The penicillinases of R+ strains have been classified into three groups by their physicochemical, enzymological, and immunological properties (*52*). Some of these enzymes are related to those from other genera of bacteria, which appear to be determined by chromosomal rather than episomal genes (*52–54*). Such similarities have prompted speculation on the origin of the penicillinases in R+ strains. However, it is difficult to make accurate phylogenetic comparisons between these enzymes and all suggested relations between R factor and similar "chromosomal" enzymes are quite tentative.

The penicillinases in R+ strains are synthesized constitutively (*54*) and are probably located in the periplasmic region of the cell (between the cell wall and the cell membrane) (*55*). The β-lactamases are the best-characterized group of R factor enzymes. With the introduction of new penicillin and cephalosporin analogs in clinical practice, studies of these enzymes will continue to be important. β-Lactamases are known which can inactivate all of the penicillin and cephalosporin derivatives in current use. R factors that determine resistance to carbenicillin (a broad-spectrum β-lactam antibiotic) have been characterized in several clinical isolates of *Pseudomonas aeruginosa* (*55a*).

Viruses

Some R+ strains are resistant to infection by several different DNA viruses, and this resistance is due to the presence of a nuclease that breaks down foreign, unmodified DNA (*56*). This phenomenon, known as restriction, is found in some R− bacteria, and specific deoxyribonucleases have been isolated from these strains (*57*). The R factor nuclease (*58*) is poorly characterized and has not been compared to the well-characterized restriction systems in R− *E. coli* (*57*).

Chloramphenicol

The mechanism of chloramphenicol resistance specified by R factors has been elucidated by Suzuki and Okamoto (*59*) and by Shaw (*60*), who found that a crude extract of an R+ strain, in the presence of acetyl coenzyme A, was capable of converting chloramphenicol to an inactive *O*-acetyl derivative. The product of the acetylation is a mixture of 3-acetoxychloramphenicol and 1,3-diacetoxychloramphenicol (*59, 60*). Both products are completely inactive as antibiotics and are apparently produced by the action of a single enzyme, chloramphenicol acetyltransferase. This enzyme is synthesized constitutively in R+ *E. coli* (*59, 60*) and is subject to catabolite repression (*61*). It is apparently not a periplasmic enzyme, since it is not released when *E. coli* is converted to spheroplasts.

Many chloramphenicol analogs have been tested as substrates for R factor chloramphenicol acetyltransferase, and only the natural D-*threo* form of the antibiotic (Fig. 4) can be modified (*60*). These studies were facilitated by a convenient and sensitive colorimetric assay for chloramphenicol acetyltransferase of Shaw (*60*), who has used this assay to screen for acetylating activity in other bacterial strains. In general, modification of chloramphenicol was not observed in extracts of strains in which chloramphenicol resistance was due to a chromosomal mutation. [Resistance to chloramphenicol in *E. coli* is believed to be a permeability defect (*62*).] Shaw detected chloramphenicol acetyltransferase in an R− strain of *Proteus* and pointed out the apparent similarity between the *Proteus* enzyme and the R factor enzyme (*63*). Chloramphenicol-resistant strains of *Staphylococcus aureus* contained a different chloramphenicol acetyltransferase that, in contrast to the *E. coli* enzyme, was synthesized only in the presence of an inducer—chloramphenicol or a chloramphenicol analog (*64*). This inducible acetyltransferase in *Staphylococcus* is determined by a plasmid gene (*39*).

Aminoglycoside Antibiotics

There are several different mechanisms of resistance to the aminoglycoside antibiotics. First, Okamoto and Suzuki (*65*) found that kanamycin and streptomycin were inactivated by cell-free extracts of R+ strains. Although this inactivation has been known for some time, only recently has the enzymology of these reactions been studied.

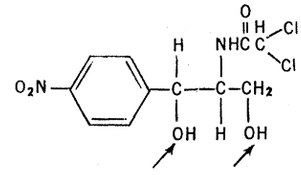

Fig. 4. The structure of chloramphenicol. The arrows indicate the 1- and 3-hydroxyl groups that are acetylated by chloramphenicol acetyltransferase.

Five enzymes that modify aminoglycoside antibiotics have been characterized (Table 3). These enzymes are (i) streptomycin phosphotransferase, which inactivates streptomycin by phosphorylation (*66*); (ii) streptomycin adenylate synthetase, which inactivates streptomycin (*67–69*) and spectinomycin by adenylylation (*68, 70*); (iii) kanamycin acetyltransferase, which inactivates kanamycin by *N*-acetylation (*71–73*); (iv) kanamycin phosphotransferase, which inactivates kanamycin and neomycin by phosphorylation (*66, 74, 75*); and (v) gentamicin adenylate synthetase, which inactivates the gentamicins, kanamycin, and tobramycin (*76, 77*).

Modification of the antibiotic can be monitored conveniently by reacting a radioactive substrate—[^{14}C]adenosine triphosphate (ATP) for adenylylation, [α-^{32}P]ATP for phosphorylation, or [^{14}C]acetyl coenzyme A for acetylation—with unlabeled antibiotic in the presence of the appropriate enzyme (*78*). The modified drug is then absorbed to phosphocellulose paper, and the transfer of radioactivity is determined. These assays are extremely sensitive and are convenient for screening many possible enzyme substrates and inhibitors. Umezawa and his associates have done most of the chemical characterizations of the products of inactivation of kanamycin and related antibiotics.

The initial experiments of Okamoto and Suzuki (*65*) suggested that streptomycin was inactivated in R factor strains by *O*-phosphorylation (Fig. 5). However, it is now apparent that streptomycin can be inactivated in two different ways by two enzymes, which transfer, respectively, the terminal phosphate (*66*) or the adenosine monophosphate residue of ATP to the 3-hydroxyl group of the L-glucosamine ring of streptomycin (*67–69*). The resulting phosphate ester or phosphodiester derivatives of streptomycin are completely inactive as antibiotics and appear un-

able to bind to the target site of the antibiotic on the ribosome (*79*).

The two enzymes, streptomycin adenylate synthetase and streptomycin phosphotransferase, are synthesized constitutively in R+ strains and are classified as periplasmic enzymes since they are released when cells washed with tris-(hydroxymethyl)aminomethane hydrochloride buffer containing ethylenediaminetetraacetic acid are subjected to osmotic shock (*80*). This convenient treatment yields partially purified enzyme preparations for studies of the enzymatic inactivation of streptomycin. The two enzymes have molecular weights of approximately 30,000 and require only ATP for inactivation of the drug. The phosphorylating enzyme can use guanosine triphosphate as alternative substrate, and the adenylylating enzyme can use deoxyadenosine triphosphate instead of ATP (*67–69*).

The two enzymes show substantial differences in the substrates that they modify. The phosphorylating enzyme can modify both *N*-methyl- and *N*-demethyldihydrostreptomycin (*68, 81*); the adenylylating enzyme does not act upon either molecule.

Neither enzyme modifies mannosidostreptomycin (streptomycin B) (*82*); this antibiotic, although a less potent antibacterial agent than streptomycin, can kill streptomycin-resistant R+ strains that produce either enzyme.

Perhaps the most striking difference between the two enzymes is that the adenylylating enzyme can modify and inactivate the antibiotic spectinomycin in addition to streptomycin. This activity is responsible for cross-resistance for these two antibiotics in certain R factor strains (*68, 70*). A D-*threo*-methylaminoalcohol moiety is apparently an important structural element of the substrate, since this residue is present in streptomycin and spectinomycin (Figs. 5 and 6). No other mechanisms of inactivation of streptomycin or spectinomycin are known, and all streptomycin- and spectinomycin-resistant R+ strains that have been examined contain one of these two inactivating enzymes.

Drug	R_1	R_2	R_3	R_4
Streptomycin	CH_3	H	H	CHO
N-Methyldihydrostreptomycin	CH_3	CH_3	H	CH_2OH
N-Demethyldihydrostreptomycin	H	H	H	CH_2OH
Mannosido-streptomycin	CH_3	H	Mannose	CHO

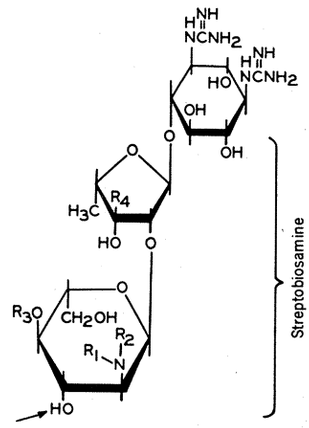

Fig. 5. The structure of streptomycin and some related compounds. The 3-hydroxyl group (arrow) on the L-glucosamine ring is modified by streptomycin adenylate synthetase and streptomycin phosphotransferase. The streptobiosamine part of the molecule is a substrate for both enzymes.

Inactivation of Kanamycin and Neomycin

N-Acetylation and *O*-phosphorylation are the common inactivation mechanisms for the neomycin and kanamycin antibiotics. Chemical studies by Umezawa and his associates (*71, 72, 74, 75*) have established the structures of the inactivated kanamycins and paromamine. Recently, these reactions have been studied in periplasmic extracts of R+ strains with the above assay in which phosphocellulose paper is used (*66, 73, 78, 83*). Many substrates have been examined, and the conclusions of the Umezawa group have been confirmed and extended. There is usually a direct correlation between the presence of these enzymes in R+ strains and resistance to the antibiotics that can be modified. The only exceptions are those compounds that are not completely inactivated by acetylation; for example, the *N*-acetyl derivative of neomycin B retains substantial biological activity (*73, 78*).

In R+ strains that possess the acetylating enzyme, kanamycin A is inactivated (*71, 72*) by enzymatic acetylation of the amino group on the 6-D-glucosamine ring (Fig. 7). However, in R+ strains that possess the phosphorylating enzyme, kanamycin A is inactivated by enzymatic phosphorylation of the 3-hydroxyl group of the same ring (*74, 75*). 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+ strain. Thus, a strain carrying the phosphorylating enzyme is resistant to neomycin, kanamycin, paromomycin, and several other related compounds. There is no apparent relation between the enzyme that phosphorylates streptomycin and that which phosphorylates kanamycin and related compounds, or between the enzyme that acetylates chloramphenicol and that which acetylates kanamycin.

Table 3. The R factor enzymes that modify aminoglycoside and aminocyclitol antibiotics. Except where noted, strains with a given enzyme are resistant to substrates for that enzyme.

Enzyme	Substrate
Streptomycin phosphotransferase	Streptomycin
Streptomycin adenylate synthetase	Streptomycin Spectinomycin
Kanamycin acetyltransferase*	Kanamycin Neomycin B
Kanamycin phosphotransferase	Kanamycin Neomycin B Paromomycin
Gentamicin adenylate synthetase	Gentamicin Kanamycin Tobramycin

* Does not confer resistance to neomycin B.

Gentamicin resistance that is mediated by R factors is a recent discovery. Three reports of transmissible gentamicin resistance of clinical orgin in Enterobacteriaceae have been made in the past 2 years (*76*). The strains are resistant to gentamicin, kanamycin, and tobramycin; and enzymatic studies with various substrates have indicated that resistance is mediated by an enzyme that inactivates these antibiotics by adenylylation (*77*). This enzyme is quite different from that which adenylylates streptomycin and spectinomycin (*67–69*).

Enzymatic inactivation of gentamicin has also been found in certain strains of *Pseudomonas aeruginosa* isolated from burn patients. Gentamicin is inactivated by acetylation on the deoxystreptamine moiety by an enzyme that shows little

reactivity with other aminoglycoside antibiotics (*83a*). It is not known if this resistance mechanism, like carbenicillin resistance, is determined by an R factor.

Enzymatic *O*-phosphorylation is a very effective—and the most common—form of inactivation of aminoglycosides and the antibiotics so modified are completely devoid of antibacterial activity. Similar enyme activity has been found in strains of *Pseudomonas* and *Staphylococcus* and is responsible for resistance to neomycin and kanamycin in these strains (*84*). In *Pseudomonas* this activity is probably determined by an R factor; and in *Staphylococcus*, by a related episome. No studies comparing these enzymes and the R factor enzymes have been done. A relation between the various phosphorylating enzymes might suggest the evolutionary origin for the R factor enzyme. The recently isolated gentamicin C components (*85*) and tobramycin (*86*) are aminoglycoside antibiotics that lack the 3-hydroxyl group that is modified by the phosphorylating enzyme. This presumably accounts for the effectiveness of these antibiotics against those R+ strains and strains of *Pseudomonas* and *Staphylococcus* that can phosphorylate antibiotics of the neomycin-kanamycin group. Similarly, antibiotics such as paromomycin, in which a hydroxyl substituent on 6-position of the D-glucosamine ring, are inert to inactivation by the *N*-acetylating enzyme (*71, 73*).

Several aminoglycoside antibiotics that are not substrates for *O*-phosphorylation or *N*-acetylation have structures similar to the antibiotic substrates and are potent inhibitors of the enzyme reactions. Table 4 lists these inhibitors. The aminoglycoside antibiotics that lack a 6-amino substituent in the D-gluosamine ring inhibit *N*-acetylation, and drugs that lack a 3-hydroxyl substituent inhibit *O*-phosphorylation, provided that the compounds are otherwise structurally similar. (Streptomycin, for example, does not closely resemble kanamycin or neomycin and does not inhibit the enzymatic inactivation of these compounds.)

These inhibition studies were carried out with isolated enzyme preparations and may not apply in vivo. Nevertheless, certain antibiotics might prevent the inactivation of other antibiotics clinically. Several antibiotics (gentamicin, tobramycin, and paromomycin) are not only inert to a particular form of R factor inactivation but can inhibit at low (equimolar) concentrations the inactivation of other aminoglycoside

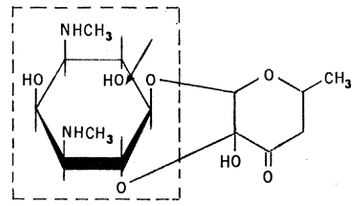

Fig. 6. The structure of spectinomycin. The arrow indicates the hydroxyl group that is adenylylated by streptomycin adenylate synthetase. The moiety in the dotted box is actinamine, which is also a substrate for this enzyme.

antibiotics. Umezawa (*87*) reported that simple sugars such as 3-amino-3-deoxy-D-glucosamine were weak inhibitors of the phosphorylation of kanamycin by the enzyme from *Pseudomonas* strains.

Molecular Nature and Replication of R factors

The structure and replication of extrachromosomal genetic elements such as R factors have been studied for many reasons. Knowledge of the base composition of R factor DNA might indicate the origin of RTF and r-determinants, since the DNA base composition of an episome or plasmid is assumed to be similar to that of the ancestral strain. Examination of the DNA of segregant R factors that have lost one or more genes might yield the base composition of the missing genes. Since many R factors of independent origin are similar genetically and physiologically, comparisons of their base composition might reveal whether R factors are closely related or whether

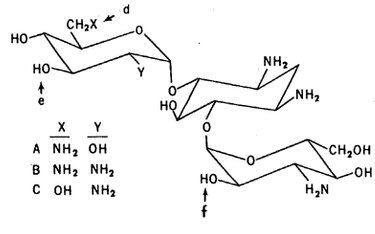

Fig. 7. The structure of the kanamycins. Substituents at positions 2 and 6 for kanamycins A, B, and C are given. The arrows indicate the sites where these antibiotics are modified by kanamycin acetyltransferase (*d*), kanamycin phosphotransferase (*e*), and gentamycin adenylate synthetase (*f*). Neomycin B has the same D-glucosamine as does kanamycin B. The gentamicin C antibiotics and nebramycin factor 6 have related structures but lack the 3-hydroxyl group that can be phosphorylated (*e*).

they are diverse episomes that have been grouped together simply because they harbor drug-resistance genes.

Episomes and plasmids offer many advantages for the study of DNA replication and its control. Since these extrachromosomal genetic elements are much smaller than the bacterial chromosome, it is usually possible to isolate episomal DNA that has not been fragmented by shear. This has permitted study of the structure of replicating and resting genetic elements.

Since R factors can be transferred to Enterobacteriaceae that differ considerably in base composition of chromosomal DNA (*16*), it is usually possible to prepare an R+ strain in which the base compositions of R factor and host DNA are quite different (*88, 89*). The R factor DNA is then manifest as a satellite band in a CsCl density gradient because the buoyant density of DNA is a function of its base composition. *Proteus mirabilis* has been used most in such experiments because the base composition of the chromosomal DNA of this species—40 percent guanine (G) + cytosine (C)—is significantly different from that of all R factors examined. The DNA of most R factors has the same density as *E. coli* chromosomal DNA, so it is not possible to visualize R factor DNA as a satellite band in this host. Recently the unique properties of covalently closed circular R factor DNA have permitted its study in *E. coli* (*15, 25, 90, 91*).

NR1 Density Profiles in Proteus mirabilis Vary with Culture Conditions

Of all R factors studied, NR1 has been characterized most thoroughly with respect to molecular structure and replication, with *P. mirabilis* the usual host strain. NR1 consists of two DNA components of density 1.712 g/cm^3 (52 percent G + C) and 1.718 g/cm^3 (58 percent G + C) (*23, 25, 88, 89*). There are multiple copies of NR1 per host cell in *P. mirabilis* (*23, 25, 88, 92, 93*).

In this species the density profile of NR1 DNA in a CsCl gradient depends in a characteristic manner on the conditions under which the host cells are cultured (*15, 23, 25, 94*). When R+ *P. mirabilis* is cultured in drug-free medium for a long period (Fig. 8A), NR1 DNA forms a single satellite band of density 1.712 g/cm^3 whose proportion is about 8 percent of the *P. mirabilis* chromosomal DNA (1.700

g/cm³) in stationary phase cultures. After prolonged growth in medium containing any of the drugs to which NR1 confers resistance (except tetracycline), a much larger satellite band of density 1.718 g/cm³ is observed; this band is usually markedly skewed toward the less dense side (Fig. 8C). These two types of density profiles are interconvertible, depending on the conditions of cell culture. From detailed kinetic analysis, it appears that a broad and diffuse band of intermediate density is intermediate in this transition (Fig. 8B); this band is apparently a collection of molecules having a broad spectrum of density between 1.712 and 1.718 g/cm³ (*15, 25, 94*).

These systematic changes in the density profile of NR1 DNA appear to reflect the dissociation and reassociation of the components of R factors under different growth conditions (*15, 25, 94*). There is now evidence that several different R factors in *P. mirabilis* dissociate to varying degrees into the transfer factor and r-determinants (*15, 23–26, 94, 95*). Each element appears to be capable of autonomous replication. In NR1, the genes for tetracycline resistance appear to reside on the transfer factor (RTF-TC), which has a density of 1.711 g/cm³. The remainder of the drug-resistance genes (CM, SA, SM and SP) are located on r-determinants (1.718 g/cm³). When RTF-TC and r-determinants are reunited to form the R factor NR1, the composite structure (1.712 g/cm³) appears to replicate under the control of the RTF-TC replication system. When NR1 in *P. mirabilis* reunites, more than one copy of r-determinants can be incorporated into individual R factors. Polygenic molecules that harbor tandemly repeated sequences of r-determinants are thus formed, as is illustrated schematically in Fig. 9. The extra copies of r-determinants required for this formation of tandem sequences appear to be provided by autonomously replicating r-determinants in the dissociated state (*15, 25, 94*).

Selection for Cells with Many Copies of r-Determinants

These considerations explain the different density profiles that are observed for NR1 DNA in *P. mirabilis* when cells are cultured in drug-free medium (Fig. 8A) or in medium containing appropriate drugs (Fig. 8C) or are in transition between these two states (Fig. 8B). In medium containing appropriate drugs, there is selection for cells that harbor the most copies of r-determinants because these cells have the highest drug resistance and grow most rapidly. Such cells result from the incorporation of multiple copies of r-determinants into individual R factors.

Table 4. Antibiotic inhibitors of the R factor enzymes that modify kanamycin. The enzymes are inhibited by 50 percent or more when the inhibitor is present at a concentration equimolar with that of substrate (*73, 78, 83*).

Enzyme	Inhibitor
Kanamycin acetyltransferase	Paromomycin Gentamicin A Gentamine A
Kanamycin phosphotransferase	Gentamicin C_{1a} Tobramycin Sisomicin

The addition of multiple copies of r-determinants (1.718 g/cm³) to an RTF-TC (1.711 g/cm³) increases the density of the R factor DNA, as is illustrated in Fig. 9. The DNA of R factors that harbor only a few copies of r-determinants is manifest as the broad band of intermediate density (Fig. 8B).

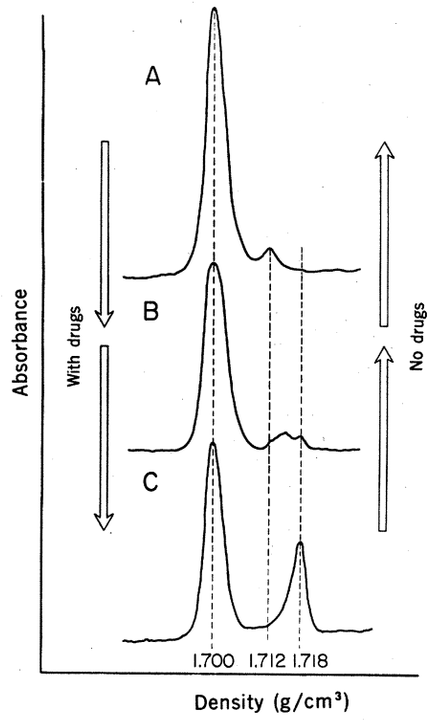

Fig. 8. Changes in DNA density profiles of R factor NR1 in *P. mirabilis*, depending on whether cells are cultured in drug-free medium (bottom to top) or medium containing appropriate drugs (top to bottom). The peak with density of 1.700 g/cm³ is host chromosomal DNA (*15, 25, 94*).

The density of R factors with many copies of r-determinants is essentially the same as that of r-determinants themselves since most of the R factor DNA is from r-determinants. Such R factors predominate after prolonged growth in medium containing appropriate drugs (Fig. 8C). In cells so grown, the proportion of DNA in the satellite band is considerably greater than that in cells grown in drug-free medium because incorporation of additional copies of r-determinants has increased the size of R factors.

The shift in the density of NR1 DNA from 1.718 to 1.712 g/cm³ appears to be essentially the reverse of the process just described. During growth of R+ *P. mirabilis* in drug-free medium, r-determinants dissociate from R factors that have tandem sequences of these elements, and smaller and less dense R factors are formed. This results in a shift in the density distribution of the NR1 DNA toward a limiting value of 1.712 g/cm³ and a decrease in the proportion of the satellite DNA band.

The decrease and eventual disappearance of the 1.718 g/cm³ band after prolonged growth in drug-free medium (Fig. 8A) indicates that r-determinants are diluted in the cells after dissociation. This finding has led to this suggestion: r-determinants replicate under more stringent control than do copies of the RTF-TC, that is, r-determinants undergo fewer rounds of replication during the bacterial division cycle (*15, 25, 94*). The extra copies produced by dissociation are thus diluted passively by cell division. This suggestion also explains why r-determinants are incorporated into R factors during growth in drug-containing medium rather than remaining autonomous. As a part of an R factor, r-determinants replicate more frequently because they are under the more relaxed control of the RTF-TC system. In any event, the behavior of R factors in *P. mirabilis* suggests several novel mechanisms by which the number of copies of specific gene segments can be regulated.

Other R Factors are Similar

Since the discovery of the systematic changes in the density profile of NR1 DNA in R+ *P. mirabilis* (*23, 25*), other R factors have been examined in this host (*24, 26, 95, 96*). Many of these consist of two components whose densities are approximately 1.712 and 1.718 g/cm³. Bands of intermediate

density also are observed; these bands appear to be composite structures of the two components. The component of lower density appears to be the RTF (*15, 23, 25, 26, 97*), although the possibility that some drug-resistance genes might normally reside on this component has not been excluded.

Nakaya and Rownd (*98*) found that when cells that contained R factors of type R(f) or R(i) were cultured in medium containing appropriate drugs, DNA's from both R factors underwent profile changes similar to those in Fig. 8. These cyclic changes in the density profile of R factor DNA are apparently a general phenomenon in *P. mirabilis* and presumably reflect the dissociation and reassociation of the RTF and r-determinants.

Recently, several R factors of independent origin have been found to consist of a component of density 1.705 g/cm^3 (45 percent G + C). One of these low-density R factors, Rtsl, whose replication is temperature-sensitive, has been characterized by Terawaki and his associates (*99, 100*). This R factor harbors resistance to only one drug (kanamycin), and the density profile of Rtsl DNA is not changed during growth in medium containing kanamycin. Several lines of evidence indicate that the transfer factor of Rtsl is quite different than the RTF's of R factors such as NR1. R factors of genotype CM SA SM TC (*101*) and AP SM (*96*) are also of the low-density variety.

As we mentioned above, most studies on R factor DNA in *E. coli* have been on the covalently closed circular form of episomal DNA. (In interpreting these experiments, one must consider that all of the R factor DNA in a cell might not be in this form.) This approach has been used primarily to fractionate R factor DNA for size estimation by electron microscopy or sedimentation analysis and to study the replication of R factors (*15, 25, 90, 91*). These studies have shown that many R factors are circular DNA molecules with molecular weights of approximately 70 × 10^6. The molecular weight of the RTF is about 60 × 10^6, and that of r-determinants is about 12 × 10^6. Experiments similar to those in Fig. 8 but with R$^+$ *E. coli* and R$^+$ *Serratia marcescens* have shown that there is no change in the density profile of NR1 DNA during growth in medium containing drugs (*15*). The available evidence suggests that NR1 exists as a composite structure in both of these species and that the transfer factor and r-determinants do not dissociate and reassociate as they do in *P. mirabilis* (*15, 25, 94*).

Replication of R Factors and Synthesis of R Factor Gene Products

Almost all experiments on replication of R factors and synthesis of R factor gene products have been carried out in *P. mirabilis*, because R factor DNA can be easily distinguished from chromosomal DNA of this host in a CsCl gradient. The study of the synthesis of R factor gene products in this host strain is of particular interest because of the variation in the number of copies of drug-resistance genes per cell.

For drugs whose resistance genes are on r-determinants (CM, SA, SM and SP), *P. mirabilis* cells with the 1.718 g/cm^3 form (Fig. 8C) of NR1 DNA have higher drug resistance than do cells with the 1.712 g/cm^3 form (Fig. 8A) of R factor DNA (*15, 25, 102*); this is as expected from the above discussion. If the average number of copies of r-determinants on polygenic R factors is estimated from the area under the peak for 1.718 g/cm^3 DNA, there is a linear dose-response relation between the number of copies of drug-resistance genes per cell and the specific activity of chloramphenicol acetyltransferase (*15, 25*).

Thus, *P. mirabilis* cells regulate resistance to several antibiotics by a novel mechanism that can vary the number of copies of drug-resistance genes. In contrast, *E. coli* is apparently unable to increase its complement of drug-resistance genes during growth in medium containing drugs, and the level of drug resistance and the specific activity of chloramphenicol acetyltransferase is the same whether drugs were present or absent in cultures (*15, 102*).

The relation between the replication of R factors and the synthesis of chloramphenicol acetyltransferase has also been examined in considerable detail in both *P. mirabilis* and *E. coli*. In most of the *P. mirabilis* experiments, cells with NR1 DNA in the 1.718 g/cm^3 form were used so that the percentage of R factor DNA (*R-DNA*) could be accurately monitored in a CsCl gradient. In Fig. 10 are results of an experiment in which *R-DNA* and the specific activity of chloramphenicol acetyltransferase were determined at frequent intervals during exponential and stationary growth phases and after restoration of a stationary-phase culture to exponential growth (*15, 25*). The *R-DNA* value is constant throughout exponential phase, a result that implies that there is a doubling of the multicopy pool of R factors for each bacterial division cycle, that is, for each duplication of the host chromosome. Thus, even though there is more than one round of R factor replication each division cycle (a round being defined as one replication of one R factor), there is a determined number of rounds (*93*). The specific activity of chloramphenicol

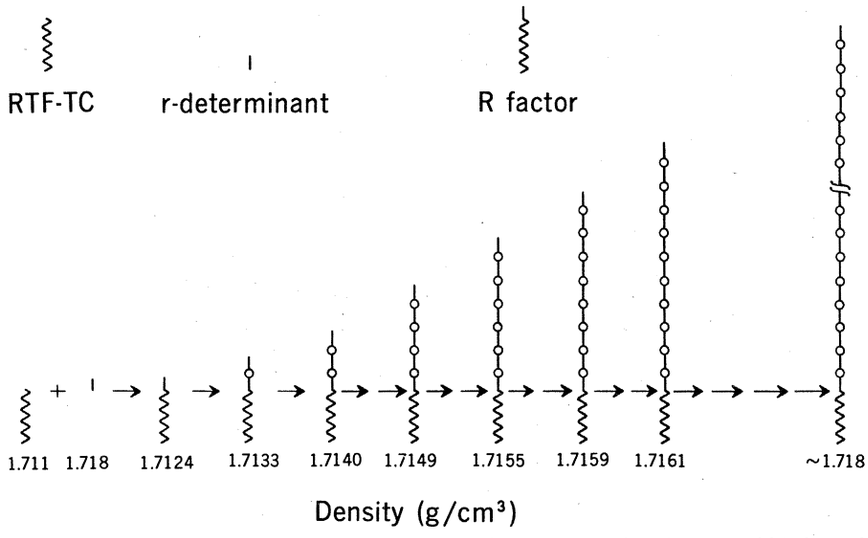

Fig. 9. Diagram illustrating the density change that accompanies the recombination of one or more copies of r-determinants with an RTF-TC to form R factors. In the calculation of the densities shown, each r-determinant copy was assumed to have one-fourth the mass of an RTF-TC copy.

col acetyltransferase is also constant throughout exponential phase.

After the culture enters stationary phase, *R-DNA* increases 2.5-fold (Fig. 10), and *P. mirabilis* chromosomal DNA increases about 2-fold. Since *R-DNA* is measured relative to host chromosome DNA, it follows that the multicopy R factor pool must increase about 5-fold due to continued R factor replication in stationary phase. The specific activity of chloramphenicol acetyltransferase also increases about 5-fold in stationary phase. Thus, although the synthesis of host chromosome gene products is arrested in stationary phase, R factor gene products continue to be synthesized.

Replication and Transcription Are Coupled

After restoration of the culture to exponential growth, *R-DNA* falls with time to the original exponential-phase value (Fig. 10). The kinetics of this process are consistent with a model in which the number of rounds of R factor replication per division cycle equals that characteristic of exponential growth (*93*). The excess R factor DNA that accumulated during stationary phase is diluted passively with each cell doubling. The specific activity of chloramphenicol acetyltransferase decreases with the same kinetics, results that indicate that the rate of enzyme synthesis after restoration of exponential growth is proportional to the number of replicating R factors rather than to the total number of R factors. Taken together, these findings suggest that R factor replication and transcription are coupled in *P. mirabilis* (*15, 25*).

The number of copies of R factor gene segments in *P. mirabilis* can apparently be regulated in two different ways: (i) the dissociation and reassociation of RFT-TC and r-determinants under different growth conditions, and (ii) the continued replication of R factors in stationary phase. As a result of these two mechanisms of gene amplification, the specific activity of chloramphenicol acetyltransferase may be varied over a 100-fold range in *P. mirabilis* (*15, 25, 103*).

In *E. coli* and *S. marcescens* NR1 appears to exist primarily as the composite structure, and the RTF-TC and r-determinants do not dissociate and reassociate as in *P. mirabilis*. However, in these two species NR1 does continue to replicate in stationary phase, and this is accompanied by a corresponding increase in the specific activity of chloramphenicol acetyltransferase.

Other aspects of the control of R factor replication have been examined in *P. mirabilis*. A culture of R+ cells was transferred from nitrogen-14 medium to nitrogen-15 medium, and the light, hybrid, and heavy DNA's synthesized were examined in a CsCl gradient to show that individual copies of the R factors NR1 (*92*) and Rtsl (*100*) are selected at random for replication during the bacterial division cycle. The initiation of the replication of the entire multicopy pool of R factors in *P. mirabilis* does not occur simultaneously, and the initiation of the replication of R factors is not simultaneous with the initiation of the replication of the host chromosome. The time required for the replication of an R factor (S period) is only a small part of the division cycle. Experiments in which auxotrophic mutants of R+ *P. mirabilis* were starved for required amino acids have shown that there is only a small amount of R factor replication after protein synthesis has been arrested (*15, 23*).

These results suggest that one or more proteins are required for the initiation of R factor replication and that there is only a small pool of initiator proteins within individual cells. It is likely that R factor replication is controlled by a positive regulation system in which the number of rounds of replication are regulated by the synthesis of a determined quantity of initiator proteins during each bacterial division cycle and that these proteins interact with individual copies of R factors in a random way (*15, 93*). Since R factor replication continues in stationary phase, synthesis of R factor initiator proteins must also continue.

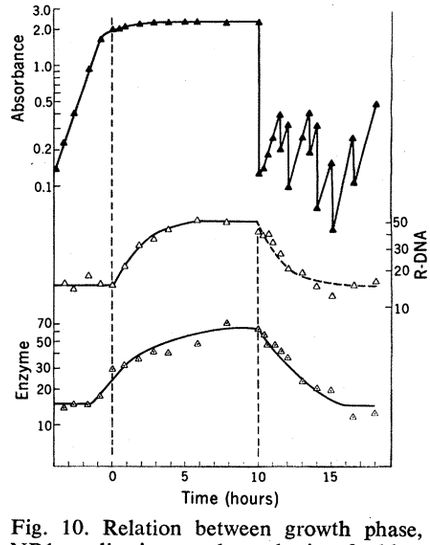

Fig. 10. Relation between growth phase, NR1 replication, and synthesis of chloramphenicol acetyltransferase in *P. mirabilis*. In the ordinate, enzyme is the specific activity of chloramphenicol acetyltransferase, and *R-DNA* is the percentage of R factor RNA. The culture went from exponential (left) to stationary growth phase (between dashed lines) and was restored to exponential growth (*15, 25*).

Concluding Remarks

The R factors are probably the most important determinants of bacterial resistance to antibiotics in the enterobacteria. With the extensive and increasing use of antibiotics in the treatment of human and animal diseases there has been a substantial increase in the incidence of antibiotic-resistant strains in the past decade (*5, 9, 10, 104*).

We have summarized the present status of work on the mechanisms of resistance specified by R factors and on the structure and mode of replication of these extrachromosomal elements. Although substantial progress has been made in these areas of research, there is undoubtedly much to be learned. With the steady introduction of new antibacterial agents it is likely that new mechanisms of resistance will be discovered (for example, the recent appearance of gentamicin resistance). We can account for the function of less than half of the genes of a typical R factor.

A very important and interesting question, which we have not discussed in any detail, is the origin of R factors. Why should such collections of prophylactic genes exist? Why are they apparently limited to a few bacterial strains? It is possible that R factors may have originated from the pickup of various resistance genes by the RTF (*1, 12, 105*). Even if one accepts the presence of sex factors in bacteria as unexceptional, the problem of the source of the r-determinants remains. It has been assumed that the r-determinants of R factors were originally chromosomal genes, which may or may not have determined antibiotic resistance in their original chromosomal state. As chromosomal genes, they may have been involved in the formation of biosynthetic intermediates or in transport or membrane processes.

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