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The genetic basis of the spread of β-lactamase synthesis among plasmid-carrying bacteria

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Despite the fact that β-lactamases from a range of bacterial species – Gram-positive and Gram-negative – show evolutionary relatedness, there is no set pattern to the genetic organization that underlies their synthesis and its regulation. Thus, for example, the enzymes of many Gram-positive species are extracellular and inducible, whereas their counterparts in Gram-negative bacteria are often produced constitutively into the periplasmic space of the cells concerned. Nor is the location of the β-lactamase genes always the same: in *Escherichia coli* and *Staphylococcus aureus*, for example, these are commonly plasmid-borne, whereas with other species the genes are chromosomal. Furthermore, the location may not be fixed: some strains of a species may, for example, have their β-lactamase genes on a plasmid, whereas others of the same species may carry the same genes as part of their chromosome.

In many cases the highly flexible genetic arrangement that underlies β-lactamase synthesis derives from two main features: first, where plasmids are involved, their ability to be transferred to related species, and the fact that they can often replicate in their new hosts, ensure that the genes specifying a given type of β-lactamase may move from species to species. Thus one finds enzymes of the same type in many distinct strains and species. The second source of flexibility is that the gene concerned is often part of a transposon: a genetic element incapable of independent replication, but which can move from one bacterial replicon to another by a mechanism independent of normal generalized recombination. Thus, with many β-lactamases – as also with enzymes that inactivate other antibiotics – their genes may move from replicon to replicon within a given bacterial cell, and from cell to cell within a bacterial population. This, then, is an arrangement of much evolutionary potential: something which is operated upon by selection pressure to give rise to the resistant bacterial populations which cause so much trouble in our hospitals. In this context, moreover, one can even think of a third level of organization where plasmid-carrying bacteria move from one host to another by a process of cross-infection.

Even though it is clear that some β-lactamase genes can spread rapidly in susceptible bacterial populations, there also exist mechanisms that limit the extent to which the spread of both plasmids and transposons occurs. For example, some strains are poor recipients for certain types of plasmid, and, at a lower level of organization, some plasmids are relatively immune to the transposition of β-lactamase transposons. Overall, therefore, as common with evolutionary systems, the performance of the system as it affects β-lactam resistance is dependent on a balance of positive and negative influences: transfer of plasmids and transposons, on the one hand, and immunity to such transfers on the other.

Introduction

Despite the fact that many β-lactamases share structural similarities (see Ambler, this symposium), there is no common feature underlying the way in which their genes are organized in bacteria. Thus, for example, the enzymes of many Gram-positive species – such as *Bacillus*

† Elected F.R.S. 20 March 1980.

cereus, *Bacillus licheniformis* and *Staphylococcus aureus* – are inducible and expressed as extracellular products, whereas in the great range of Gram-negative species the enzymes are liberated constitutively into the periplasmic space of the bacteria (Richmond & Curtis 1974).

The extracellular expression of enzymes in bacteria often implies specific mechanisms for achieving their liberation into the surrounding environment, and an examination of the mechanisms involved often shows the production of a 'proto' form of the enzyme, which is subsequently tailored to give rise to the extracellular product. The contribution by Lampen *et al.* in this volume deals with some specific examples of this. From the point of view of the genetic basis of enzyme expression, these mechanisms for enzyme liberation imply the presence of genetic information, closely linked to the structural gene of the extracellular product, since the initial product of gene expression is often larger than the liberated product which is derived from its precursor by proteolytic action. Similarly the specific inducibility of an enzyme system implies the presence of regulatory genes, some to code for the production of high molecular mass regulatory products, and some that make no protein product but which act as the site to which the regulatory macromolecules bind to exert their effect (Imsande 1978). It follows from this, therefore, that β-lactamase expression in many Gram-positive species is the consequence of the activity of a complex of genes; but whether these genes are always organized as a single operon is still undecided.

In contrast, expression of the β-lactamases of Gram-negative species seem frequently to be controlled by less complex genetic systems. When strains of *Escherichia coli* express the so-called TEM or type IIIa β-lactamase (which is usually so among ampicillin resistant variants of this species) the genetic organization that underlies the phenomenon is commonly a transposon of the TnA type (see later) carried on a bacterial R-plasmid (Heffron *et al.* 1975*b*). Analysis of the structure and size of TnA suggests strongly that the β-lactamase gene is unlikely to have extensive regulatory regions linked to it in the transposon (Heffron *et al.* 1978), even if there may be leader sequences to facilitate its liberation into the periplasmic space from the bacterial inner membrane where it appears to be synthesized (Richmond & Curtis 1974). Furthermore, the constitutive nature of the synthesis of this enzyme certainly implies no need for additional regulatory genes.

Even though the genetic organization that underlies the expression of these two broadly different types of β-lactamase system are so different, one cannot assume that the structure of the enzymes that are expressed are also very different. The β-lactamase of *Staphylococcus aureus* and that specified by R_{TEM} in *E. coli* have considerable similarities in primary sequence (Ambler, this volume), even though the extent of linked regulatory regions is markedly different in the two cases (Richmond & Sykes 1973).

The existence of a common primary sequence whose expression is organized in widely different ways implies that the β-lactamase enzyme itself is evolutionarily more primitive than the supplementary regulatory systems that govern its expression; indeed from an argument *a priori* this would seem to be probable. We have little information so far and cannot say by what route the various evolved forms of β-lactamase expression have actually emerged. Much more comparative work on the details of β-lactamase expression in a range of bacterial species is needed before anything categorical can be said in this respect.

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The location of β-lactamase genes

The genetic information in bacterial cells is primarily organized in one of two forms: either as part of a single large linkage group known as the chromosome, or as small self-replicating linkage groups known as bacterial plasmids. Both types of organization are to be found in Gram-positive and Gram-negative species, and β-lactamase genes are to be found in both locations in each group of microorganisms. Indeed, β-lactamase genes are sometimes found to be plasmid-linked in some variants of a species while being chromosomal in others (Asheshov 1966); and occasionally bacteria carry two β-lactamase genes at once, one chromosomal and the other plasmid linked (Jack & Richmond 1970). Nor are β-lactamases necessarily species-specific: staphylococcal β-lactamase seems to be exclusively associated with that species even though its expression is plasmid-mediated (Richmond 1968); but the TEM (or type IIIa) β-lactamase discussed in detail by Ambler (see his paper in this symposium) has been encountered in a large number of distinct Gram-negative bacterial species, such as *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Neisseria gonorrhoeae* and *Klebsiella aerogenes* (to mention only a few) as well as in *Escherichia coli* – the species in which it was originally detected.

The existence of a given type of β-lactamase in a range of distinct bacterial species, and indeed the presence of a given β-lactamase gene as part of the chromosome in one strain, but on a plasmid in another, argues strongly that mechanisms exist whereby genes of this type can move from one genetic location to another and between bacterial species. Nor is such a situation confined to the β-lactamase gene. The β-galactosidase of *Escherichia coli*, for example, is also found in other bacterial species, even to the point at which *Lac*$^+$ *Salmonella typhi* can cause clinical problems (Falkow & Baron 1962).

Plasmids and β-lactam resistance

Wherever an identical enzyme occurs in a range of bacterial species, modern microbiology suggests that the genes concerned may be carried on bacterial plasmids; this has proved to be true of β-lactamase genes in a number of cases. This is not the place to give a detailed description of the structure and properties of bacterial plasmids. Several reviews (Clowes 1972; Bennett & Richmond 1978*a*) and one or two recent books (Falkow 1975; Broda 1979) do this at length. Suffice it to say that plasmids are self-replicating pieces of DNA that are commonly about 1–2% of the size of the bacterial chromosome. The particular importance of plasmids in the context of antibiotic resistance is that they are well adapted to facilitate the movement of resistance genes – once they are located on a plasmid – to other bacteria. The features of plasmid structure that allow this are several. First, as mentioned earlier, they are able to replicate in bacteria independently of the chromosome. Thus they are, in a sense, adventitious pieces of DNA which do not need to undergo recombination with an existing replicon once they have gained access to a new bacterial cell. Secondly, the size of many bacterial plasmids is about that needed to become incorporated in the head of a bacterial virus. This makes plasmids particularly well adapted to transfer from one bacterial cell to another by transduction. In particular this method of transfer seems important among Gram-positive species. However, by far the most important feature of plasmids – at least in the context that concerns us here – is that many carry, as part of their own genetic makeup, genetic information that makes them infectious: in other words, they carry genes that mediate the production by the host cell of

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apparatus specifically adapted to allow the transfer of a copy of the plasmid to a bacterial cell that does not yet carry it. This process, known as bacterial conjugation, seems to be of particular importance to Gram-negative bacteria. The ability of a plasmid specifying resistance to antibiotics to catalyse its own transfer to other bacterial cells that are not at that time resistant clearly has enormous potential for achieving the spread of antibiotic resistance through susceptible populations (Falkow 1975). Moreover, the operation of selection pressure on such an evolutionary situation magnifies the effect by selecting the new resistant bacteria once they are formed.

TABLE 1. THE CHARACTERISTICS OF β-LACTAMASES SHOWN TO BE PLASMID-MEDIATED IN GRAM-NEGATIVE BACTERIA

(At least the first three have been shown, in some strains, to be part of a transposon. Data from various sources, including Glaxo Allenbury's Research Ltd.)

plasmid	trans-poson	substrates									
		pen. G	amp.	carb.	oxa.	meth.	clox.	CER	CET	CFX	CXM
R1	+	100	106	10	5	0	0	76	20	< 10	< 2
RP1	+	100	107	10	5	0	0	74	20	< 10	< 2
p453	+	100	212	8	0	< 2	< 2	56	8	14	4
R977	–	100	253	14	< 2	< 2	2	183	3	< 2	< 2
RGN238	–	100	382	30	197	332	190	30	15	0	< 2
R46	–	100	179	15	646	23	200	37	25	< 5	< 2
R57b	–	100	178	10	336	29	350	44	10	< 5	< 2
RPL11	–	100	90	97	< 2	< 2	< 2	18	< 2	0	< 2
R151	–	100	267	121	317	803	371	32	< 2	11	4
Rms149	–	100	101	253	·	·	3	10	·	< 1	·
pMG19	–	100	88	150	8	16	< 2	40	4	8	< 2

All values are adjusted to a rate of hydrolysis for benzyl penicillin of 100. Abbreviations: pen. G, benzyl penicillin; amp., ampicillin; carb., carbenicillin; oxa., oxacillin; meth., methicillin; clox., cloxacillin; CER, cephaloridine; CET, cephalothin; CEX, cephalexin; CXM, cefuroxime. Missing values: data not available.

At present, most penicillin-resistant strains of *Staphylococcus aureus* owe their resistance to the presence of plasmids (Richmond 1968). For these elements, transfer is normally by transduction, since the plasmids do not carry the genes that would specify infectious self-transfer. A number of distinct groups of such staphylococcal plasmids are known, and this leads to the possibility of finding bacterial cells that produce more than one type of β-lactamase. This is rare in natural isolates, but is something that can be established readily enough in the laboratory (Novick & Richmond 1966).

A wide range of other characters may be found associated with β-lactam resistance in the staphylococcal plasmids. Perhaps the most common is resistance to metal ions – often Hg^{2+} and Cd^{2+} (Richmond & John 1964; Novick 1969) – but, on occasion, resistance to other antibiotics, such as fusidic acid, is also plasmid-mediated (Lacey & Grinsted 1972).

In Gram-negative bacteria a wider range of β-lactamases is to be found which are specified by plasmid-carried genes (Richmond & Sykes 1973; Sykes & Matthew 1976). By far the most common is the so-called TEM (or type IIIa) enzyme, but others are to be encountered in clinical isolates. Table 1 summarizes the situation with respect to plasmid-mediation among β-lactamases to be found in Gram-negative species.

Unlike the situation in *S. aureus*, the plasmids specifying resistance to penicillins and cephalosporins in Gram-negative bacteria are often self-transmissible. Thus, in these organisms,

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transfer seems to be more commonly by conjugation than by transduction. This would seem to have an important practical corollary: spread of given types of β-lactamase genes in Gram-negative bacteria is likely to be more widespread than is the case with Gram-positive bacteria; and experimental observations tend to support this view (Bennett & Richmond 1978).

Transposons

A recent development in the genetics of bacterial β-lactam resistance has been the realization that β-lactamase genes are themselves often – but not always – organized as part of a *transposon*. Transposons are genetic units that can move between bacterial replicons independently of the normal generalized recombination systems of the cell (see Starlinger & Saedler (1976), Kleckner (1977) and Bukhari *et al.* (1978) for reviews). In this respect, therefore, they fulfil a role analogous to self-transmissible plasmids in bacteria, but at a lower level of organization: plasmids facilitate the transfer of resistance genes between bacteria, but transposons play the same role with respect to the transfer of genes between plasmids (Richmond 1979).

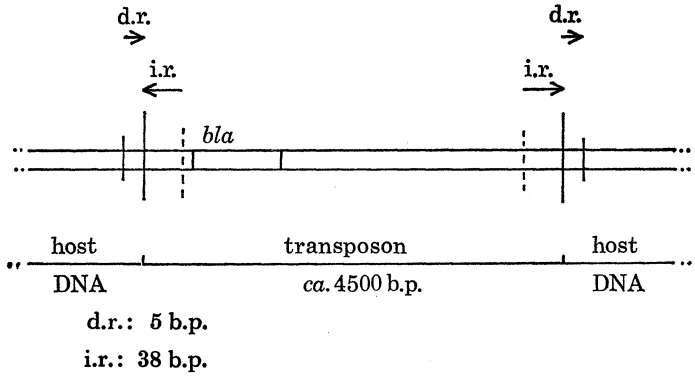

Figure 1. The overall organization of the transposon that mediates β-lactamase expression. Abbreviations: d.r., direct repeat; i.r., inverted repeat; *bla*, β-lactamase gene. Note that the figure is not drawn to scale: the transposon itself is about 4500 nucleotide pairs (b.p.) in length, with the direct repeats at 5 and the inverted repeats at 38 nucleotide pairs in length.

Unlike bacterial plasmids, transposons are not self-replicating units, and as a result they have to rely on functional replicons for replication. Thus they are specialized DNA sequences that exist as part of the chromosome, or of plasmids, or as bacteriophages.

Intensive investigation of the structure of transposons has recently revealed several unique features. Perhaps the most striking is that the transposon always seems to be bounded by so-called 'inverted repeats' (Kopecko & Cohen 1975; Heffron *et al.* 1975*b*): DNA sequences where the order of nucleotides at one end of the transposon is balanced by a sequence at the other end that is closely similar when written in the opposite orientation from the first (figure 1). With several ampicillin resistance transposons that have been investigated (see later) the length of the inverted repeats is 38 base pairs; but longer inverted repeats are to be found in other transposons. For example, the tetracyline transposon commonly encountered in *E. coli* plasmids seems to have inverted repeats of about 1000 base pairs (Kleckner 1977).

Another feature of a transposon – or at least of that part of a replicon where a transposon is present – is that there is always a short duplication of the host sequence on each side of the transposon (N. Kleckner, personal communication). Thus ampicillin resistance transposons

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(TnAs) are commonly bounded by a five base pair *direct* repeat of a host DNA sequence immediately outside the 38 base pair *inverted* repeat of the transposon (see figure 1). Further analysis shows that the five base pair repeat is derived from the host DNA sequence at the particular site of insertion of the transposon and it seems inescapable that this arrangement must be a consequence of the mechanism whereby the transposon is inserted into the host sequence during the transposition process.

Analysis of the base sequence of the inverted repeats that span a transposon shows that the sequence at each end is not necessarily precisely the same. In Tn501, for example (see figure 2), there are three bases in one inverted repeat that are not complementaro to the bases in the equivalent position of the other inverted repeat.

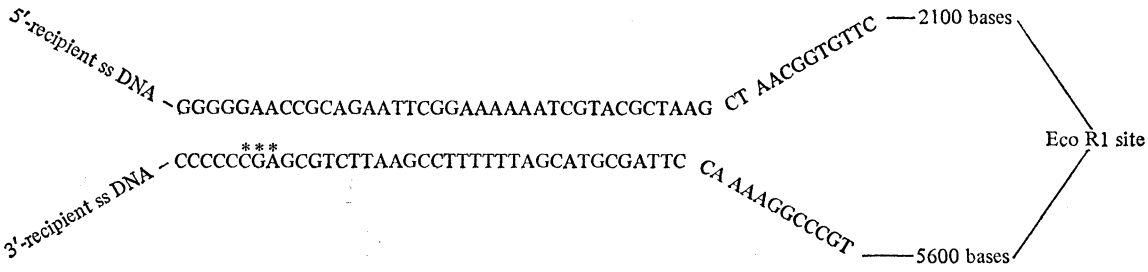

FIGURE 2. The nucleotide sequence of the inverted repeats of the mercuric ion resistance transposon Tn501. In this figure, the structure is drawn as an intramolecular reanneal of a single strand from the transposon. As well as having an EcoR1 site as shown in the figure, there is an EcoR1 site (5′-GAATTC-3′) in each of the inverted repeats. Asterisks indicate mismatched bases.

Although the β-lactamase gene responsible for the expression of the TEM type β-lactamase in many Gram-negative species is part of a class of transposons (TnAs), this is not so for all plasmid mediated genes. Thus there is no evidence as yet to suggest that the β-lactamase gene complex carried on staphylococcal plasmids is part of a transposon, even if the erythromycin resistance gene, which may be present on the same plasmid, can be so (Novick *et al.* 1978). Similarly, there is no evidence that some of the chromosomally mediated β-lactamases of Gram-negative species are part of transposons, even though laboratory experiments make it clear that transposition of TnA onto the chromosome can occur (Richmond & Sykes 1972).

Merely because a particular β-lactamase gene is part of a functional transposon in one species, it does not necessarily follow that the same gene is always part of an identical structure in another species, even though there may be strong circumstantial evidence that the gene in question reached the new species by gene transfer. Studies described later show that the *bla* gene in some strains of the gonococcus have all the characteristics of being present as a fragment of a transposon that has lost one end of its structure and thus its transpositional potential.

TRANSPOSITION

As stated earlier, transposition is the process whereby a section of DNA of specialized structure moves from one replicon to another without involving the generalized recombination systems of the host bacterium (see Kleckner (1977) for a review). There has been much discussion of the rules that determine the possible sites of insertion of the transposon in the recipient replicon, and also what are the essential requirements for transposition to take place.

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Studies on transposition of TnA both to small plasmids such as RSF1010 (Heffron *et al.* 1975*a*), and to larger plasmids of the P and W incompatibility groups (Bennett & Richmond 1976), show that there are many possible sites of insertion in a recipient replicon (figure 3*a*). Examination of the sites of insertion of a given transposon into a common recipient replicon show that these frequently cluster in certain regions of the recipient element. This is partly for technical reasons, because insertion of a transposon into replication genes of a plasmid is likely to block replication and therefore lead to the loss of the plasmid from the growing population.

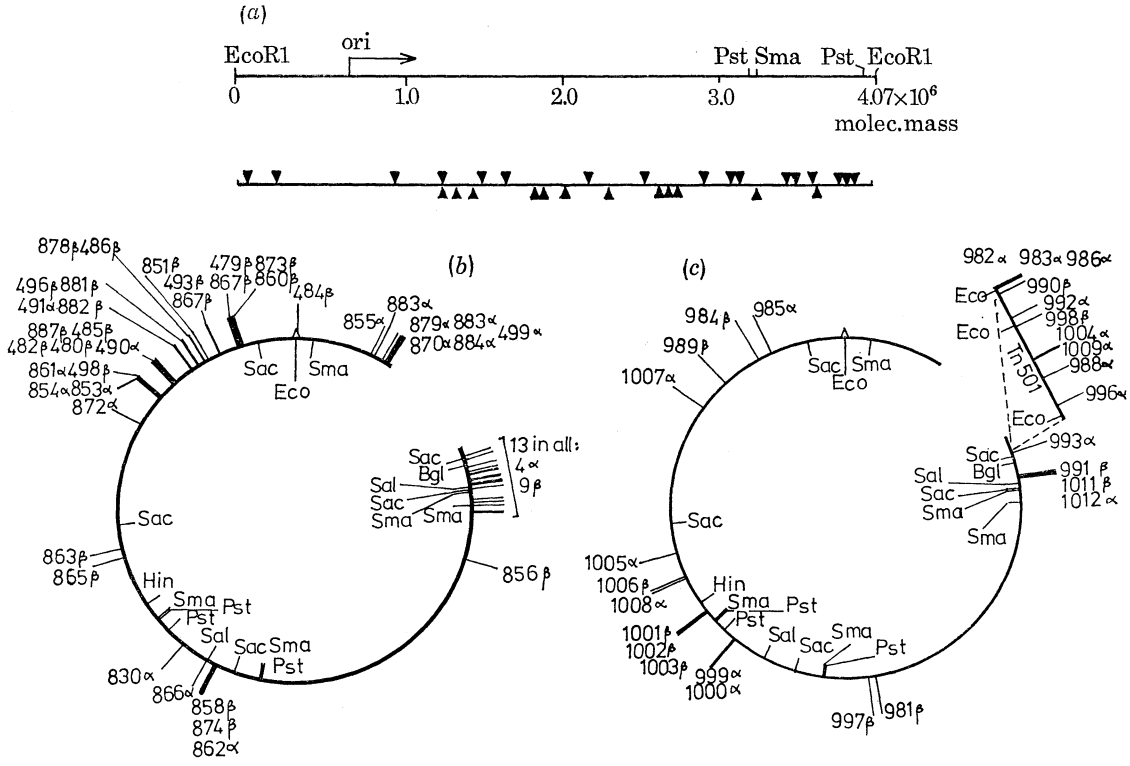

FIGURE 3. Some sites of insertion of TnA into ColE1 (*a*) and two derivatives of RP1 (*b*, *c*). The two derivatives of RP1 are the plasmid pUB307 (*b*) and pUB937 (*c*), the latter being a derivative of the former to which the mercuric ion transposon has been transposed. The plasmid pUB307 is a deletant of RP1 which lacks most, if not all, of TnA.

On the other hand, there do seem to be 'hot-spots' for TnA insertion in some replicons which are nothing to do with the lethal effect of the process (Grinsted *et al.* 1978). Indeed, so many are the potential sites for insertion of a transposon in a recipient plasmid, it is clear that there cannot be stringent sequence requirements in the recipient replicon for insertion of the incoming DNA. This conclusion is reinforced by a further set of observations. If a recipient plasmid is examined for the location of its hot-spots for the insertion of TnA, this pattern of events can be significantly affected by the generation of deletions, or indeed the insertion of further transposons, in the same replicon (Grinsted *et al.* 1978). Figure 3 compares the sites of insertion of TnA into two plasmids, pUB307 and pUB937, which differ only in the fact that pUB937 is a derivative of pUB307 in which the mercuric ion transposon TnM has been inserted by a preliminary transposition event. It is clear that the change involved in making pUB937 from pUB307 has changed the hot-spots in the recipient plasmid. At present it is unclear what causes the change in position of these hot-spots: clearly it is not the DNA base

sequence of the recipient; a more likely possibility is that it is due to promoter sequences in the recipient that allow interaction with enzymes – probably nucleases – that are needed for the transposition phenomenon (Grinsted *et al.* 1978).

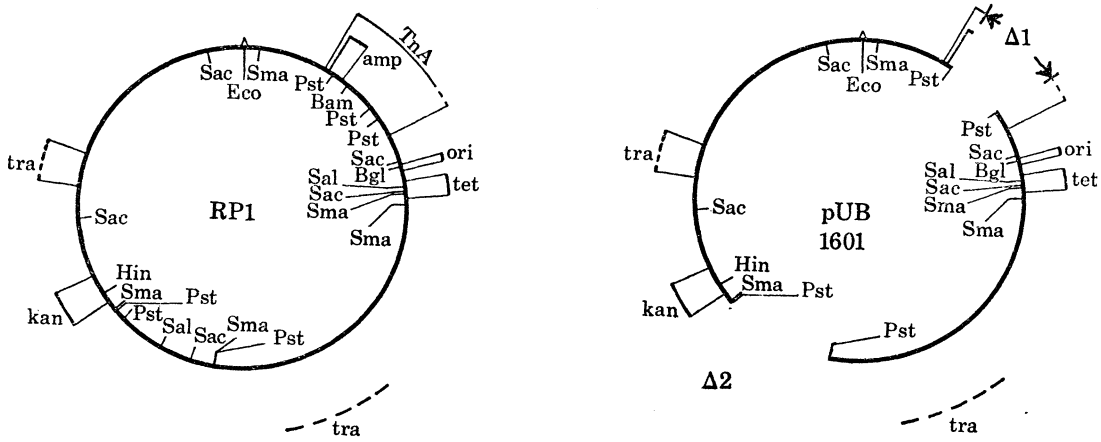

FIGURE 4. The derivation of pUB1601 from RP1 by digestion with Pst restriction endonuclease followed by religation. The initial plasmid which has five Pst sites is reduced to a structure with only two.

TRANSPOSITION IMMUNITY

The transposition of TnA between certain pairs of bacterial plasmids occurs at such a frequency that TnAs would be seen as highly mutagenic agents were the frequency of insertion in the recipient plasmid to be reflected in transposition to the chromosome. But in fact it is not always easy to transpose a transposon from a bacterial plasmid to the chromosome of the bacterial cell in which the donor plasmid is carried (Richmond & Sykes 1972). It follows from these observations that there is likely to be some process which may render a replicon relatively refractory to the uptake of transposons, but there is still much doubt as to what the molecular basis of this process might be.

One clear-cut phenomenon that can be demonstrated with TnA units is the fact that the transposition of a second TnA on to a replicon that already carries a TnA occurs at a much lower frequency, if it occurs at all, than when the recipient, otherwise identical, contains no TnA (Robinson *et al.* 1978*a*). This phenomenon has been termed 'transposition immunity' and certain features of the phenomenon are clear. First, the failure to get two copies of TnA into the same plasmid is not due to recombinational instability of the product (Robinson *et al.* 1978*b*). Two TnAs can be inserted into the same replicon by *in-vitro* manipulation techniques and this conformation is stable provided the two units are in opposite orientation with respect to one another. Secondly, as a rare event, two TnAs can be inserted in the same replicon, but – with Tn801 at least – this is only observed when the two events occur close together in time (Robinson *et al.* 1978*b*). Thus it seems that immunity to transposition is a property which takes some time to establish after the insertion of the first TnA. Thirdly, the immunity effect is only manifested in a *cis* configuration; that is the presence of a second TnA on a second compatible plasmid in the same cell has no effect on the frequency of transposition of a TnA unit from the chromosome to a plasmid which does not yet carry TnA. Fourthly, it is not only derivatives of TnA that show this phenomenon. Transposons with different resistance genes can react in the same way as two TnAs, although the immunity may not be so absolute in such cases.

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Analysis of the regions of TnA that are necessary for transpositional immunity suggests strongly that much of the DNA between the inverted repeats of the transposon is irrelevant from the point of view of the immunity phenomenon. Reduced derivatives of the plasmid RP1 can be obtained by *in-vitro* religation following digestion of RP1 with PstI restriction endonuclease. These reduced plasmids contain all of the DNA of RP1 save for a small amount between the close Pst cuts near coordinate 20 and a substantial section of TnA including part of the β-lactamase gene (figure 4). Such a reduced plasmid still shows its immunity properties (L. Wallace, personal communication).

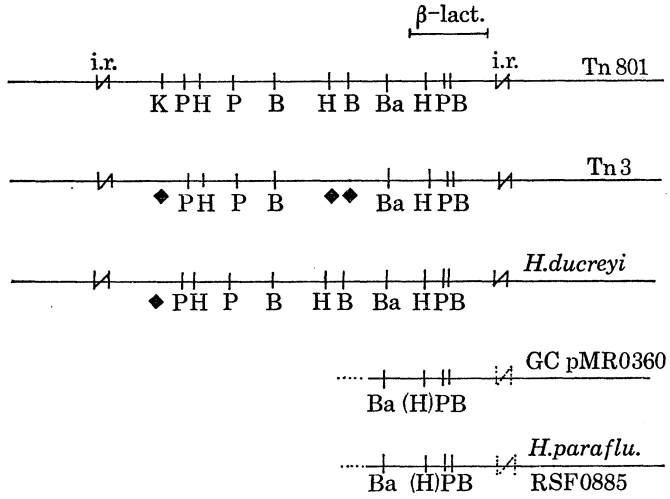

FIGURE 5. A comparison of the restriction endonuclease maps of various TnAs, and derivatives, from different bacterial sources. K, Kpn; P, Pst; H, *Hin*d II; B, Bgl; Ba, Bam.

THE EVOLUTION OF TRANSPOSONS

Just as there is evidence that bacterial isolates sometimes carry mutational variants of the β-lactamase structural gene – see for example the differences between the so-called TEM1 and TEM2 β-lactamases (Ambler, this symposium) – so also one finds evolutionary variation in the structure of TnAs. Figure 5 shows some examples. Tn801 and Tn3 are two well studied TnAs originally isolated from naturally occurring clinical strains of *E. coli*. In both cases the TnA is very similar in size and in the nature of the β-lactamase it specifies, but the restriction endonuclease digestion patterns of the two transposons is not identical.

Examination of plasmids that mediate β-lactamase production in isolates of *N. gonorrhoeae* and in certain strains of *Haemophilus influenzae* and *H. parainfluenzae* show that only 30–40 % of the TnA sequence is present (Roberts 1977; de Graaff 1976). In contrast a β-lactamase-specifying plasmid of molecular mass 6×10^6 found in *Haemophilus ducreyi* carries the whole TnA sequence (J. Brunton, unpublished data) as do the conjugative β-lactamase-specifying plasmids of molecular mass 30×10^6 found in *Haemophilus influenzae* (de Graaff 1976).

We do not know whether the deletion in TnA occurred as a result of nuclease action (presumably at the time of transfer to the new host) or whether it was due to recombination at the time of transfer to the new 'carrier' replicon.

Detailed sequence studies on transposons are now beginning to reveal similarities between different transposons that are not apparent from a study of the phenotypic effects that they mediate. For example, comparison of the nucleotide sequences of the inverted repeats of one

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of the ampicillin resistance transposons (Tn3) (R. P. Novick, personal communication) and one of the transposons conferring resistance to mercuric ions (Tn501) shows that they have much in common (figure 6). Furthermore, the differences between the sequences show one very interesting feature. Certain regions of the inverted repeats of the two transposons seem to be conserved, whereas other sections are widely different. The significance of these similarities and differences is impossible to judge at present. It does seem inescapable, however, that the inverted repeats of these two transposons must be evolutionarily related.

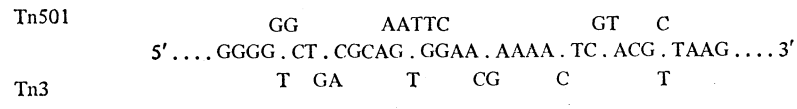

FIGURE 6. A comparison of the nucleotide sequences of one inverted repeat from Tn3 and the analogous structure of Tn501.

CONCLUSION

When one comes to consider the genetic organization that underlies the expression of β-lactamase production in bacteria, one is struck by the prevalence of systems with a powerful evolutionary potential. The location of genes on plasmids allows their transfer between bacterial cells, and the organization of the *bla* region as part of a transposon facilitates the movement of the gene complex from one plasmid to another. Were this undoubted potential for evolutionary change to be allowed to operate in an uncontrolled manner, it seems that it must be very disruptive to the overall genetic strategy and organization of the microorganisms concerned. In many microbiological systems one is struck with the balance that occurs between positive and negative effects: mutation is balanced with repair, and restriction with modification, for example. In the field that we have been discussing, it seems that we may now identify further sets of countervailing processes: transposition is set against transposition immunity, and conjugational transfer against immunity to plasmid transfer (Richmond 1979). It is perhaps only by a careful balancing of positive and negative effects in biological systems that changes can be made without so disrupting the living system that the changes are disadvantageous. The evolution of bacterial populations resistant to penicillin and cephalosporins is no exception, and a careful analysis of the basis of change in this system gives us a first-hand view of microbial evolution in action.

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