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Extrachromosomal Elements and the Spread of Antibiotic Resistance in Bacteria

THE FOURTH COLWORTH MEDAL LECTURE

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Historical

It was apparent, even to the workers who pioneered the use of antibiotics as chemotherapeutic agents, that organisms resistant to the very antibacterial agents they were developing could be isolated with disturbing ease from the immediate vicinity of the experimental work and even from the workers themselves. Although these observations were somewhat depressing for those who saw antibiotics as 'wonder drugs' capable of protecting mankind from pathogenic bacteria for all time, there were those who realized that the interaction of pathogenic bacteria with antibiotics in localized environments, such as hospitals, could provide a wonderful, if rather antisocial and unfeeling, opportunity of watching evolution in action.

In the early stages of this encounter between bacteria and antibiotics, it was generally assumed that the emergence of drug-resistant bacteria must be based on a process of stepwise mutation and selection; and, indeed, some of the experimental evidence obtained in the early days tended to support this view. For example, resistance of some types of bacteria to sulphonamide—admittedly not an antibiotic in the strict sense of the word—could be achieved by a single-step mutation (Oakberg & Luria, 1947). Sulphonamide, of course, interferes with the function of the tetrahydropteroic acid synthetase enzyme in the folic acid-biosynthetic pathway in sensitive organisms by competing with one of the normal substrates, *p*-aminobenzoic acid, for the active centre of the enzyme (Scheme 1) (Woods, 1940; Brown, Weisman & Molnar, 1961), and it has now been shown that mutation to sulphonamide-resistance in pneumococci, at least, involves a change in the structure of the synthetase enzyme so that its active centre has a considerably lower affinity than normal for sulphonamide whereas its affinity for *p*-aminobenzoic acid is almost unaffected (see Table 1) (Wolf & Hotchkiss, 1963).

Despite the few examples where resistance could be shown to be based on mutation followed by selection of the mutants, it soon became clear that such a relatively simple process was unlikely to account in full for the widespread isolation of resistant organisms from patients treated with antibiotics. A number of lines of reasoning led to this conclusion. First, if one or a few mutations, followed by selection, could give rise to effective resistance, then it should be possible to reproduce the phenomenon in the laboratory. But whenever such an experiment was attempted, although resistant organisms appeared, the type of resistance found was almost invariably different from that encountered naturally. For example, staphylococci resistant to penicillin could easily be obtained by mutagen treatment, or even by growing organisms on penicillin gradient plates, but the type of resistance generated in this way never involved the production of penicillinase, which is so characteristic of the penicillin-resistant staphylococci of hospital origin (Barber, 1957).

The second line of evidence that suggested that the emergence of antibiotic-resistant strains might not be mutational in origin, at least not in the immediate past, came from studies in which the distribution of different types of antibiotic resistance was observed among different strains of a given species. In a number of such cases, the type of resistance in question was initially only found in one or two distinct serotypes or phage patterns within a species, but as the use of the antibiotics was continued so the distribution widened, until ultimately some isolates from all distinct types from within a species were resistant (Guinée, Scholtens & Willems, 1967). An example of this process was the spread of ampicillin-resistance in *Salmonella typhimurium*. Initially, resistance was apparently confined to serotype 1a, but soon afterwards penicillin-resistant members of type 29 were also detected, and from that time on penicillin-resistance has become relatively common in almost all *Salm. typhimurium* serotypes (Anderson, 1965, 1967). Examination of the β-lactamase enzyme, which is responsible for penicillin-resistance in these cells, has shown that the enzyme molecule

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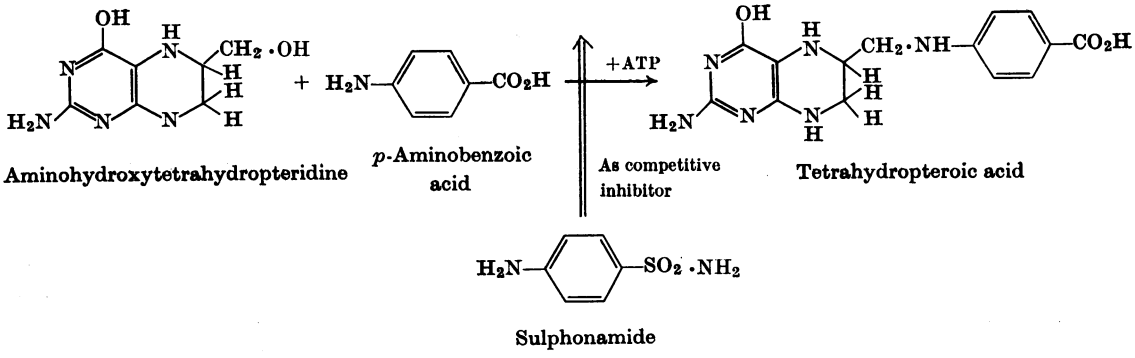
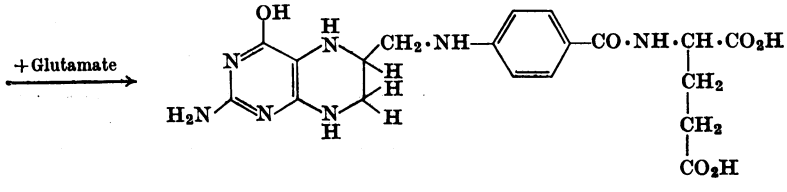
Step 1



Step 2



Scheme 1. Point of the inhibitory action of sulphonamide in the biosynthetic pathway to tetrahydrofolic acid.

Table 1. *K_m, K_i and $V_{max.}$ values for the wild-type and mutant versions of the tetrahydropteroic acid synthetase enzyme from parental and mutant strains of* Pneumococcus

The K_m and K_i values are given as molarities, and the $V_{max.}$ values in n-equiv./min./mg. of protein. The data are from Wolf & Hotchkiss (1963).

Substrate or inhibitor	*p*-Aminobenzoic acid		Sulphonamide
	$V_{max.}$	10^3K_m	10^3K_i
Wild-type enzyme	1·3	0·1	0·06
Mutant enzyme	0·4	0·5	65

itself is closely similar in properties in many cases (M. H. Richmond, unpublished work) and it seems more likely, therefore, that the appearance of penicillin-resistance in an ever-widening range of *Salmonella* types is due to the spread of the β-lactamase structural gene from cell to cell by some infective process, rather than to the possibility that such a complex molecule as an enzyme could have arisen repeatedly by mutation in different strains and yet have such similar properties on each occasion.

Of course, the idea that whole genes, or blocks of genes, can be transferred from one bacterial cell to another, is now a genetic commonplace. Three types of intercell genetic transfer—transformation, conjugation and transduction—are recognized, all of which can transmit antibiotic-resistance determinants, just like any other genes, from cell to cell. In all cases the essence of the transfer is the passage of DNA from one cell to another. In transformation, naked double-stranded DNA passes from cell to cell, apparently unprotected against the nucleolytic activity commonly found in bacterial-culture media, whereas in transduction the DNA is protected by being wrapped in a bacteriophage coat and, moreover, can use the specifically adapted apparatus of the phage for inserting the DNA into the recipient cell. During conjugation, the apposition of the mating pair of cells affords some protection to the DNA during its passage from the donor to the recipient cell.

Despite the undoubted potential of these methods of gene transfer, however, there is some doubt whether they are the only factors that are important in the spread of antibiotic-resistance from strain to strain. Certainly they are not all equally important

for a given pair of strains at all times. In this lecture, therefore, I will first briefly review the existing evidence for the significance of transduction, transformation and conjugation as a means of gene transfer under natural circumstances (taking special note of the occasions on which these routes of transfer must have limited importance) and then go on to discuss other factors that may be important. In particular, I will describe how a combination of generalized transduction and the extrachromosomal nature of the resistance genes may provide an unexpectedly flexible and efficient method for the spread and evolution of antibiotic-resistance in bacteria.

Transformation, transduction and conjugation as means of gene transfer

Transformation. Although a great deal is known about the laboratory conditions necessary for effective gene transfer by transformation, and indeed for transfer by all these three basic routes, less is known about their relative importance as a means of gene transfer in the natural environment and thus of the role they play in the spread of antibiotic resistance. Griffith's (1928) original experiments on transformation showed that the phenomenon could indeed occur in a living mouse, but little attempt was made, at that time, to quantitate the process, largely because it was not until Avery's pioneering work a decade and a half later that anyone had any clear idea of what it was that achieved the 'transformation' (Avery, MacLoed & McCarty, 1944; McCarty & Avery, 1946). Even now attempts to assess the efficiency of the process must rely on laboratory experiments that hardly reproduce the conditions found naturally. In laboratory strains of pneumococci, Hotchkiss (1957) showed that with a culture of 10^8 bacteria/ml. the highest incidence of transformants amounted to about 10% of the culture when the transforming-DNA concentration in the test was about 0·1 μg./ml. (Fig. 1). At this concentration the relative mass of DNA to cell material is about 1:100, and the process seems to be reasonably efficient. But these experiments were carried out under the most favourable conditions that could be devised; under natural conditions one would expect the process to be markedly less effective.

Although Griffith's (1928) experiments show clearly that transformation can be important *in vivo*, two considerations seem to greatly restrict its importance as a general means of gene transfer in bacteria. First—apart from certain *Haemophilus* strains—transformation is confined to Gram-positive bacteria, and among the Gram-positive bacteria themselves it only occurs in strains in which no potent extracellular deoxyribonuclease is produced (Ravin, 1961). Thus, to have any hope of transforming staphylococci, for example, one must first obtain deoxyribonucleaseless mutants. In practice gene transfer by transformation seems to be confined to species of *Bacillus*, *Streptococcus* and *Haemophilus*.

Conjugation. Transfer by conjugation seems to be by far the most effective method found in bacteria, at least in Gram-negative species. The process has been studied extensively and is undoubtedly of the greatest importance for the spread of antibiotic-resistance in enteric bacteria (Watanabe, 1963; Meynell, Meynell & Datta, 1968). Under laboratory conditions, the efficiency of gene transfer by conjugation is high and the process relies greatly for its potency on the fact that cells that have recently received a gene by conjugation have, themselves, a greatly enhanced ability to pass it on to uninfected cells (Lederberg, Cavalli & Lederberg, 1952; Cavalli-Sforza, Lederberg & Lederberg, 1953; Stocker, Smith & Ozeki, 1963). This enhanced ability to transfer newly acquired genes only lasts for a few hours under normal growth conditions, but this is long enough to ensure the wildfire spread of a genetic character (e.g. an antibiotic-resistance determinant) through a sensitive culture.

Although all the problems are not yet solved, the molecular processes underlying this fluent spread of genetic material within a culture seem to involve a disproportionate multiplication of the incoming piece of DNA before replication of this material is synchronized with the synthesis of other genetic material in the recipient (de Haan & Stouthamer, 1963). In this latter synchronized phase, the

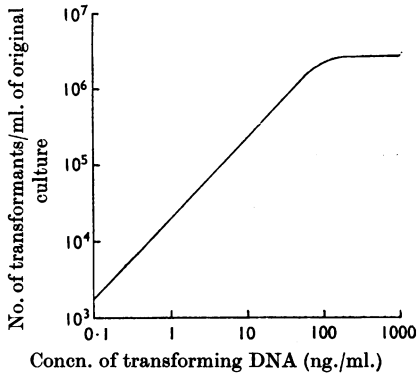


Fig. 1. Relation between the concentration of transforming DNA and the number of transformants obtained. The data are from Hotchkiss (1957); the culture originally contained 10^8 bacteria/ml.

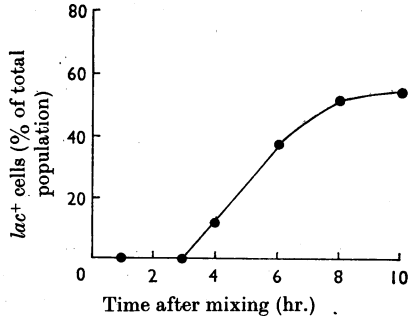


Fig. 2. Spread of the F′.*lac*+ episome in a population of growing *lac*− *Escherichia coli* cells. Initially, one *lac*+ cell was added per 10^4 recipient cells.

number of introduced gene copies seems to fall from many to one, or at least a few, per cell. Whatever the final situation, however, the initial accumulation in the recipient allows the infection of many susceptible recipient bacteria in a relatively short time. For example, Fig. 2 shows the kinetics of acquisition of a *lac*+ gene after the introduction of a few F′.*lac*+ cells into a predominantly *lac*− population. Initially one *lac*+ cell was added for every 10^4 *lac*− recipient cells, yet within 8hr. of mixing, about 50% of the population could be shown to have acquired the *lac*+ gene (M. H. Richmond, unpublished work). Both the *lac*+ and *lac*− cells grow at a closely similar rate (de Haan & Stouthamer, 1963), so there is no chance of the *lac*+ outgrowing the *lac*− fraction of the culture. Similar experiments have recently been performed with resistance-transfer factors (R-factors) in enteric bacteria and this explosive spread of genetic material after conjugation is undoubtedly of the utmost practical significance (Meynell *et al.* 1968). Moreover, the ability for transfer to occur between strains from different species means that a non-pathogenic donor, which might be a normal component of a healthy gut, may act as an effective source of genetic resistance determinants for a superinfecting pathogen (Watanabe, 1963).

Although conjugation can be such an effective means of gene transfer, it does not occur universally among bacteria. For successful transfer to occur, one of the strains involved must carry and the other lack the genetic determinant responsible for initiating the conjugation process. Such genes are only found in the enteric bacteria (Harada, Suzuki, Kameda & Mitsuhashi, 1960) and a few closely related genera such as *Serratia* (Rownd, Nakaya Nakamura, 1966) and *Vibrio* (Baron & Falkow, 1961). As a consequence, gene transfer by conjugation is restricted to this range of bacteria, and is never found among Gram-positive species.

Transduction. The term transduction covers any transfer of genetic material from one bacterial cell to another in which a bacteriophage acts as a vector. Although the term is used widely and often indiscriminately, it comprises three types of transfer that differ in their molecular basis and in their efficiency (see Hayes, 1964, for a general account). In phage conversion the uptake of the phage particle by a recipient bacterial cell always leads to the transfer of the gene in question; in localized (or restricted) transduction the phage can transfer only a very limited range of genetic markers, but these markers are transferred with high efficiency—maybe with a frequency as high as 1/10 or $1:10^2$ recipients; in generalized transduction, the phage seems capable of transferring practically any genetic marker from the donor to the recipient, but the efficiency of the process is low: normally transfer frequencies of about $1/10^8$ recipients can be obtained, but occasionally the frequency may be as high as $1/10^5$ recipients.

Although phage can act with a wide range of efficiency as a vector of genetic material between bacterial strains, it must be stressed that the quoted values for transfer frequencies are usually based on experiments in which the phage and the recipient bacteria are mixed in approximately equal proportions. This situation is most unlikely to arise naturally, and consequently the actual efficiency of transduction as a means of gene transfer under natural conditions must be a good deal less than is implied by the laboratory experiments. In particular it seems unlikely that generalized transduction by itself can ever be an effective mechanism of gene transfer. With a phage/cell ratio of $1/10^4$—which is not unrealistically low in the wild state—the net transfer frequency by generalized transduction could be as little as $1/10^{12}$ recipients, and gene transfer by this means would, consequently, be an inefficient process, to say the least.

On statistical grounds, phage conversion and restricted transduction are more likely to play some part in the spread of antibiotic resistance markers than is generalized transduction. There is, however, no evidence that conversion is ever responsible in practice for this process, despite its undoubted potential as a means of gene transfer. The conversion of non-toxinogenic cells of *Corynebacterium diphtheriae* to toxin production is a case in point. In this case lysogenization of the non-toxinogenic strain with a converting phage invariably results in the appearance of cells that produce toxin; and the process occurs sufficiently freely to be a readily detectable and practically important process (Barksdale, 1959).

Restricted transduction, although apparently a less efficient means of gene transfer than conversion,

has been reported in a limited number of cases to be responsible for the transfer of resistance markers *in vivo*. For example, Novick & Morse (1967) showed that lysogenic phages in staphylococci may, under rare circumstances, become restricted transducing phages for the extrachromosomal elements that carry the penicillin-resistance determinants in these strains, and staphylococcal cells carrying such a restricted transducing phage can, when inoculated into mice, transfer penicillin-resistance to a hitherto sensitive strain of *Staphylococcus aureus* that had been previously established in the animals.

Summary. To summarize the possible significance of these methods of gene transfer, therefore, it seems that most of the evolution and spread of resistance determinants in the enteric bacteria, which have caused so much trouble in hospital infections in the recent past, is due to transfer by conjugation. In other bacterial genera, however, the situation is less clear. Transformation can achieve gene transfer in pneumococci and restricted transduction can, in certain rare cases, be effective in staphylococci, but neither process has the efficiency shown by conjugation among the enteric bacteria. In particular, generalized transduction seems, at first sight, to be of doubtful efficiency, despite its undoubted ability to achieve gene transfer under laboratory conditions. Nevertheless, examination of the transfer of the extrachromosomal genetic elements that mediate penicillin-resistance in staphylococci, particularly in the period immediately after transfer by generalized transduction, suggests that the combination of an extrachromosomal state for the gene concerned with phage-mediated gene transfer may result in a genetic situation of great potential for the evolution and spread of antibiotic-resistance markers. It is this possibility that will be described in detail in the following section.

Penicillinase plasmids and their genetic interaction in Staph. aureus

Penicillin-resistance in *Staph. aureus* is caused by the inducible production of penicillinase, and the genes responsible both for the nature of this enzyme and for the regulation of its synthesis are frequently present on extrachromosomal elements, or plasmids as they are usually called in staphylococcal strains (for review see Novick, 1967; Richmond, 1968). A number of genetic determinants other than those involved in penicillinase synthesis and regulation are also carried on the same plasmids, and some idea of the range of plasmid types that have been encountered to date is shown in Table 2.

All the evidence available suggests that penicillinase plasmids are replicated and distributed to daughter cells at division independently of the chromosome, and in many ways they appear to behave as small chromosomes in their own right. Although the underlying molecular processes involved in plasmid replication and distribution are uncertain as yet, it does seem certain that the plasmids—in a manner analogous to the chromosome *sensu stricto*—have specific attachment sites whose responsibility it is to initiate replication of the plasmid and to ensure its accurate distribution to the daughter cells on division (Novick & Richmond, 1965; Richmond, 1969).

Examination of the behaviour of the penicillinase plasmids shows that they may be classified into two main groups on the basis of a number of distinct properties. These groups are known as 'compatibility groups', and most of the penicillinase plasmids, at least those commonly encountered in hospital staphylococci, fall into two. Originally plasmids were placed in these groups on the basis of their ability to co-exist in a single bacterial cell: pairs of plasmids that could co-exist were said to be compatible and to be members of different groups, while plasmids that could not co-exist—for any length of time at least—were said to be incompatible and members of the same group (Novick & Richmond, 1965). Further experiments have now shown, however, that the compatibility of penicillinase plasmids should be based on the nature of the maintenance site the plasmids use to ensure their replication and distribution: plasmids using the same site are incompatible and those using different sites are compatible (Richmond, 1969).

The fact that plasmids use specific maintenance sites to ensure their survival in the cell means that two distinct genetic situations can arise when a second plasmid is transferred, by transduction in this case, to a cell that already contains one. If the incoming plasmid is a member of a different compatibility group from the resident, then a biplasmid cell is formed with each plasmid attached to its own specific site. Such a genetic situation has, in the past, been rather loosely called a 'dissociated plasmid diploid', since in most of the experiments the genetic markers on the plasmids have been

Table 2. *Genetic markers carried by some of the penicillinase plasmids detected to date in staphylococci of phage groups I and III*

+, Character present; −, character absent.

Character	Plasmid type					
	α, θ	γ	β	ζ	η	ι
A-type penicillinase	+	+	−	−	−	+
C-type penicillinase	−	−	+	+	+	−
Erythromycin-resistance	−	+	−	−	−	−
Resistance to Cd^{2+} ions	+	+	+	−	−	−
Resistance to Hg^{2+} ions	+	+	+	−	+	−
Resistance to arsenate ions	+	+	+	−	−	−

similar enough to give rise to a partially diploid situation. Such dissociated biplasmid cells, which are shown diagrammatically in Fig. 3(*a*), give rise to a characteristic spectrum of segregants as they grow. The two plasmids are lost independently, giving first bacteria carrying a single plasmid and then, in turn, plasmidless cells. Moreover, the haploid segregants arising by this process are usually parental, recombination between the two plasmids before segregation being rare (Richmond, 1965).

The other type of genetic situation that may be encountered—an 'associated' or 'double-plasmid' cell—usually arises when the incoming plasmid is a member of the same compatibility group as the resident (Fig. 3*b*). Under these circumstances no maintenance site is available—it is already occupied by the resident plasmid—and since there appears to be only one site available to members of a given compatibility group in each cell, the only means of survival in the cell for any length of time open to the incoming plasmid is to recombine with the resident to form a double-sized plasmid (Richmond, 1969). The actual molecular mechanism whereby this process of association occurs is as yet uncertain, but it seems that the process must be analogous to that proposed by Campbell (1962) for the integration of lysogenic phage and adapted by Broda, Beckwith & Scaife (1964) to the integration of an extra-chromosomal element (such as an F-particle) into the bacterial chromosome. According to this model, the outcome of a recombination between two plasmids is a single 'open-eight' structure containing all the markers carried on the two parental plasmids. This 'associated' structure is free to break down once again to form two dissociated plasmids that can be either parental or recombinant in nature (see Fig. 4, and Richmond, 1967).

Cells carrying an associated diploid formed by the

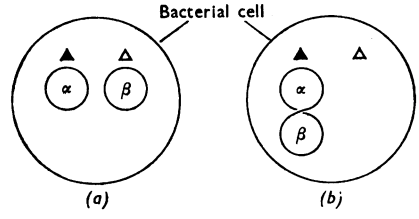


Fig. 3. Diagrammatic representation of 'dissociated' and 'associated' biplasmid cells. (*a*) 'Dissociated' biplasmid cell. The two plasmids α and β are held separately at two distinct maintenance sites designated ▲ and △. (*b*) 'Associated' biplasmid cell. The α- and β-plasmids have formed a single 'open-eight' structure and are held at the type 1 maintenance site (▲).

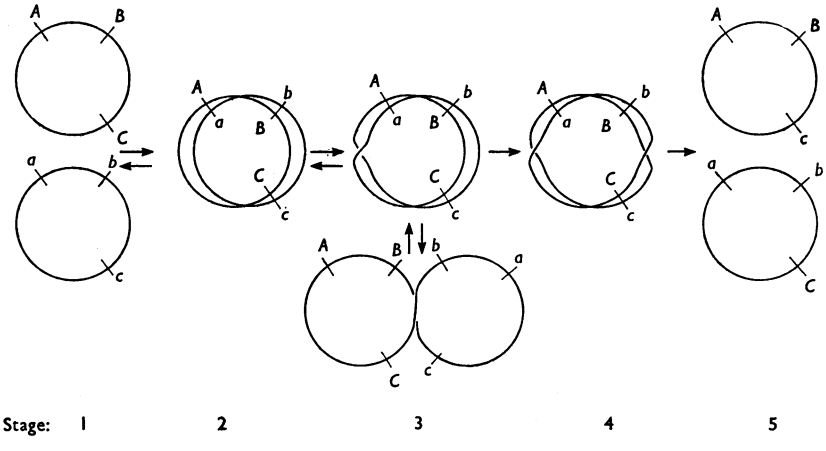


Fig. 4. Reversible association and dissociation of two plasmids. Stage 1: two parental plasmids with allelic markers *ABC* and *abc*. Stage 2: apposition of the two plasmids, *A* opposite *a*, *B* opposite *b* and *C* opposite *c*. Stage 3: formation of a single crossover between the two plasmids to form a single structure of genotype *ABCabc*. This 'open-eight' structure is also shown in an alternative form in which the apposition between the markers has been broken. Stage 4: re-formation of two separate plasmids by a crossover in a different place, and in the opposite direction, from the first. Stage 5: separation of the component plasmids. It should be noted, in the case shown, that these plasmids are now recombinant in nature and have the genotypes *ABc* and *abC*. In practice the genotype of the single plasmids emerging at the end of this procedure depends on the position of the first and second crossovers. Parental plasmids may reappear by a reversal of stages 1 and 2. These genetic interactions are derived from a procedure originally proposed by Campbell (1962) to describe the integration of phage λ into the chromosome of *Escherichia coli*.

Table 3. *Characteristics of 'dissociated' and 'associated' biplasmid cells*

(*a*) Segregation patterns in clones grown from single cells

Type of biplasmid cell	Proportion of segregants (%)		
	Parental	Recombinant	Plasmidless
Dissociated	98	2	0
Associated	47	31	22

(*b*) Transductants obtained when biplasmid cells are used as the source of the phage used to transduce a plasmidless recipient.

Type of biplasmid cell	Proportion of all transductants (%)		
	Haploid parental	Haploid recombinant	Diploid
Dissociated	95	5	1
Associated	26	21	53

Table 4. *Composition of a clone arising from the growth of a single associated biplasmid cell*

The category of biplasmid cells is inferred from the presence of a clone of mixed parental or recombinant genetic constitution.

Total no. of cells examined	Haploid segregants (%)		Biplasmid (%)	Plasmidless (%)
	Parental	Recombinant		
607	42	23	27	8

recombination of two plasmids produce a clearly different segregation spectrum from those in which the two plasmids are maintained separately in the cell. First, because of the physical connexion between the two plasmids, segregation tends to yield plasmidless cells at a single step, and haploid segregants are rare. Further, propagation of a transducing phage on a cell carrying a double plasmid leads to the isolation of a phage preparation capable of transducing both plasmid components of the double structure as a single event. This again is presumably a reflection of the recombinant nature of the structure. Typical behaviour of an associated plasmid cell is compared with that of a dissociated biplasmid cell in Table 3.

From the point of view of the spread of antibiotic-resistance in bacterial populations, it is the formation of the associated plasmid structure and its subsequent behaviour that concerns us most. Table 4 shows the composition of a single clone of staphylococcal cells that arose from a single transductant obtained when a compatibility group 1 plasmid, bearing the markers $i^-p^+.Cd^s.ero^r$, was transferred by transduction to a cell already carrying a plasmid of the same compatibility group with the markers $i^+p^-.Cd^r.ero^s$. The transductants were selected for a marker both on the donor (ero^r) and on the recipient (Cd^r). Apart from the haploid segregants (which do not concern us for the moment) there were still, after about 27 generations from the initial transduced cell, 27% of cells present in the clone that carried all the markers derived from both the donor and the recipient plasmid. Further experiments showed that these cells mostly carried associated plasmids, and consequently it is clear that this stage can be sustained in the cell over a very large number of generations. In fact, picking and recloning the cells demonstrated that the associated plasmid, which has the constitution ($i^-p^+.Cd^s.ero^r.i^+p^-.Cd^r.ero^s$), can survive for at least 100 cell generations in the strain in question. Now, as shown in Fig. 4, associated plasmids are free to break down into single plasmids, but when they do so the probability is immensely high that they will be recombinant, or not parental, since it is extremely improbable that the first and second crossovers involved in the process will occur at precisely the same point in the DNA (cf. Campbell, 1962). This implies that the formation and breakdown of an associated plasmid can potentially give rise to a great variety of recombinant single plasmids. Fig. 5 shows the possibilities for the progeny of two parental plasmids *ABC* and *abc*.

Now let us consider the fate of cell that is carrying a plasmid to which a second plasmid of the same compatibility group is transferred by transduction. After the insertion of the new plasmid, a single associated structure may be formed. As implied by the model shown in Fig. 5, this associated structure is free to break down and re-form. However, only if cell division intervenes between breakdown and re-formation will stable segregants be produced. If the probability of stable segregation is fairly low (10^{-3}/division is a not improbable value for such a clone in practice) then no stable segregants would be likely to arise on statistical grounds until the original transduced cell was well on its way to multiplying by the nine or so generations needed to bring its progeny to a total of 1000. At this point (from the probability of 10^{-3}/division mentioned above), but not before, stable segregants would begin to appear and to accumulate as the culture grew. But the majority of cells at this point would still carry the associated double plasmid. As the growth of the clone proceeded stable segregants would accumulate, but the culture would only revert completely after very many cell generations; and the crux of the matter is that in the course of this process examples of every type of possible recombinant plasmid would arise in the clone arising from the simple transduced cell.

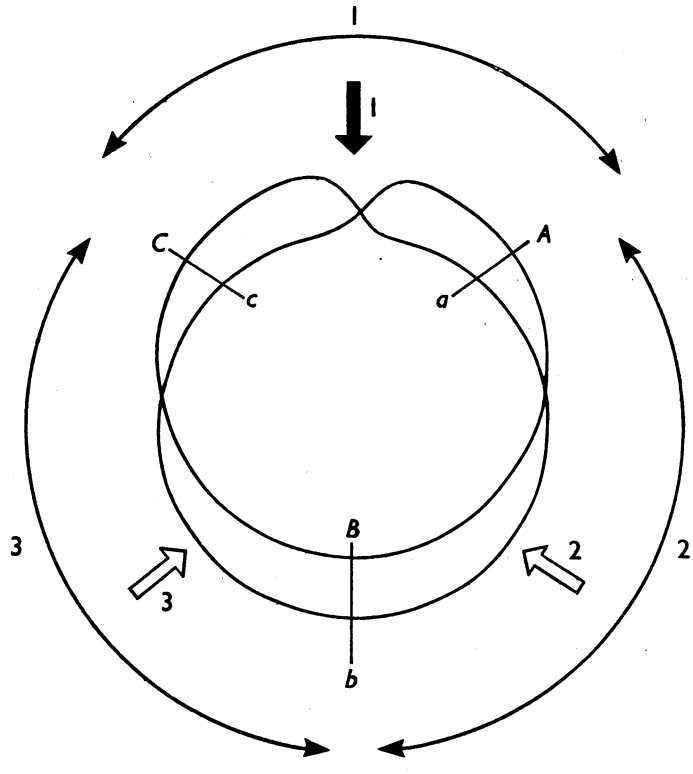


Fig. 5. Possible recombinant plasmids arising from two circular parental plasmids *ABC* and *abc*. The diagram shows the associated structure formed by a crossover at position 1 (➡). Two alternative classes of associated structure can be formed by a crossover at position 2 or 3 (⇨). Single plasmids may arise from each of the associated structures, and also by crossovers at positions 1, 2 or 3. The outcome of all these possibilities is nine pairs of single plasmids of four distinct types: *ABC*/*abc*, *ABc*/*abC*, *ABc*/*aBC* and *AbC*/*aBc*. For further details, see the text. The 'Stages' are defined in Fig. 4.

Stage 1: Parental plasmids:	*ABC abc.*					
Stage 3: Associated structure:						
Formed by crossover at position 1:	*ABCabc*					
Formed by crossover at position 2:	*AbcaBC*					
Formed by crossover at position 3:	*ABcabC*					
Stage 5: Emergent plasmids:						
Position of initial cross-over	1		2		3	
Formed by second cross-over at position 1:	*ABC*	*abc*	*Abc*	*aBC*	*ABc*	*abC*
Formed by second cross-over at position 2:	*Abc*	*aBC*	*ABC*	*abc*	*AbC*	*aBc*
Formed by second cross-over at position 3:	*ABc*	*abC*	*AbC*	*aBc*	*ABC*	*abc*

This behaviour of associated plasmids may well, therefore, have great importance from the point of view of the spread and evolution of antibiotic-resistance patterns in bacteria. Moreover, this sequence goes some way to overcoming the objections that have been raised to generalized transduction as means of gene transfer in bacteria. Although the initial probability of the transduction occurring remains unaltered by these considerations, once the associated structure has been formed, it has two properties that are vital for the subsequent evolution of the clone: first, the associated structure can multiply, and, secondly, it is a totipotent source of all the genetic combinations of markers from both parent plasmids (see Fig. 5). Of course, if the two plasmids carry essentially the same genetic determinants, the scope for evolution is restricted; but if two plasmids that have some regions in common and some that are not shared find themselves in the same cell, then the formation of a new

plasmid is possible. So, for example, when a plasmid with markers *DEF* is transferred to a cell already carrying a plasmid *EFG*, two new types of plasmid can be formed: *DEFG* and *EF* (Fig. 6); and the formation of an associated plasmid that is capable of multiplication ensures that both these new plasmid types will be present among the progeny arising from the original transduced cell.

Although genetic interactions of this type can occur commonly in cells, the evolutionary potential of the process is closely associated with the extra-chromosomal nature of the plasmids. It is possible to think of plasmids as small chromosomes, but they can be distinguished from the true chromosome on the basis of size and the fact that they can be lost from the cell without impairing its ability to grow in the great majority of environmental conditions. In fact, only media containing antibiotics whose resistance determinant is carried on the plasmid will be affected by loss of the plasmid. Thus all the genetic interactions that involve the plasmids—association, dissociation and loss of the plasmid from the cell—can occur without affecting the cell's basic ability to survive under most growth conditions. A similarly flexible genetic situation is much less likely to occur if the antibiotic-resistance determinants are part of the chromosome. Adaptation of the sequence of events with extra-chromosomal elements to the case where the determinants are chromosomal shows that, on segregation, one class of segregants is always likely to lack some chromosomal genes that are necessary for growth under any conditions and will consequently be unable to survive.

In summary, therefore, a picture emerges in which the various methods of gene transfer seem to be important in different groups of bacteria: with enteric bacteria conjugation is almost certain to be the most important, with pneumococci and various *Bacillus* species it may be transformation, and the route outlined above may apply solely to staphylococci. However, the fact that, once formed, extra-chromosomal 'double plasmids' can survive in cells for a relatively long period suggests that such structures may be present, at least transiently, in a significant proportion of bacterial cells isolated under natural conditions. Such cells may readily escape detection since the genetic instability of the biplasmic state will ensure that, by the time the cell has been cultured by conventional techniques, the vast majority of cells in the clone will carry only one plasmid. However, if any biplasmid cells do exist in a bacterial population occupying a given ecological niche, such cells are ideally poised genetically to respond rapidly and flexibly to changes in their environment, such as those caused by the intermittent use of antibiotics.

Until recently the author was an honorary member of the Medical Research Council Group for the Study of Bacterial Enzyme Variation in the Department of Molecular Biology, University of Edinburgh.

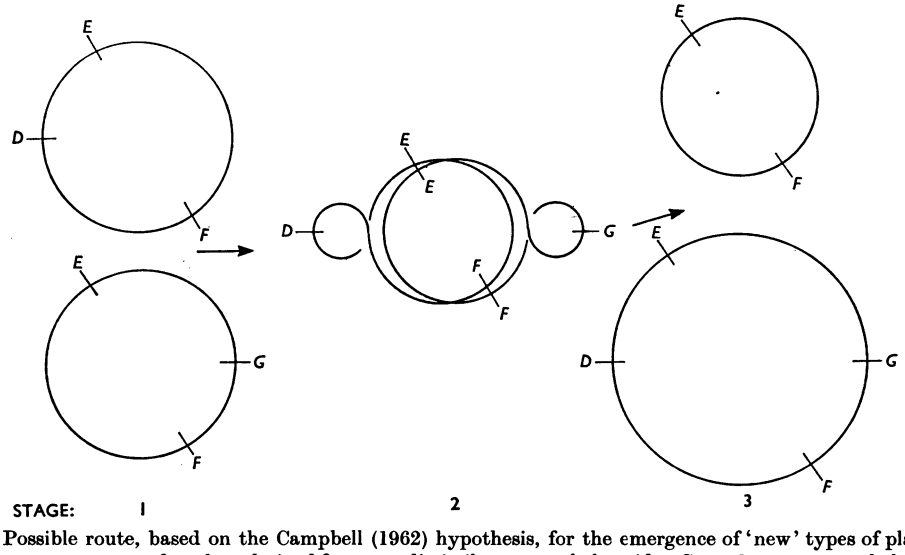


Fig. 6. Possible route, based on the Campbell (1962) hypothesis, for the emergence of 'new' types of plasmids carrying an assortment of markers derived from two dissimilar parental plasmids. Stage 1: two parental plasmids, *DEF* and *EFG*. Stage 2: arrangement of the two parental plasmids to give maximum apposition of homologous regions. Stage 3: two of the possible recombinant plasmids (with constitutions *EF* and *DEFG*) that can arise from the parents *DEF* and *EFG*. For the nature of the intermediate steps between stages 2 and 3, see Fig. 4.

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