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Genetic Interactions between Mixed Microbial Populations [and Discussion]

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Genetic interactions between mixed microbial populations

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Of the various mechanisms by which DNA has been shown to be transferred between bacteria in the laboratory, the combination of promiscuous plasmids and transposable DNA seems the most likely means by which this occurs in Nature. Not only can this lead to the direct transmission of the associated genes, but it can also lead to various structural and genetic changes in other DNA, particularly chromosomal, which might play an important role in microbial evolution. The changes in the DNA in a *Pseudomonas* strain that has evolved to degrade a novel substrate are considered in this context.

Genetic transfer

In one way, bacterial geneticists have been more concerned with interactions between individual organisms than have other microbiologists. Gene transfer has been the staple methodology for both mapping genes and for studying the function of genes by complementation (see Sherratt 1981). Any standard genetics text for undergraduates will detail the uses of sex factor F in *Escherichia coli*, of the transducing phage P1 in *E. coli* and *Salmonella*, and of transformation methods in *Bacillus* spp. It has therefore been long established that DNA, and hence genes, can be passed from one bacterium to another in the laboratory. In addition to the classical genetic procedures of conjugation, transduction and transformation, the past decade has revealed a further perspective to the role of gene transfer. In addition to their classical role as sex factors, as exemplified by F, plasmids have now been shown to be determinants of many of the specialized phenotypic characters found in isolates from Nature, and present indications are that this list will be extended further. In addition to the early discovery that resistance to antibacterial drugs in hospital isolates was invariably plasmid-coded, plasmids have now been described with functions as diverse as phytopathogenicity, resistance to heavy metals, resistance to ultraviolet, virulence towards animals, catabolism of compounds varying from sugars to hydrocarbons, and production of surface antigens (see Reanney 1976). In addition, many isolates from natural environments have been found to contain plasmids, and often a multiplicity of different plasmids, without a functional role having been ascribed to them. Plasmids appear to be important genetic components of natural populations.

Plasmid DNA can be transferred between different bacteria by three separate mechanisms (Clark & Warren 1979):

(1) conjugative plasmids carry transfer genes that determine a physical and biochemical apparatus by means of which a copy of the plasmid can be transferred into a suitable recipient;

(2) non-conjugative plasmids can make use of the transfer apparatus of other conjugative plasmids coexisting in the same host (mobilization);

(3) plasmids, usually the smaller ones, can be transferred by transducing phage.

By means of any of these mechanisms it is easy to visualize that the epidemic spread of a

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plasmid might play an important role in the adaptation of mixed populations to an environmental stress against which the plasmid provided some protection. The extent of this spread of a particular plasmid is severely limited. Many plasmids have a limited host range and even within hosts to which they are normally accessible the presence of other plasmids can limit transfer. The reason for the barriers have not always been distinguished, but can be either at the outside surface of the cell or once the plasmid has entered due to its failure to replicate or its destruction by nucleases; in either case the net effect is the same and transfer cannot be detected. Certain classes of plasmid, however, appear able to overcome both species and generic barriers. In particular the plasmids classified as belonging to the IncPI group appear to be able to transfer between individuals of virtually any two genera of Gram-negative bacteria. These so-called promiscuous plasmids are potential vectors for carrying their own DNA throughout very mixed populations, and the evidence is that they do so, since they have been isolated from a wide variety of different genera. One of the original isolates of one of these plasmids (RP1) was originally found as a result of its intergeneric transfer in a burns unit (Sykes & Richmond 1970).

In addition to carrying their own DNA across intergeneric boundaries, certain promiscuous plasmids, in particular R68.45, can act as sex factors in mobilizing chromosomal DNA in a wide range of Gram-negative organisms (Holloway 1979), and can also form stable R′ plasmids (analogous to F′ plasmids of *E. coli*) in which a region of chromosome has been inserted. These R′ plasmids have been successfully used to transfer regions of chromosome across species and generic boundaries (Hedges *et al.* 1977; Holloway 1978): because of the lack of homology of the insert with the recipient chromosome, it is retained as a stable part of the plasmid in the recipient cell.

Perhaps the most revolutionary discovery of modern molecular biology is the existence of DNA sequences that can move (transpose) from one location to another (Kleckner 1977; Starlinger 1980). Where these DNA sequences carry a gene or genes that confer a selectable phenotype, they are referred to as transposons; in the absence of any such marker they are referred to as insertion sequences (IS). Transposition appears to require little or no specificity in the target DNA sequence, and is unlike the higher requirement for a single specific target sequence (*att*B) required for integration of bacteriophage into the *E. coli* chromosome, or the requirement for sequence homology before transformed or transduced DNA can integrate. In addition, transposition does not use the recombination system necessary for homologous recombination: the apparatus required for this illegitimate recombination appears to be coded for by transposable elements themselves (Arthur & Sherratt 1979). In addition to their ability to become relocated into plasmid, virus or chromosome, transposable elements can have a major effect upon the DNA into which they have been inserted. This can involve (i) inactivation of a gene or operon into which they are inserted (ii) activation of gene(s), since some elements carry internal promoters, and (iii) production of structural rearrangements including deletions, inversions, duplications and fusions (Kleckner 1977).

By extrapolating from laboratory experiments it is possible to construct a network of bacteria through which gene transfer could occur in Nature (Reanney 1976, 1978; Kelly *et al.* 1981). Bacteriophage and narrow host-range plasmids could be responsible for transfer of chromosomal genes and plasmid genes respectively between taxonomically related species. Promiscuous plasmids could act as wide-ranging vectors spanning a much larger spectrum of organisms. Transposable elements could move through the entire network by a series of steps, using other

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genomes as temporary hosts. Furthermore, they are capable of leaving in their wake a variety of new DNA arrangements that might well be important determinants of genetic variability upon which evolutionary selection could act.

Since all the steps of such a network can be demonstrated in the laboratory, one may speculate whether such gene exchange actually occurs in natural populations. This area has been reviewed by Kelly *et al.* (1981).

It seems unlikely that transformation is of very great environmental significance, except perhaps in a limited range of genera such as *Bacillus*, *Haemophilus* or *Pneumococcus*. Apart from the likely short half life of extracellular transforming DNA because of physical, chemical and biological breakdown, the range of organisms that can undergo transformation is severely limited. For example in a survey of a large number of *Acinetobacter* isolates, only one was found that could undergo transformation with linear DNA (Juni 1978). *E. coli* strains can be transformed with plasmid DNA in the laboratory, but the conditions required are such as to be unlikely to be found in Nature.

The generally accepted impression of transduction is that phages have a very limited host range, even though some of the generalized transducing phages used in laboratory genetics such as P1 do transduce in a range of species. By isolating phages and strains of *Bacillus* fresh from soil, Reanney (1976) has shown that most of the phages have a host range that crosses species lines, and some are quite non-specific, lysing strains from six taxonomically distinct species. The survival of transduced DNA in a recipient cell requires that it is not destroyed by restriction endonucleases and that it can undergo homologous recombination with its chromosome of *Bacillus*. However, since there is evidence that transformation does occur between the species used in Reanney's experiments (1976), then transduction via these non-specific phages is also a distinct possibility. However, the requirement for homology is always likely to be the factor that limits generalized transductional exchange to within taxonomically close organisms. Even this might well be sufficient to cause the blurring of species boundaries that we and workers in other laboratories find when selective enrichment from soil often results in strains of indeterminate taxonomy.

The most likely vectors for any widespread dissemination of genes are the combination of plasmids and transposons: this is not only because of the wide host range of the promiscuous plasmids but also because of the autonomy of plasmids and the illegitimacy of recombination of transposons; DNA exchange by this route is thus freed from the limitations of homologous recombination. The evidence that gene transfer via these vectors is significant rests on a number of pieces of evidence.

1. The widespread occurrence of particular DNA sequences, for example the 5 kilobase penicillin resistance transposon Tn1: this has been found as part of a wide variety of plasmid types isolated from many different genera from different geographical locations (Heffron *et al.* 1975).

2. From the very early observations on drug-resistant bacteria it became apparent that new resistances were readily acquired under antibiotic selection. Subsequently it was shown that in many cases the multiple resistance genes were located on single plasmids and, with the development of structure analysis by restriction digestion and heteroduplexing, it became apparent that these plasmids were modular in construction with IS elements and transposons as components (Cohen 1976).

3. If the assumption is made that all plasmids within any one compatibility group share a

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common ancestry, at least as far as their replication machinery is concerned, then the occurrence of different phenotypic genes carried on plasmids of the same group (for example IncP9 resistance and catabolic plasmids (Bayley *et al.* 1979)) further indicates the acquisition of the different functional genes by the plasmids after their evolution as replicons.

All the above evidence is, however, circumstantial and relies on making assumptions about the evolution of isolated plasmids. Taken in combination with laboratory studies that demonstrate transposition of genes, recombination between plasmids and structural reorganizations of DNA as a result of the action of transposable elements, this must be fairly strong evidence that gene transfer occurs in Nature and is significant in producing variability in bacterial genomes (Reanney 1976).

What does appear to be lacking is good experimental evidence that plasmids transfer in the environment. In an extensive review, Kelly *et al.* (1981) have assembled the available evidence. In certain environments where the concentration of cells is high, plasmid transfer has been demonstrated. For example the transfer of R plasmids (Smith 1970) and plasmids determining enterotoxin production (Gyles *et al.* 1978) have been shown to transfer in the gut, R plasmids transfer in infected burns (Roe *et al.* 1971) and the Ti plasmids of *Agrobacterium* transfer to non-infective strains in infected crown gall tissue (Kerr 1969). However, because of the high concentration of cells in these particular environments, these demonstrations are more like laboratory conjugation experiments. There is far less direct experimental evidence about the occurrence of transfer in environments that are more heterogeneous and have lower bacterial contents, such as soil, sediment or natural waters (Kelly *et al.* 1981).

The evolution of plasmids

It has recently been suggested (Doolittle & Sapienza 1980; Orgel & Crick 1980) that DNA sequences that have the capacity to mediate their own replication need no further explanation for their existence and survival, i.e. they are selfish genes. In prokaryotes IS sequences and transposons would fall into this category, as also would plasmids, which have an additional survival advantage in being able to maintain an autonomous existence unlinked to any other genome. This suggestion removes the necessity of arguing that elements that are apparently functionless (as far as the host cell phenotype is concerned) must confer some selective advantage on the host, such as making them more adaptable by promoting genetic variability. Viewed in this way, plasmids are symbionts of bacteria, at the opposite end of a spectrum from the parasitic phages. However, it seems fairly obvious that the acquisition by a plasmid of functional genes that can confer on its bacterial host some selective advantage, such as drug resistance or the ability to degrade a particular organic carbon source, will give the plasmid DNA itself an additional survival potential. The role of the plasmid in conferring this adaptability to the microbial population should be seen only as a secondary consequence of its self-replication strategy.

What is not clear is why certain phenotypic characters seem almost always to be plasmid-coded when strains are isolated from Nature. Why is the drug resistance of hospital isolates almost ubiquitously due to plasmids, why are the genes responsible for degrading toluene and the xylenes in *Pseudomonas* strains found on plasmids when other peripheral catabolic pathways such as phenol degradation are chromosomally determined, or why is the crown gall-producing phenotype of *Agrobacterium tumefaciens* only plasmid-determined? These examples are even more

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puzzling when many of these genes can be shown in the laboratory to transpose on to the chromosome. They might therefore be expected to do so under natural conditions, especially if the selective pressure were sustained over a long period, when it would be to the advantage of the bacterium to have the genes linked to its chromosome. Is it perhaps that there are far more bacterial phenotypes that are plasmid coded than we are yet aware of, or do those particular characters determined by plasmids possess some feature that ensures their autonomy?

The TOL plasmid pWWO in halobenzoate-degrading *Pseudomonas*

The behaviour of the TOL plasmid in the chlorobenzoate-degrading *Pseudomonas* sp. B13 is a good example of how plasmids can participate in and initiate changes of DNA organization under conditions of selection as might be exerted on a saprophytic soil population in Nature. Strain B13 was isolated by selective enrichment on 3-chlorobenzoate as sole carbon source

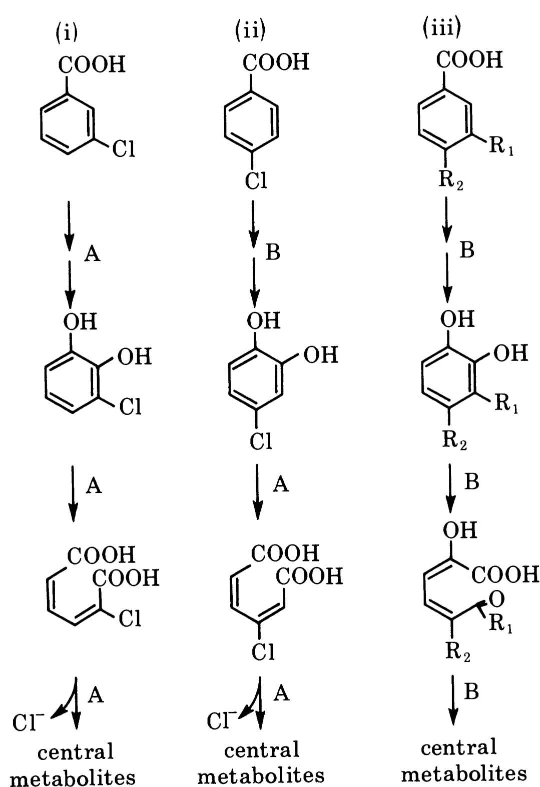

Figure 1. Pathways for the metabolism of chlorobenzoates and methylbenzoates (toluates) in *Pseudomonas* strains: (i) 3-chlorobenzoate metabolism in *Pseudomonas* sp. B13 (WR1); (ii) 4-chlorobenzoate metabolism in WR 216; (iii) *m*-toluate ($R_1 = CH_3$, $R_2 = H$) and *p*-toluate ($R_1 = H$, $R_2 = CH_3$) metabolism in *P. putida* mt-2 (PaW 1) determined by the TOL plasmid pWWO. A, enzymes determined by WR1, B, enzymes determined by TOL plasmid. Adapted from Reineke *et al.* (1982) and Jeenes *et al.* (1982).

(Dorn *et al.* 1974). Its ability to utilize other chlorinated benzoates is limited solely by the high substrate specificity of one of the catabolic enzymes, benzoate dioxygenase (Reineke & Knackmuss 1978). Axenic cultures of B13 cannot be adapted to grow on other analogues (Reineke & Knackmuss 1980). Hartmann *et al.* (1979), however, produced a derivative strain WR912, capable of utilizing both 4-chloro- and 3,5-dichlorobenzoate. Their strategy was to adapt a mixed culture of soil organisms successively to 3-chloro-, 4-chloro- and 3,5-dichlorobenzoate

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in a chemostat. This culture was initially seeded with both *Pseudomonas* B13 and with *Pseudomonas putida* mt-2, which carries an 115 kilobase conjugative catabolic plasmid pWWO (TOL), which encodes the complete utilization of toluene, *m*- and *p*-xylenes, one step of which uses a non-specific benzoate (or toluate) dioxygenase. The dominant strain at the end of adaptation, WR912, expressed the chlorobenzoate pathway of B13 but contained a benzoate dioxygenase with a specificity similar to that of pWWO. Although no genetic characterization or physical investigation of the pathway genes in WR912 was undertaken, it was assumed (Hartmann *et al.* 1979) that gene transfer and possible recombinational events had occurred between the two seeded strains and possibly others in the mixed culture to give the hybrid pathway of WR912.

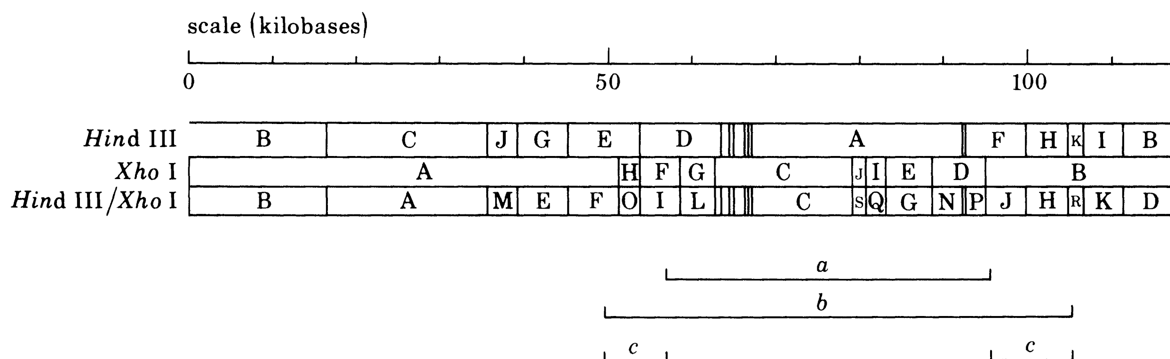

Figure 2. The TOL plasmid pWWO. The plasmid is presented in a linear form from the *Xho*I cutting site between fragments XA and XB. The cutting sites for *Hin*d III and *Xho*I, acting both singly and together, are shown; *a* delineates the 39 kilobase section containing the genes for the catabolic enzymes, which is deleted from the plasmid in WR 211; *b* delineates the 56 kilobase section that integrates into the chromosome in WR 211, and the arrow marks the position where the 3 kilobase DNA insert of unknown origin occurs; *c* represents the 17 kilobase of residual DNA resulting from deletion of the 39 kilobase from the 56 kilobase region. Adapted from Jeenes *et al.* (1982).

This experiment is similar to the adaptation of natural mixed populations exposed to a xenobiotic such as one of the chlorinated phenoxyacetate herbicides, MCPA (2-methyl 4-chlorophenoxyacetate) or 2,4-D(2,4-dichlorophenoxyacetate), where there is often a considerable delay of weeks or even months before a strain capable of its total degradation appears, apparently spontaneously (Audus 1960). It has been proposed that a possible explanation is that processes involving gene transfer might be required before the total catabolic function is assembled into one organism (Williams & Worsey 1976). Subsequently one organism capable of chlorophenoxyacetate degradation has been described that contains a catabolic plasmid, pJP1 (Fisher *et al.* 1978), apparently encoding only part of the pathway (J. Shapiro, personal communication).

The conclusions about the origin of WR912 were given added strength by the construction of two derivatives of B13, WR211 and WR216, the latter capable of 4-chlorobenzoate degradation, by conventional conjugation techniques between only *P. putida* mt-2 as donor and B13 as recipient (Reineke & Knackmuss 1979, 1980). The direct transconjugant of the mating, WR211, was selected for its ability to grow on *m*-toluate (a metabolite of *m*-xylene on the TOL pathway) but had not acquired the ability to grow on 4-chlorobenzoate; WR216 was a spontaneous derivative of WR211 selected for its ability to grow on 4-chlorobenzoate. In both of these strains the plasmid DNA of pWWO had been structurally modified. Three events

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appear to have taken place in the formation of WR211 (Jeenes & Williams 1982; Jeenes *et al.* 1982):

(1) a region of 56 kilobases of pWWO, including all the catabolic genes, had become transposed elsewhere in the genome of WR211, almost certainly in its chromosome;

(2) an insertion sequence of about 3 kilobases had become inserted into this 56 kilobase transposon-like region inactivating the top half of the pathway;

(3) a region of 39 kilobases, corresponding to the central section of the 56 kilobase region, was specifically excised from pWWO to leave a cryptic 78 kilobase plasmid, pWWO-1211, as the sole plasmid DNA in the cell.

The 56 kilobase region, containing the 3 kilobase insert, can be rescued from its presumed chromosomal site by expelling the resident plasmid, pWWO-1211, by mating in an incompatible R plasmid, pMG18 or R2, and then using the resultant R^+ transconjugant as a donor in a mating, selecting for transconjugants with the ability to grow on *m*-toluate (Jeenes & Williams 1982). In all cases a recombinant plasmid is found in these strains in which the 56 kilobase transposon-like region of pWWO was integrated into the R plasmid. Furthermore, upon selection for loss of the catabolic function from the strains carrying these recombinant plasmids, the 39 kilobase easily excisable region was lost from the 56 kilobase insert to leave 17 kilobases of DNA, originally from pWWO, but now apparently functionless, in the R plasmid.

We have strong evidence that the same 17 kilobase of DNA region is present in the chromosome of a strain PaW 340, which is a plasmid-free derivative of *P. putida* mt-2: this region appears to be able to transpose into resident plasmids to produce mutations (P. A. Cane & P. A. Williams, unpublished results). This suggests that the 56 kilobase region has been part of the chromosome of PaW 340 at some time in its past history, and that the 39 kilobase region has been excised from it, leaving the residual 17 kilobase region apparently still transposable.

In order that the TOL plasmid can confer the ability of growth on 4-chlorobenzoate to *Pseudomonas* B13, there are two biochemical requirements: one is that the non-specific TOL benzoate dioxygenase must be expressed, but in addition the bottom of the TOL *meta* pathway must not be expressed, since it can channel the carbon from 4-chlorobenzoate into intermediates that cannot enter the central metabolism (Reineke *et al.* 1982). Hence WR 211 fulfills the first condition but not the second and as a result does not grow on 4-chlorobenzoate. The selection of derivatives of WR 211, such as WR 216, that grow on 4-chlorobenzoate, requires the inactivation of the TOL pathway and in WR 216 this is achieved by a complex structural change in the plasmid DNA. The 78 kilobase plasmid in the parental WR 211 regains some of the 56 kilobase chromosomal insertion, and deletes some additional DNA and gains two additional insertion sequences of about 3 kilobases each. Why the particular nutritional selection should result in such a complex change has not been explained except in one feature: one of the 3 kilobase inserts in the plasmid in WR 216 is in *xyl*E, the gene for catechol 2,3-oxygenase, thus producing the required metabolic block in the *meta* pathway (Jeenes *et al.* 1982).

All the DNA structural changes described occur in the strains upon selection for the ability to grow on particular carbon sources. We do not understand why the selections produce the particular changes, nor what are the structural features of the plasmid that facilitate those changes. It is possible that they represent the kind of structural modifications that occur in enrichments of mixed cultures such as in the formation of WR 912 (Hartman *et al.* 1979) and therefore also in natural mixed populations subject to novel selective stresses.

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Discussion

J. M. Lynch (*Agricultural Research Council Letcombe Laboratory, Wantage, U.K.*). The study of soil microorganisms has often been hampered because experiments to study their activities have been difficult to replicate; the experiments by Dr Williams demonstrating plasmid transfer now provide a possible explanation for the variations. However, the use of percolation of columns of soil, as is often used to isolate strains capable of degrading xenobiotics such as MCPA, has limited relevance to the natural environment because the water and oxygen relations are quite different.

P. A. Williams. Except perhaps to soils in North Wales in the winter! The point made is quite valid, not only regarding water and oxygen but also the concentration of substrate, which is normally much lower in Nature and of intermittent occurrence. The same applies to the other methods usually used to study degradation in the laboratory, namely enrichment in batch culture or in chemostats, as described by Slater & Bull (this symposium). I am not sure that these objections necessarily invalidate the conclusions drawn from such experiments, but I would agree with its implication that the time is ripe for a serious experimental assessment of gene transfer and its role in the evolution of metabolic capabilities under conditions prevalent in the natural environment.

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