Molecular Structure and Relationships of Pseudomonas Plasmids

by

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I would like to take this opportunity to thank all the people who have contributed to my work and have helped make my stay in Edinburgh a happy one. In particular, my thanks go to Dr P. Broda, my supervisor, and Dr R. Hayward, my advisor, for their constant guidance, understanding and enthusiasm throughout the three years. I am grateful to many members of the Broda team, both past and present, for their valuable help, and especially Clive Duggleby with whom some of this work was done in collaboration.

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Finally, I would like to thank my parents and Chris for their moral support, encouragement and tolerance throughout my postgraduate studies.
Plasmid DNA was isolated from thirteen *Pseudomonas* strains judged on genetic criteria to carry plasmids coding for the degradation of toluene and m- and p-xylenes (TOL plasmids). Most strains harboured a single plasmid species, but two strains carried two size classes, and a third strain (MT 14) carried plasmids that fell into a range of size classes. Five of the thirteen strains harboured plasmids that were indistinguishable on the basis of size and electrophoresis pattern of endonuclease-generated fragments. Two other strains carried plasmids that were indistinguishable from each other but different from those of the first group. The remaining strains carried unique plasmids. Some plasmids could be transformed into a *P. putida* strain to give Tol' progeny.

A set of independently-arising Tol' derivatives of strain *P. putida* (arvilla) mt-2 were studied. Some had lost the unique plasmid carried by the parent strain. In others this plasmid had suffered a deletion of a specific region of about 27 Md.

Plasmid DNA from m-toluate grown cells of strain MT 14 ranged in size from 25 to 202 Md. However, after serial subculture of such cells on L-broth, plasmid DNA from a Tol' clone fell into a single size class of 48 Md. After serial subculture of this clone on m-toluate the original distribution was again observed. Endonuclease digestion patterns suggest that the larger plasmid species may be multimers of the 48 Md species.
The relationships between plasmids of the same, and of different, incompatibility groups were examined by hybridisation of radioactively labelled plasmid DNA probes to nitrocellulose bound fragmentation patterns of endonuclease digests of plasmid DNA. Plasmids of the same group showed a considerable amount of homology even when the group contained both drug resistance plasmids and degradative plasmids. However, plasmids of different groups had few common sequences.
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CHAPTER 1

Introduction

1.1 The genus Pseudomonas

Pseudomonads have interested researchers in numerous fields including medicine, agriculture, industry and mining, principally because they are robust organisms that can live in a number of different habitats, and are therefore widely distributed in nature. Stanier et al (1966) have proposed the following definition for aerobic pseudomonads:

"Unicellular rods, with the long axis straight or curved, but not helical. Motile by means of one or more polar flagella. Gram-negative. Do not form spores, stalks or sheaths. The energy-yielding metabolism is respiratory, never fermentative or photosynthetic. All use molecular oxygen as a terminal oxidant". Guanine and cytosine constitute between 58 and 69% of the DNA (Mandel, 1966).

A large number of rather diverse bacteria constitute the aerobic pseudomonads, and many belong to the genus Pseudomonas. The taxonomy of the genus is controversial but the most extensively studied subgroup, the fluorescent pseudomonads, is well defined (Stanier et al, 1966). These bacteria can produce water-soluble, yellow-green, fluorescent pigments of unknown chemical nature and this property has been important in their characterisation. Flügge (1886) was the first to describe fluorescent pseudomonads, and identified two biotypes which can be distinguished by the characteristic of gelatin liquefaction. The liquefying biotype was given the name Pseudomonas
fluorescens, and the non-liquefying biotype, Pseudomonas putida. Simple fluorescent pseudomonads are common inhabitants of soil and water; they are nutritionally versatile and can use a very wide range of organic compounds as sole source of carbon and energy, as was first reported by den Dooren de Jong (1926).

Other species can produce a type-specific phenazine pigment in addition to the group-specific fluorescent pigment. Pseudomonas aeruginosa, which can synthesise both types of pigment, can be distinguished from other fluorescent pseudomonads because it can grow at higher temperatures, and it is the only member of the group that is pathogenic for mammals (Haynes, 1964). Pseudomonas is difficult to kill with many common disinfectants, and it is resistant to many chemotherapeutic agents, so treatment of P. aeruginosa infections is difficult. P. aeruginosa is not highly pathogenic, and infection usually only occurs when the natural resistance mechanisms of the body are lowered, for instance in burns (Lowbury, 1975). In recent years there has been a marked increase in the frequency of P. aeruginosa infections and concomitantly the spectrum of antibiotic resistance patterns has widened. Both the antibiotic resistances and the nutritional versatility of species of Pseudomonas have provoked considerable interest in the genus (see Clarke and Richmond (1975) for an excellent discussion of many aspects of the genetics and biochemistry of Pseudomonas).
1.2 The catabolic versatility of pseudomonads

The pseudomonads are important ecologically since they represent the major group of micro-organisms that are involved in the catabolic aspects of the carbon cycle in nature. Moulds are responsible for the primary attack on organic waste and matter resulting from death. However, once soluble or monomeric units are produced the faster growing and nutritionally versatile pseudomonads take over.

Stanier et al (1966) have determined the nutritional profiles of 267 strains which represented many of the principal biotypes among aerobic pseudomonads. Of the 16 organic compounds that were screened, most pseudomonads could use more than half as sole source of carbon and energy. The most versatile was a strain of *P. multivorans* which could utilise over 100 compounds. Strains of *P. aeruginosa* and *P. putida*, the species that have been used more frequently in genetic studies, are slightly less versatile. Strains of other species could only utilise a few compounds. The rate of growth on the more unusual compounds is often faster than on glucose. Rovira and Sands (1971) have suggested that the abundance of pseudomonads in soil is directly correlated to the proximity of a carbon source. It is clear that pseudomonads owe their success to their ability to catabolise almost any carbon source, rather than merely to the ability to compete for readily metabolised compounds, or to maintain viability when not actively growing.

In marked contrast to the pseudomonads, the enteric bacteria can utilise only a limited range of compounds as
sole carbon and energy source. Gutnick et al (1969) have shown that a strain of Salmonella typhimurium can utilise only 73 compounds out of 600 tested. The genetic basis of the nutritional versatility of the pseudomonads has been a recurring problem (Gunsalus et al, 1968) since the genome of this organism is no larger than that of Escherichia coli (Pemberton, 1974). Gunsalus et al (1974) have suggested that while commonly occurring compounds are degraded by chromosomally-encoded enzymes in most or all strains of Pseudomonas, nutritional versatility of individual strains may be increased by the possession of plasmids which code for the enzymes that degrade novel carbon sources.

1.3 **Plasmids**

Plasmids are extrachromosomal genetic elements which can replicate autonomously, and although they are not an absolute requirement for growth or viability, they often code for secondary functions that allow the bacteria to inhabit ecological niches where growth would otherwise be impossible. Some are transmissible to other bacteria, and may at times integrate into the bacterial chromosome.

The first reports that bacteria can harbour genes that are plasmid-encoded were made in the 1950s. However, in this early work only the plasmids of E. coli were studied. Before 1969, the only well-characterised plasmid of Pseudomonas was the sex factor FP2 which promotes the transfer of bacterial chromosome in P. aeruginosa (Holloway and Jennings, 1958). In 1969 Lowbury et al isolated highly carbenicillin resistant strains of P. aeruginosa from
patients in a Birmingham burns unit, and it was subsequently shown that these strains carried plasmids which encoded resistance to carbenicillin and other antibiotics (Fullbrook et al, 1970; Sykes and Richmond, 1970). Since that time many different drug resistance plasmids (R plasmids) have been isolated from, and characterised for, Pseudomonas.

*P. aeruginosa* is naturally resistant to many common antibiotics. Therefore because of its pathogenicity, new antibiotics have been developed that are active against this strain. This in turn has led to the appearance of strains of *P. aeruginosa* harbouring R plasmids which encode resistance to such antibiotics. Iyobe et al (1974) have carried out epidemiological studies in Japan which showed that 40% of the resistant strains they examined carried R plasmids, and it has been estimated that between 5 and 10% of all clinical *Pseudomonas* isolates carry R plasmids (Bryan et al, 1973; Kawakami et al, 1972). Characterisation of R plasmids which confer resistance to the new, powerful antibiotics carbenicillin, gentamicin and tobramycin (Knothe et al, 1973; Korfhagen et al, 1975; Roe et al, 1971) has made an understanding of the mechanism of evolution of R plasmids essential if *P. aeruginosa* infections are to be minimised. Most R plasmids confer resistance to a number of antibiotics and a range of resistance patterns are known. Generally, while the chromosomally encoded intrinsic resistance of *Pseudomonas* to antibiotics is a property of the cell membrane (Meadow, 1975), R plasmids usually code for enzymes which inactivate the antibiotics by phosphorylation, adenylation, acetylation or a
combination of these modes. Some R plasmids are promiscuous and transfer not only to other strains of Pseudomonas, but also to other genera including Acinetobacter, Rhizobium, Neisseria and the Enterobacteriaceae (Datta et al, 1971; Olsen and Shipley, 1973).

Besides drug resistance and chromosome mobilisation, plasmids have been isolated from strains of Pseudomonas which encode other functions. These include catabolic functions (which will be discussed later), restriction and modification systems (Jacoby and Sutton, 1977), and heavy metal resistance (Nakahara et al, 1977). Some strains of Pseudomonas also carry cryptic plasmids (Finley and Punch, 1972; Pemberton and Clark, 1973).

Plasmids can greatly increase the genetic capability of a bacterial population. Many plasmids are transmissible but it is difficult to assess the importance of this in nature. It has been suggested that, in response to an environmental change, plasmids may transfer to many cells in a bacterial population. Alternatively, there may be little transfer and the plasmid may simply confer an advantage on the host cell, which then becomes predominant in the population. However, in the long term, genetic recombination and plasmid transfer between strains must occur and novel variants of bacteria may be selected in response to new environmental challenges including new antibiotics and unusual carbon sources such as industrial waste products.
1.4 The ortho and meta pathways for catechol dissimilation

The diversity of carbon sources that can be degraded by species of Pseudomonas has led to extensive biochemical investigation of their oxidative pathways. Most species of Pseudomonas can grow on benzoate but few can grow on more complex aromatic compounds. Strains of Pseudomonas that can utilise naphthalene, salicylate or m-toluate were isolated after selective enrichment with these compounds and submitted to detailed genetic and biochemical analysis. In each case, catechol, an intermediate in the degradation of these compounds, was degraded via the divergent meta (or ketoacid) pathway (Murray et al, 1972). Catechol is cleaved in the meta position and the cleavage product, 2-hydroxymuconic semialdehyde, is converted to pyruvate and acetaldehyde (Fig. 1.1). This sets them apart from other strains of Pseudomonas that cannot utilise such compounds, since when such strains are grown on benzoate, the intermediate, catechol is cleaved in the ortho position (Fig. 1.1). An example is P.putida (arvilla) mt-2 which was isolated after selective enrichment with m-toluate (Nozaki et al, 1963). This strain carries the genes for both pathways; m-toluate is degraded via the meta pathway but when the strain is grown on catechol (in the absence of m-toluate) it is cleaved in the ortho position (Murray et al, 1972). The ortho pathway is not normally expressed in cells carrying the genes for the meta pathway because of differences in regulation (Feist and Hegeman, 1969). The meta pathway is induced by the primary substrates (for
Fig. 1.1 The ortho and meta pathways for the degradation of catechol by *P. putida* mt-2. Benzoate is metabolised by the meta pathway, catechol by the ortho pathway; m-toluate and p-toluate are metabolised by the meta pathway and the corresponding methyl derivatives. The intermediates of the pathways are: 1, benzoate; 2, catechol; 3, 2-hydroxy-muconic semialdehyde; 4, 4-oxalocrotonate (enol); 5, 4-oxalocrotonate (keto); 6, 2-oxopent-4-enoate; 7, 4-hydroxy-2-oxovalerate; 8, cis, cis-muconate; 9, muconolactone; 10, β-ketoadipate. (Reproduced from Williams and Murray, 1974.)
Fig. 1.1
example, the toluates), whereas the ortho pathway is induced by \textit{cis}, \textit{cis}-muconate, the product of its first enzyme (Ornston, 1966). The benzoate oxidase system that is associated with the ortho pathway is very specific in its catalysis and will not oxidise methylbenzoates; therefore only cells containing the less specific benzoate oxidase of the meta pathway can utilise m- and p-toluate (Fig. 1.2). However, o-toluate cannot be dissimilated via either pathway. The dissimilation of m-toluate proceeds via the hydrolytic branch of the meta pathway whereas benzoate and p-toluate are degraded via the 4-oxalocrotonate branch (Fig. 1.1).

When strain \textit{P. putida} mt-2 is grown on benzoate mutants spontaneously arise that have lost simultaneously and irreversibly the ability to grow on the toluates and all the enzymes of the meta pathway. This genetic loss also takes place, although to a lesser extent, when cells are grown on nonaromatic compounds in the presence of mitomycin C. The cells retain the ability to grow on benzoate using the ortho pathway (Williams and Murray, 1974). These results suggest that, like several other pathways (Section 1.5), the enzymes of this pathway may be plasmid-specified.

1.5 Degradative plasmids

Genetic evidence has suggested that the ability of certain strains of \textit{Pseudomonas} to catabolise specific substrates is due to the possession of plasmids coding for degradative pathways. The plasmids are naturally occurring and some are transmissible while others are not. Until recently, it was thought that the transmissible
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Fig. 1.2 Metabolism of toluene and the xylenes by P. putida mt-2. The enzymes involved are: 1, toluene oxidase; 2, benzyl alcohol dehydrogenase; 3, benzaldehyde dehydrogenase; 4, benzoate oxidase; 5, catechol 2, 3-oxygenase. (Reproduced from Williams and Worsey, 1976.)
plasmids could only transfer to other Pseudomonas species. However, Benson and Shapiro (1978) have now shown that the TOL plasmid can be transferred to E.coli but is unable to express the degradative function in this species. A carbenicillin resistance marker was inserted into the TOL plasmid. The resistance, replicative and conjugative functions of the recombinant plasmid were expressed in both Pseudomonas species and E.coli but the ability to degrade m-toluate was only expressed in Pseudomonas species. A number of plasmids which specify enzymes involved in the biodegradation of complex organic compounds have been described. The properties of these are summarised in Table 1.1.

The first report of a degradative plasmid was made in 1972 when Chakrabarty showed that the enzymes involved in salicylate degradation were plasmid-specified in a strain of P.putida. All the genes of the complete salicylate degradative pathway, which includes a meta cleavage pathway, are plasmid-specified. The evidence presented includes: the spontaneous loss of the ability to degrade salicylate is enhanced by mitomycin C treatment, all the enzymes of the pathway are lost simultaneously and there is no reversion to wild type. SAL can be transmitted by conjugation into other strains of Pseudomonas.

Some strains of Pseudomonas can metabolise naphthalene, which is another aromatic compound, and it is degraded via the meta pathway. Salicylate is an intermediate between naphthalene and catechol so such strains can also utilise this compound (Davies and Evans, 1964). The ability to utilise naphthalene and salicylate has been shown to be
Table 1.1 Properties of degradative plasmids

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<tr>
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<th>Degradative pathway</th>
<th>Molecular weight (Md)(^{(a)})</th>
<th>Transfer proficiency</th>
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<tr>
<td>SAL</td>
<td>salicylate</td>
<td>40, 55 ((1))</td>
<td>+</td>
</tr>
<tr>
<td>NAH</td>
<td>naphthalene</td>
<td>42 ((2))</td>
<td>+</td>
</tr>
<tr>
<td>OCT</td>
<td>octane</td>
<td>29 ((3))</td>
<td>-</td>
</tr>
<tr>
<td>CAM</td>
<td>camphor</td>
<td>150 ((1))</td>
<td>+</td>
</tr>
<tr>
<td>TOL</td>
<td>(\beta)- and (p)-xylene, toluene</td>
<td>See Chapter 4</td>
<td>+</td>
</tr>
<tr>
<td>XYL</td>
<td>(m)- and (p)-xylene, toluene</td>
<td>10 ((1))</td>
<td>-</td>
</tr>
</tbody>
</table>

Md = Million daltons

(a) References are given in parentheses as follows:
(1) Chakrabarty (1976); (2) Johnston and Gunsalus (1977); (3) Palchaudhuri (1977).
plasmid-specified in one strain of \textit{P. putida} biotype A (Dunn and Gunsalus, 1973). Since the NAH plasmid specifies enzymes which are analogous to all the enzymes of the pathway encoded by the SAL plasmid, it was possible that these two plasmids may be closely related to each other. Heinaru \textit{et al} (1978) have recently demonstrated that, indeed, they are related. The NAH plasmid is transmissible and curing of this plasmid too is enhanced by mitomycin C treatment. However, in a strain of \textit{P. putida} biotype B, which does not appear to be closely related to the biotype A strain which has already been discussed (Palleroni \textit{et al}, 1972), the genes involved in the catabolism of naphthalene appear to have a chromosomal location (Dunn and Gunsalus, 1973). Therefore, a particular degradative function need not be exclusively located on either plasmids or the chromosome in all strains of \textit{Pseudomonas}.

In \textit{P. putida} octane is degraded via octanol, octanaldehyde and octanoic acid (Baptist \textit{et al}, 1963) in cells harbouring the OCT plasmid. The OCT plasmid does not carry the genes for the complete dissimilation of octane but codes for the five enzymes involved in its conversion to octanoic acid, which is further degraded by the chromosomally-encoded 2-oxidation pathway that results in the formation of acetyl- and propionyl-coenzyme A (Neider and Shapiro, 1975). Besides octane, cells which harbour the OCT plasmid are enabled to utilise other \textit{n}-alkanes including hexane and decane. The OCT plasmid was originally isolated in a strain of \textit{P. oleovorans} (Chakrabarty, 1973), and genetic evidence suggests that it is a plasmid aggregate.
Transfer of the aggregate to *P. putida* results in its dissociation into three separate replicons: OCT, MER and factor K (Chakrabarty and Friello, 1974; Chakrabarty, 1974). The OCT replicon is a nontransmissible plasmid which is rendered transmissible by the presence of the transfer plasmid, factor K. Besides mobilising OCT, factor K can also mobilise other nontransmissible plasmids and the chromosome (Chakrabarty, 1974). The presence of MER, which is also a self-transmissible plasmid, confers on the host cells resistance to toxic concentrations of mercuric ions.

Rheinwald et al (1973) have suggested that some of the enzymes involved in the dissimilation of D- and L-camphor are plasmid-specified in a strain of *P. putida*. The evidence presented includes the frequencies of curing in the presence and absence of mitomycin C, and the self-transmissible nature of the *cam* genes. As in the case of the OCT plasmid not all the enzymes of the degradative pathway are plasmid-specified, but only the genes involved in the conversion of D- or L-camphor to isobutyrate. Iso-butyrurate is then degraded via chromosomally-encoded pathways. Several intermediates in this degradative pathway remain to be isolated and characterised but six plasmid-specified enzymes have been identified so far (Gunsalus, 1974). CAM and OCT cannot exist stably in the same cell; that is, they are incompatible (see Section 1.7), which suggests that they may be related to each other (Chakrabarty, 1974). However, OCT can be transferred by K to CAM* strains to give CAM* OCT* clones, although the
reverse cross does not give this phenotype. These clones are unstable, and one of the plasmids is lost after several generations (Chakrabarty, 1973). They are unable to transfer either plasmid, but the surviving plasmid becomes transfer proficient as soon as the other is lost. The plasmids therefore interfere with the expression of each others transfer functions. By selecting for the transfer of OCT from such a strain to *P. aeruginosa*, Chakrabarty (1973) has isolated CAM-OCT fusions. The host range, transfer frequencies and mitomycin C sensitivity indicate that the replication of the chimeras is probably controlled by CAM. The fused plasmids are stable, and on curing both phenotypes are lost simultaneously.

One other pathway which is specified by genes which have a plasmid location has been studied extensively. *P. putida* mt-2 degrades benzoate, m- and p-toluuate by the divergent meta pathway (Section 1.4) (Murray et al., 1972). Genetic evidence suggests that the pathway is plasmid encoded (Wong and Dunn, 1974; Williams and Murray, 1974). The ability to utilise these compounds is lost at a high rate, although the rate is not significantly enhanced by mitomycin C treatment. The TOL plasmid is transmissible, and can be transferred into cured strains to give Tol+ clones. It was reported later by Williams and Worsey (1975) that the TOL plasmid confers on the host cells not only the ability to degrade m- and p-toluuate but also toluene, m- and p-xylene (Fig. 1.2). The toluates are intermediates in the degradation of the xylenes. Friello et al. (1976) have described in a strain of *Pseudomonas* another plasmid,
termed XYL, which also specifies the enzymes involved in the degradation of this group of compounds. XYL and TOL appear to specify very similar catabolic enzymes, and the regulation of the two pathways appears to be the same (Chakrabarty, 1976). However, XYL is nontransmissible.

Studies of several other peripheral metabolic pathways have suggested a plasmid location for the genes coding for the enzymes of these pathways, and there are probably many more plasmid-specified pathways that have not been identified yet. However, in comparison to the criteria used for plasmids in the Enterobacteriaceae, the genetic evidence presented for establishing the plasmid-coded nature of the degradative pathways has been rather superficial. Recently, several of the plasmids have been isolated and shown to exist as autonomous, extrachromosomal, covalently closed circular DNA (Palchaudhuri and Chakrabarty, 1976; Johnston and Gunsalus, 1977; work described in this thesis). This finding strongly supports the genetic evidence. Most of these plasmid preparations were shown, by electron microscopy, to contain a single size class of circular molecule (Table 1.1), but Chakrabarty (1976) observed two size classes, of 40 Md and 55 Md, in the plasmid preparation from a strain of P.putida that carried the salicylate degradative pathway. The significance of this is unknown but it is possible that only one of these plasmid species is involved in salicylate degradation.

Richmond (1970) has discussed the genome of Pseudomonas in terms of three classes of genes: essential, often useful and occasionally useful. He has suggested that the genes
of the first two classes are located on the chromosome, whereas those of the third class, which includes those of the catabolic pathways, may be carried by a plasmid in one strain, but be located on the chromosome or absent in others. Continual transfer of plasmid and chromosomal loci between strains ensures that there is continual reassortment of nutritional types. Only in the case of naphthalene dissimilation has a plasmid location and a chromosomal location been shown for the genes of the same degradative pathway. Generally, for the other pathways which are claimed to be plasmid-specified, only one or two strains have been examined, and no attempt has been made to determine whether the genes are plasmid-borne in all strains that have the pathway. Williams and Worsey (1976) have isolated independently from soil after enrichment culture, thirteen strains of *Pseudomonas* that can utilise m-toluolate as sole source of carbon and energy. The genetic evidence suggests that the ability to degrade m- and p-toluolate, m- and p-xylene, and toluene (the Tol function) is plasmid-specified in every case. In this thesis the isolation and characterisation of plasmid DNA from these strains is described. The TOL plasmids are compared to determine the relationships between them, and the molecular organisation of two quite different TOL plasmids is examined.

1.6 The classification of plasmids

A number of different criteria have been used to classify plasmids. One of these is the function they
determine, for example, hydrocarbon utilisation for
degradative plasmids, mobilisation of the host chromosome
for sex factors, or antibiotic resistance for R plasmids.

R plasmids which are found in hospital \textit{P. aeruginosa}
isolates can be subdivided on the basis of spectrum of
resistances, mechanism of resistance, and transmissibility
among \textit{Pseudomonas} species as well as to members of the
\textit{Enterobacteriaceae}. Other criteria used in the classifi-
cation of plasmids include DNA homology, plasmid size and
effect on the sensitivity of host cells to certain
bacteriophage and on the production of pyocin. The last
two have important implications in the treatment of
\textit{P. aeruginosa} infections since certain R plasmids change the
pyocin typing or bacteriophage typing of the host, and
these characteristics are used in the identification of
the infecting strain. Plasmids are also classified by
plasmid-plasmid interactions, and in particular by
incompatibility relationships. The division of plasmids
into groups which have similar properties represents an
important step in understanding the relationships between
plasmids and in studying the development of new types.

1.7 Incompatibility

Strains of bacteria that carry a plasmid resist
infection by a related or isogenic plasmid. There are two
separate barriers; surface exclusion and incompatibility
(\textit{Novick}, 1969). Surface exclusion is the interference, by
a resident plasmid, with the entry of genetic material
through conjugation and is associated with the transfer
function of the plasmid. Surface exclusion in *E. coli* can be abolished (Sneath and Lederberg, 1961) but the superinfecting plasmid may then be incompatible with the resident. Compatible plasmids can coexist stably in the same cell, but if the plasmids are incompatible either one or the other is eliminated from the host cell or they recombine to form plasmid hybrids. Plasmids which are incompatible do not necessarily exclude each other.

The mechanism of incompatibility is complex and poorly understood, but in the case of the sex factor *F* of *E. coli* it has been shown to depend on the inhibition of replication of one of the plasmids (Dubnau and Maas, 1968; Falkow *et al*, 1971). The plasmid that is not replicated is diluted out during subsequent cell growth and division (Pritchard *et al*, 1969). Two types of models have been suggested for the mechanism of incompatibility. One model proposes that every autonomous replicon must be attached to a structural component of the cell, a replicational or segregational site, which is required for replication and distribution of replicas. Incompatibility can then be explained in terms of competition between the resident and superinfecting plasmids for the maintenance site (Jacob *et al*, 1963). The other model suggests that plasmid replication is under the control of repressor molecules and that the repressors of incompatible plasmids are either identical or cross-reacting (Pritchard *et al*, 1969).

Recent work has supported the latter hypothesis (Uhlin and Nordström, 1975; Cabello *et al*, 1976; Taylor and Grant, 1977). Uhlin and Nordström (1975) have isolated a
number of mutants of the R plasmid R1 that have an increased ratio of plasmid copies per chromosome. The compatibility relationships of these mutants with R plasmid R100 were studied in E.coli K-12. The mutants fell into two classes; one increasing and one decreasing the incompatibility exerted towards R100. These results do not support the maintenance site model since, according to this model, an increase in copy number would result in more competition for the maintenance site and therefore increased incompatibility. However, applying the repressor model, incompatibility would be increased or decreased depending on whether the mutation was in the repressor target or the repressor gene. These observations suggest that plasmid incompatibility is a quantitative, and not just a qualitative, property. In addition, they indicate that incompatibility is linked not only to the prevention of replication of related plasmids, but also to the regulation of copy number and possibly to other aspects of the control and mechanics of the replication of plasmid DNA.

1.8 Classification of plasmids by incompatibility

Chabbert et al (1972) studied the stability of 12 plasmids in pairs in E.coli. They found that incompatibility between pairs of plasmids is a clear-cut phenomenon and that each plasmid belonged to a single group. Incompatibility is easy to observe and provides a reliable method for classifying plasmids. In E.coli more than twenty incompatibility groups have been defined to date (Datta, 1974).
The degree of incompatibility between members of the same group can differ. For instance, the plasmids of the H incompatibility group of E. coli can be divided into two subgroups. Incompatibility between plasmids of the same subgroup was strong, but weaker interactions were evident between plasmids of different subgroups (Taylor and Grant, 1977). Uhlin and Nordström (1975) have shown that the degree of incompatibility can be changed by mutation (Section 1.7), and the genetic background of the host can also affect incompatibility (Pfister et al, 1976). The genetic basis of incompatibility is therefore complicated and both plasmid-linked and host chromosome-linked genetic determinants are involved.

Initially, since many Pseudomonas plasmids can be transferred to E. coli, their compatibility characteristics were studied in relation to E. coli plasmids. However, some Pseudomonas plasmids cannot be transferred to E. coli; therefore a new classification system based on the interaction of plasmids in Pseudomonas was devised (Shahrabadi et al, 1975; Sagai et al, 1976). Sagai et al (1976) and Jacoby (1977) have shown that the plasmids found in Pseudomonas can be divided into at least ten incompatibility (Inc) groups. Classification using this characteristic agrees well with classification using other criteria, and plasmids of a particular incompatibility group often have other properties in common. For instance, plasmids of the IncP-1 group inhibit the propagation of phage G101, confer susceptibility to the phages PRR1, PRD1, Pf3, PR3 and PR4, and confer tolerance to some bacteriocins (Jacoby and
Shapiro, 1977). IncP-1 and most IncP-3 plasmids can be transferred by conjugation to *E. coli*, but few other *Pseudomonas* plasmids have this ability (Jacoby and Shapiro, 1977). Plasmids of the same incompatibility group often have similar sizes and drug resistance profiles. Most of the plasmids that have been classified in this manner are R plasmids, although the sex factor FP2, and several degradative plasmids have also been assigned to incompatibility groups (Korfhagen *et al.*, 1978).

In order to understand the basis of incompatibility and the mechanisms of emergence of new types of plasmids, it is important to determine the relationships between plasmids of the same, and of different, incompatibility groups and between plasmids which carry common or related functions. So far, little work has been done to determine the extent and localisation of homology between such pairs of *Pseudomonas* plasmids, and indeed, with the exception of IncP-1 plasmids, the physical and molecular properties of few *Pseudomonas* plasmids have been reported. In this thesis the homology between ten plasmids is examined, and the homology is assigned to particular endonuclease-generated fragments. Of the ten plasmids, six belong to different incompatibility groups and four belong to the same group. They include seven R plasmids, two degradative plasmids and one sex factor.
2.1 Bacterial strains

A list of strains used and their phenotypes is given in Table 2.1. The strains which are able to metabolise toluene and the xylenes were isolated from Welsh soil samples by Williams and Worsey (1975) and their properties are discussed in Section 4.1. The B1 derivatives of these strains are spontaneously arising Tol⁻ segregants.

2.2 Resistance plasmids

The resistance profiles of the resistance plasmids used are recorded in Table 2.2. These were kindly provided by G. Jacoby in the host strains described.

2.3 Media

L-Broth contained 10 g of tryptone (Difco), 5 g of NaCl and 5 g of yeast extract (Difco) per litre, adjusted to pH 7.2. Minimal medium was made by the addition of sterile 1 M carbon source (see Section 3.1) to 80 ml of 4 x concentrated M9 medium (Adams, 1959) to give a final concentration of 10 mM. This mixture was added to 300 ml of sterile water to which had been added 0.5 ml of stock salts solution (Bauchop and Elsden, 1960), 0.25 ml of 1 M MgSO₄ and 0.25 ml of 36 mM FeSO₄. This method of preparation causes little precipitation of phosphates and hydroxides of heavy metals, and avoids the need to use an organic chelating agent.

For plates and slants these media were solidified by the inclusion of 1.5% New Zealand agar.
<table>
<thead>
<tr>
<th>Classification</th>
<th>Strain designation</th>
<th>Phenotype</th>
<th>Parent strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. putida (arvilla) mt-2</td>
<td>PaW 1</td>
<td>Tol+</td>
<td></td>
<td>Williams and Worsey, 1976</td>
</tr>
<tr>
<td></td>
<td>PaW 8</td>
<td>Tol-</td>
<td>PaW 1</td>
<td></td>
</tr>
<tr>
<td>P. putida</td>
<td>MT 1</td>
<td>Tol+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MT 1-B1</td>
<td>Tol-</td>
<td>MT 1</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas species</td>
<td>MT 3</td>
<td>Tol+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MT 3-B1</td>
<td>Tol-</td>
<td>MT 3</td>
<td></td>
</tr>
<tr>
<td>P. putida</td>
<td>MT 5</td>
<td>Tol+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MT 5-B1</td>
<td>Tol-</td>
<td>MT 5</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>MT 12</td>
<td>Tol+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MT 12-B1</td>
<td>Tol-</td>
<td>MT 12</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>MT 13</td>
<td>Tol+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MT 13-B1</td>
<td>Tol-</td>
<td>MT 13</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>MT 14</td>
<td>Tol+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MT 14-B1</td>
<td>Tol-</td>
<td>MT 14</td>
<td></td>
</tr>
<tr>
<td>P. putida</td>
<td>MT 15</td>
<td>Tol+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MT 15-B1</td>
<td>Tol-</td>
<td>MT 15</td>
<td></td>
</tr>
<tr>
<td>Classification</td>
<td>Strain designation</td>
<td>Phenotype</td>
<td>Parent strain</td>
<td>Reference</td>
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<td>----------------</td>
<td>-------------------</td>
<td>-----------</td>
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</tr>
<tr>
<td><em>P. putida</em></td>
<td>MT 16</td>
<td>Tol⁺</td>
<td>MT 16</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>MT 16-B1</td>
<td>Tol⁻</td>
<td>MT 16</td>
<td>&quot;</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>MT 17</td>
<td>Tol⁺</td>
<td>MT 17</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>MT 17-B1</td>
<td>Tol⁻</td>
<td>MT 17</td>
<td>&quot;</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>MT 18</td>
<td>Tol⁺</td>
<td>MT 18</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>MT 18-B1</td>
<td>Tol⁻</td>
<td>MT 18</td>
<td>&quot;</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>MT 19</td>
<td>Tol⁺</td>
<td>MT 19</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>MT 19-B1</td>
<td>Tol⁻</td>
<td>MT 19</td>
<td>&quot;</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>MT 20</td>
<td>Tol⁺</td>
<td>MT 20</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>MT 20-B1</td>
<td>Tol⁻</td>
<td>MT 20</td>
<td>&quot;</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>MT 21</td>
<td>Tol⁺</td>
<td>MT 21</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>MT 21-B1</td>
<td>Tol⁻</td>
<td>MT 21</td>
<td>&quot;</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>ac 34</td>
<td>Ade⁻</td>
<td>PpG1</td>
<td>Gunsalus et al, 1974</td>
</tr>
</tbody>
</table>
### Table 2.1 (continued)

<table>
<thead>
<tr>
<th>Classification</th>
<th>Strain designation</th>
<th>Phenotype</th>
<th>Parent strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td>PU21</td>
<td>Ilv&lt;sup&gt;-&lt;/sup&gt; Leu&lt;sup&gt;-&lt;/sup&gt; Rif&lt;sup&gt;r&lt;/sup&gt; Str&lt;sup&gt;r&lt;/sup&gt;</td>
<td>PAO</td>
<td>Pemberton and Clark, 1973</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>PA0303</td>
<td>Arg&lt;sup&gt;-&lt;/sup&gt;</td>
<td>PAO</td>
<td>Isaac and Holloway, 1972</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>PAC</td>
<td></td>
<td>PAO</td>
<td>Clarke, 1970</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>ED678&lt;sup&gt;(a)&lt;/sup&gt;</td>
<td></td>
<td>JC6256</td>
<td>Alfaro and Willetts, 1972</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>UB1139&lt;sup&gt;(b)&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>Bennett and Richmond, 1976</td>
</tr>
</tbody>
</table>

(a) Strain ED678 carries CoIE1. It was obtained by the transfer of CoIE1 into strain JC6256.
(b) Strain UB1139 carries RP1.

Phenotype abbreviations: Tol<sup>+</sup>, the ability to metabolise m-xylene, p-xylene, toluene, m-toluate and p-toluate; Nah<sup>+</sup>, the ability to metabolise naphthalene and salicylate; Ade<sup>-</sup>, Arg<sup>-</sup>, Ilv<sup>-</sup> and Leu<sup>-</sup>, requirement for adenine, arginine, isoleucine and leucine respectively; Str<sup>r</sup> and Rif<sup>r</sup>, resistance to streptomycin and rifampicin respectively.
# Table 2.2 Resistance plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Host strain</th>
<th>Resistance profile</th>
<th>Reference to plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP1</td>
<td>PU21</td>
<td>Cb Km Tc</td>
<td>Grinsted <em>et al.</em>, 1972</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lowbury <em>et al.</em>, 1969</td>
</tr>
<tr>
<td>R527</td>
<td>PU21</td>
<td>Cb Cm Gm Km Sm Su Tc Hg</td>
<td>Stanisich and Ortiz, 1976</td>
</tr>
<tr>
<td>R679</td>
<td>ac 34</td>
<td>Sm Su</td>
<td>Bryan <em>et al.</em>, 1973</td>
</tr>
<tr>
<td>Rms148</td>
<td>PA0303</td>
<td>Sm</td>
<td>Kremery <em>et al.</em>, 1975</td>
</tr>
<tr>
<td>FP2</td>
<td>ac 34</td>
<td>Hg</td>
<td>Loutit, 1970</td>
</tr>
<tr>
<td>R2</td>
<td>PU21</td>
<td>Cb Sm Su</td>
<td>Kawakami <em>et al.</em>, 1972</td>
</tr>
<tr>
<td>pMG18</td>
<td>ac 34</td>
<td>Cb Gm Km Sm Su Hg</td>
<td>H. Matsumoto via G. Jacoby unpublished.</td>
</tr>
<tr>
<td>R91</td>
<td>PAC</td>
<td>Cb</td>
<td>Stanisich and Holloway, 1971</td>
</tr>
</tbody>
</table>

Abbreviations: Cb, carbenicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Su, sulphonamide; Tc, tetracycline; Hg, HgCl₂.
Bacterial buffer contained 3 g of KH₂PO₄, 7 g of Na₂HPO₄, 4 g of NaCl, and 0.2 g of MgSO₄·7H₂O per litre.

2.4 Buffers

TES buffer, 0.05 M tris (hydroxymethyl) aminomethane (Tris), 0.005 M ethylenediaminetetraacetic acid (EDTA) and 0.05 M NaCl, pH 8.0.

TE buffer, 0.01 M Tris-hydrochloride and 0.001 M EDTA, pH 7.2.

20 x SSC buffer, 3.0 M NaCl and 0.3 M Na citrate.

TNE buffer, 10 mM Tris-hydrochloride, 10 mM NaCl and 2 mM EDTA, pH 8.0.

SSCP buffer, 120 mM NaCl, 15 mM Na citrate, 15 mM KH₂PO₄ and 1 mM EDTA, pH 7.2.

Electrophoresis buffer, 40 mM Tris-acetate, 20 mM Na acetate and 5 mM EDTA, pH 8.2.

2.5 Enzymes

Egg white lysozyme (grade I, lyophilised), Sigma Chemical Company, St Louis, Mo, USA.

Bovine pancreatic DNase I (crude grade), Sigma Chemical Co.

E. coli DNA polymerase I (highest purity), Boehringer - Mannheim, W. Germany.

2.6 Radiochemicals

(α-³²P) dGTP, 350 Ci/mM and 1 mCi/ml in ethanolic solution (dried for 1 hr in vacuo over conc H₂SO₄ before use).

Radiochemical Centre, Amersham, Buckinghamshire.

2.7 Antibiotics

Carbenicillin (Pyopen), Beechams Research Laboratories, Brentford, Middlesex.
Chloramphenicol (Chloromycetin), Parke, Davis and Company, Pontypool, Monmouthshire.

Gentamicin (Genticin), Nicholas Laboratories Limited, Slough, Gloucestershire.

Kanamycin (Kannasyn), Winthrop Laboratories, Surbiton-upon-Thames, Surrey.

Streptomycin sulphate BP, Glaxo Laboratories Ltd, Greenford, Middlesex.

Tetracycline (Achromycin), Lederle Laboratories Division, Cyanamid of Great Britain, Gosport, Hampshire.

2.8 Reagents

Adenine Sulphate, Sigma Chemical Company, St Louis, Mo., USA.

Albumin bovine (Fraction V), (BSA), BDH Chemicals Ltd, Poole, Dorset.

Caesium Chloride, Analytical Reagent, Fisons Scientific Apparatus, Loughborough, Leicestershire.

Chloroform, Analytical Reagent, Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire.

Deoxyribonucleoside triphosphates, Boehringer - Mannheim, West Germany.

EDTA, Analar, BDH Chemicals Ltd.

Ethidium bromide, Sigma Chemical Company.

Ficoll, Pharmacia.

β-mercaptoethanol, Pure, Koch-Light Laboratories Ltd.

Polyvinyl pyrrolidine, Laboratory Reagent, BDH Chemicals Ltd.

Propan-2-ol (anhydrous), Analytical Reagent, Koch-Light Laboratories Ltd.
Sodium chloride, Analytical Reagent, Fisons Scientific Apparatus.

Sodium citrate, Analytical Reagent, Fisons Scientific Apparatus.

Sodium hydroxide (pellets), Analytical Reagent, Koch-Light Laboratories Ltd.

D(+)−Sucrose, Analytical Reagent, Koch-Light Laboratories Ltd.

Trichloroacetic acid, Pure, Koch-Light Laboratories Ltd.

Tris-hydrochloride, Analytical Reagent, Koch-Light Laboratories Ltd.

Yeast RNA (Type III), Sigma Chemical Company.

2.9 Miscellaneous

Agarose, Koch-Light Laboratories Ltd, Colnbrook, Bucks.


Dowex 50W-X8 20-50 U.S. Mesh (H), BDH Chemicals Ltd, Poole, Dorset.

Nitrocellulose DHX 30/50, Nobel Division, ICI Ltd, Stevenston, Ayrshire.

Nitrocellulose filters (type BA85), Schleicher and Schüll, Dassel, West Germany.

Polypropylene tubes (No. 39/10, capacity 1.5 ml), W. Sarstedt (UK), Leicester.

Repelcote, Hopkin and Williams, Chadwell Heath, Essex.
CHAPTER 3

Methods

3.1 Culture conditions and determination of growth rate

When it was necessary to select for and to ensure retention of the Tol\(^+\) phenotype in bacterial strains, a minimal salts medium containing 10 mM \(m\)-toluate as sole carbon source was used. Other prototrophic strains were maintained on minimal agar slopes containing either 10 mM succinate or 10 mM benzoate as the carbon source. Auxotrophic strains were maintained on L-broth agar slopes.

For the isolation of degradative plasmid deoxyribonucleic acid (DNA), initially cell cultures were grown to stationary phase in 25 ml of selective medium containing \(m\)-toluate. Cells grown under these conditions on L-broth medium supplemented with antibiotics were used for the isolation of plasmid DNA from strains carrying \(R\) plasmids. Such cultures were then inoculated into 1 litre volumes of minimal medium containing glucose, in 2 litre conical flasks. Cell density was measured turbidimetrically with a Klett-Summerson colorimeter with a red filter. Bacterial cultures were grown in rotary shakers at 30\(^\circ\)C for \(P\).\(putida\) strains, and at 37\(^\circ\)C for \(P\).\(aeruginosa\) and \(E\).\(coli\) strains.

3.2 Isolation of plasmid DNA

ColE1 and RP1 DNA for electron microscopy were prepared from \(E\).\(coli\) strains ED678 and UB1139 respectively, as described previously (Thompson et al., 1974). Chloramphenicol was added to exponentially growing cultures (3 \(\times\) 10\(^8\) cells/ml) of the former strain to a final
concentration of 60 μg/ml, in order to increase the number of copies of ColEl DNA per cell (Clewell, 1972).

3.3 Isolation of degradative plasmid DNA and R plasmid DNA

One litre batches of bacterial cultures were grown to late exponential phase (approximately $10^9$ cells/ml or 180 Klett units) in minimal medium and harvested by centrifugation at 4°C. This pellet was resuspended in 21 ml of cold sucrose solution (25% sucrose in 0.05 M Tris-hydrochloride, pH 8.0) and stored in this form at -20°C. After thawing, spheroplasts were formed by adding lysozyme solution (3 ml of freshly prepared solution at 10 mg/ml in 0.25 M Tris-hydrochloride, pH 8.0) and shaking at 37°C for 2 min, followed by storage on ice. After 5 min, 13 ml of 0.25 M EDTA solution (sodium salt, pH 8.0) was added, and after a further 5 min the cells were lysed by adding 27 ml of an aqueous solution of 2% Triton X-100, 0.05 M Tris-hydrochloride (pH 8.0), and 0.0625 M EDTA. After allowing up to 1 h for lysis, the DNA in the resulting viscous solution (approximately 60 ml) was sheared by slow passage through the Luer nozzle of a 50-ml disposable syringe for 45 to 60 s. The sheared lysate was denatured (to pH 11.5) by the addition of 2 ml of freshly made 4 N NaOH solution (or a solution that had been stored in a stoppered bottle) while stirring with both a magnetic follower and glass rod. When the mixture appeared homogeneous, the glass electrode of a pH meter was used to ascertain the homogeneity of pH (i.e. that there were no pockets of extreme pH). The solution was held at pH 11.5 for 60 s while being mixed
gently; the pH was then rapidly restored to between 8.0 and 8.5 by the addition of 25 ml of saturated (at room temperature) Tris-hydrochloride solution, pH 7.0. The single-stranded DNA from denatured chromosome and open circular plasmid molecules was removed by adsorption to nitrocellulose. Approximately 20 g (wet weight) of nitrocellulose, which had been ground to fibrous mats with a porcelain mortar and pestle and washed extensively with distilled water, was sprinkled into the DNA solution and gently rotated with it for 1 h at 4°C. The nitrocellulose with the associated single-stranded DNA was removed by centrifugation at 5,000 rpm for 10 min at 4°C; this extraction step was then repeated by a further addition of nitrocellulose to the supernatant. After centrifugation, each solution was filtered through glass wool into a polycarbonate centrifuge bottle, underlayered with 4 ml of saturated CsCl (at room temperature) in TES buffer, and centrifuged (for 15 to 20 h) at 18,000 rpm (32,000 x g at r<sub>av</sub>) in a fixed-angle type 21 rotor (Beckman-Spinco) at 4°C after the centrifuge bottle was completely filled by careful overlayering with distilled water. After gentle aspiration of the upper 90 ml, the lowest 9 ml of each tube was collected, gently mixed, and filtered through a glass wool plug into polyallomer centrifuge tubes. Solid CsCl was added to bring the refractive index to 1.402, and the tubes were each filled to within 1 cm of the top with CsCl solution of refractive index 1.402. Last, 0.2 ml of ethidium bromide solution (20 mg/ml in water) was added and gently mixed with the tube contents by inversion.
From this step onward, the tubes were protected from light whenever possible to minimise light-activated dye nicking of covalently closed DNA molecules. The centrifuge tubes were overlaid with liquid paraffin and centrifuged at 40,000 rpm (100,000 x g at r_{av}) for 40 h in a fixed-angle type 50 Ti rotor (Beckman-Spinco) at 15°C. The DNA banded within the density gradient to form (i) an upper viscous band comprising linear chromosomal and open circular plasmid DNA and (ii) a lower band comprising covalently closed circular plasmid DNA. These were apparent under long-wavelength ultraviolet illumination and, if present in high yield, were also visible against a white background in daylight. The plasmid band was collected by piercing the centrifuge tube immediately underneath the band with a disposable syringe needle and allowing plasmid DNA to drip out. When necessary, the material from the plasmid band was pooled and recentrifuged to concentrate the plasmid DNA and to obtain a purer preparation. However, it was generally found that a single ethidium bromide-CsCl density gradient centrifugation step was adequate to isolate plasmid DNA in sufficiently pure form to allow subsequent characterisation by electron microscopy and agarose electrophoresis after endonuclease digestion.

Ethidium bromide was removed from the plasmid preparation by gentle extraction with propan-2-ol (presaturated with CsCl solution) followed by dialysis against the sodium form of Dowex 50 W-XB resin in 50 ml of buffer (0.8 M NaCl, 0.05 M Tris-hydrochloride, 0.01 M EDTA, pH 8.0) (Sharp et al, 1972). The DNA solution was
finally dialysed against several changes of TE buffer before storage at 4 °C. The DNA concentration was determined spectrophotometrically, assuming that an absorbance at 260 nm of 1.0 corresponds to 50 µg/ml. Yields of up to 50 µg of plasmid DNA per preparation were obtained.

3.4 Electron microscopy

Plasmid DNA was visualised by the formamide method. (Davis et al, 1971).

3.5 Restriction endonuclease digestion of plasmid DNA

Digestion was carried out in sterile 1.5 ml polypropylene tubes at 37 °C for up to 6 h, depending upon the activity of the enzyme preparation. DNA preparations in TE buffer containing approximately 1 µg of DNA were each dried down to 20 µl in a vacuum desiccator over concentrated H₂SO₄ at a reduced pressure of 12 mm of Hg. The digestion mixture was adjusted to 10 mM Tris-hydrochloride (pH 7.5), 10 mM 2-mercaptoethanol, 100 mM NaCl and an excess of 10 mM MgCl₂ over the EDTA concentration. Endonuclease EcoRI (Hedgpeth et al, 1972) prepared by the method of Yoshimori (1971) in 50% glycerol, was added in requisite amounts, usually 1 to 2 µl, to the digestion mixture. Endonuclease Xma I, purified from Xanthomonas malvacearum was used in a similar manner (Endow and Roberts, 1977). Digestion with the endonuclease Pst I, which was purified from Providencia stuartii (Smith et al, 1976), was carried out at 30 °C; this digestion mixture contained 100 mM Tris-hydrochloride (pH 7.5), and an excess of 10 mM MgCl₂ over the EDTA concentration.
In each experiment phage \(\lambda\) DNA was digested as a control. The reactions were terminated by heating in a water bath at 70\(\degree\)C for 10 min, after which the tubes were rapidly cooled in ice water; this also prevents the cohesive ends of phage \(\lambda\) DNA from reannealing. The contents of each tube were mixed with 5 \(\mu\)l of loading mixture, comprising 10% Ficoll and 0.04% bromophenol blue, and the liquid volume was reduced to about 10 \(\mu\)l by evaporation in a vacuum desiccator before the concentrated solution was loaded into the sample wells of the agarose slab gel.

3.6 Agarose gel electrophoresis

Electrophoresis was carried out on vertical slab gels according to the method of Hayward and Smith (1972). 2.5 g of agarose was refluxed with 250 ml electrophoresis buffer until dissolved. After cooling to 50\(\degree\)C the gel was poured to within 3 cm of the top of a space (0.3 cm x 17 cm x 40 cm) between two glass plates, one of which had been coated with 0.1% agarose solution which was dried onto the glass at 70\(\degree\)C to prevent gel slippage. The plates were separated by perspex strips at the sides and a perspex slot-former giving 1 cm x 0.2 cm slots at the bottom. When the 1% agarose had set the space was filled with 3% agarose in electrophoresis buffer, so that when inverted into the running position the lowest 3 cm of the slab gel were formed from 3% agarose, again to prevent gel slippage. The slot-former was removed and after filling with electrophoresis buffer samples were layered into the slots. After electrophoresis at a constant
voltage of 150 V for 15 to 20 h, the agarose gel was removed from the glass plates and stained for 30 min in aqueous ethidium bromide solution (2 µg/ml), after which the gel was washed in water for up to 3 h before photographing. The gels were photographed against a black polythene sheeting background under short-wavelength ultraviolet illumination through a x 4 red filter onto Ilford FP4 film.

3.7 Densitometry

Densitometry scans of photographic films of gels were made on a MK III double-beam recording densitometer (Joyce, Loebl and Co Ltd, Gateshead, England).

3.8 Transfer of R plasmids from the PU21 background to strain ac 34

R plasmids were transferred from the Leu⁺ Ilv⁻ P.aeruginosa strain PU21 to the plasmid-free Ade⁺ P.putida strain ac 34 using a modification of the method of Nakazawa and Yokata (1977). An inoculum of cells from overnight cultures of each donor and the recipient was diluted into L-broth to a cell density of $5 \times 10^7$ cells/ml. Strain ac 34 was incubated at 30°C, and the donor strains were grown at 37°C until a cell density of $2 \times 10^8$ cells/ml was reached. To initiate conjugation, 2 ml volumes of donor and recipient cultures were mixed in 100 ml Erlenmeyer flasks and incubated for 2 h at 30°C without shaking. Concurrently, separate flasks containing 4 ml volumes of donor or recipient culture were incubated as controls. 0.1 ml volumes of the mating mixture (undiluted and diluted tenfold) were plated on minimal plates supplemented with
adenine (50 µg/ml) and carbenicillin (2 µg/ml) for the detection of exconjugants.

R plasmid Rms148 was transferred from the P. aeruginosa strain PA0303 to ac 34 in a similar manner; however in this case exconjugants were selected for on minimal plates supplemented with adenine and streptomycin (100 µg/ml).

3.9 Detection of specific sequences among the endonuclease-generated fragments of degradative plasmids and R plasmids

a) Agarose gel electrophoresis

Electrophoresis was carried out on vertical slab gels as described in Section 3.6, but with the modification that a slot-former was used that gave slots which were the full width of the gel (0.3 cm). The gel was poured in the running position, and the glass plates were separated by perspex strips at the sides, the bottom being sealed with parafilm and autoclave tape. 3 cm of 3% agarose were poured into the bottom of the space between the glass plates and allowed to set. The space was then filled with 1% agarose and the slot-former was placed in the top. Use of this type of gel ensured that after electrophoresis the DNA in the bands was spread across the full width of the gel, and this was found to aid transfer.

b) Transfer of DNA fragments to nitrocellulose filter

Endonuclease-generated fragments of plasmid DNA were transferred from 1% agarose gels to nitrocellulose filters using the method of Southern (1975) as modified by Heinaru et al (1978). DNA was denatured by submersion of
the gel in 0.5 M NaOH, 1.5 M NaCl for 30 min. The gel was rinsed with neutralisation solution (3.0 M NaCl, 0.5 M Tris-hydrochloride, pH 7.0) and then immersed in neutralisation solution for 40 min. Occasional mixing ensured that the gel was submerged at all times. Transfer of DNA from the gel was carried out in an apparatus constructed from a horticultural polystyrene seed tray (36 x 22 cm) upon which a sheet of glass was laid. Five sheets of Whatman 3 MM paper were draped over the glass to form a wick in contact with the 20 x SSC buffer held in the tray. The neutralised gel was laid upon this, and, under the peripheral few millimeters of the shorter sides of the gel, 2 cm wide strips of heavy gauge polythene sheeting were inserted between the paper and the gel. This prevented liquid short-circuits caused by the sagging of the Whatman paper which was subsequently laid over the gel. Perspex support strips (3 mm thick) were laid parallel to the longer sides of the gel and several millimeters from it. A sheet of nitrocellulose filter, 0.5 cm longer and 1.5 cm wider than the gel, was moistened with 2 x SSC buffer and laid carefully upon the gel, so that it overlapped all the edges of the gel and rested on the longitudinal support strips. All air bubbles between the gel and the paper, and between the nitrocellulose filter and the gel, were expelled. Several sheets of Whatman paper were moistened with 20 x SSC and laid upon the nitrocellulose filter in a similar manner; in turn, sheets of dry Whatman paper were laid upon these to form a pile 10 cm high. The apparatus was covered with cling.
film and a glass plate placed on top to compress the stack of filter paper. After 16 h most of the Whatman paper was removed, leaving a pile 1 cm high. The perspex support-strips were then replaced by thinner ones (1.5 mm thick) to compensate for the shrinking gel, and a new pile of Whatman paper placed on top. Transfer was continued for a further 6 h, when, due to further gel shrinkage, it was necessary to replace each perspex strip by three strips of heavy gauge polythene sheeting. Transfer was completed over a further 16 h. After transfer the nitrocellulose filter was peeled off the gel and washed in 2 x SSC buffer for 10 min. The filter was dried at 37°C for half an hour before baking in vacuo for 2 h at 80°C. Confirmation that DNA had transferred from the gel was provided by restaining it in ethidium bromide solution and photographing it as previously. DNA fragments larger than about 5 Md transferred poorly and substantial proportions of these species remained behind in the gel.

c) Radioactive labelling of plasmid DNA by "Nick translation"

The plasmid DNA probe was radioactively labelled to a high specific activity by "nick translation" (Maniatis et al, 1975) after initial random nicking of the covalently-closed plasmid DNA by pancreatic DNase. The "nick translation" reaction mixture contained 50 mM Tris-hydrochloride, 5 mM MgCl₂, 10 mM β-mercaptoethanol pH 7.8, and was made up with approximately 1 µg plasmid DNA in sterile 1.5 ml polypropylene tubes using 10 µCi (α-³²P) dGTP, and unlabelled deoxyribonucleoside triphosphates of
the other three bases. Plasmid DNA was nicked by adding 2 µl of 1 µg/ml pancreatic DNase I and incubating at room temperature. After 2 min one unit of *E. coli* DNA polymerase I was added and incorporation of radioactive label was allowed to proceed for 5 h. Samples were taken at hourly intervals to measure uptake of $^{32}$P into acid-precipitable material. The reaction was terminated by the addition of 0.1 ml of pre-equilibrated (TNE buffer) phenol (distilled under nitrogen and preserved from peroxidation by storing with approximately 0.01% 8-hydroxyquinoline at 4°C in the dark). 10 µl yeast RNA was added as carrier nucleic acid. The reaction mixture was extracted twice with phenol, twice with chloroform-propan-2-ol (24:1) and twice with diethyl ether, and the remaining ether was removed by gentle aspiration at 37°C. The nucleic acid was separated from the reaction substrates by chromatography on a 7 ml column of Sephadex G-50 in TNE buffer. The position of the reaction substrates was indicated by the co-chromatography of Orange G dye added to the sample.

The position of the nucleic acid was detected with a Geiger-Müller monitor and 2-drop fractions were collected. Fractions were counted by Cherenkov radiation and the peak activity, comprising approximately 20% of the total labelled nucleic acid, was pooled and stored at -20°C. Total $^{32}$P counts were determined by liquid scintillation (butyl PBD, 0.5% in toluene) as acid precipitable (5% trichloroacetic acid) material. Samples of this labelled nucleic acid, containing $10^6$ cpm, were denatured for 5 min in a boiling water bath and cooled rapidly in iced water before use as
single-stranded probe for hybridisation with the DNA bound to the nitrocellulose filter.

d) Hybridisation of plasmid probe to nitrocellulose-bound single-stranded DNA

Nitrocellulose filters were prepared for hybridisation by incubation in 0.02% BSA, 0.02% ficoll, 0.02% polyvinylpyrridone in 3 x SSC, in 100 ml measuring cylinders for 6 h (Denhardt, 1966). The hybridisation solution contained 10^6 cpm of single-stranded DNA probe, the ingredients of the pre-incubation solution in 4 x SSCP and 0.2% (W/v) sodium dodecyl sulphate (to reduce non-specific binding of DNA to the nitrocellulose filter). Hybridisation was carried out in siliconised 250 ml measuring cylinders with the nitrocellulose filter wrapped around a nylon plug insert. In this way the volume of the solution was kept to about 5 ml. Most hybridisation occurred if the side of the filter to which DNA was bound faced inwards, since the filter had a tendency to cling to the glass. A few drops of paraffin oil were layered over the hybridisation mixture to reduce surface evaporation and the whole was incubated in a covered water bath at 65°C for 40 h. After half an hour, and at 12-hourly intervals throughout the incubation, the nylon plug was rotated to dislodge air bubbles from the filter. The filter was washed in 0.05% SDS in 4 x SSC buffer at 65°C for 2 h and then in 4 x SSC buffer alone at 65°C for 30 min. The filter was then rinsed in 2 x SSC buffer at room temperature and dried at 37°C for an hour prior to exposure to pre-exposed photographic film (XHL, Kodak-Eastman) next to an intensification screen (Ilford Fast Tunstate) at -70°C (Laskey and Mills, 1977).
4.1 Introduction

Williams and Worsey (1976) have isolated thirteen strains of bacteria from nine soil samples by selective enrichment culture on m-toluate minimal medium. These strains were classified as pseudomonads; they included representatives of both P.putida biotypes A and B, and others which did not fit precisely into the putida group. Genetic evidence suggested that these strains carried plasmids which were superficially similar to the TOL plasmid of P.putida mt-2 which has been described by Williams and Murray (1974), Wong and Dunn (1974) and Worsey and Williams (1975) (Section 1.5). Each strain could also utilise toluene, m-xylene and p-xylene by a pathway similar to that found for P.putida mt-2. The hydrocarbons were degraded via benzoate, m-toluate and p-toluate respectively, and subsequently by the meta pathway (Section 1.4). Cured derivatives of the strains arose spontaneously in which, of the aromatic compounds, only benzoate could be utilised; this was then metabolised by the ortho pathway. However, only eight of the isolates were able to transfer their degradative functions into their own cured derivatives, and of these, only five were transfer proficient in interstrain conjugation with the cured derivative of P.putida mt-2. Differences between the degradative functions were also suggested on the basis of differences in the efficiencies with which the various substrates could be
utilised, in the ability of some of the strains to give rise to 'B3' derivatives in which the ability to degrade p-xylene, m-toluate and p-toluate is lost, in the ease of curing and in the regulation of the enzymes. In this chapter the isolation and molecular characterisation of plasmid DNA from these strains is described. This work has been published (Duggleby et al, 1977).

4.2 Isolation of plasmid DNA

Most of the techniques which have been developed for the isolation of plasmid DNA depend upon the existence of plasmids as covalently closed circular (CCC) DNA in the cell, and their consequent resistance to sedimentation compared to the chromosome and the cellular membrane. In these techniques chromosomal DNA and the cellular membrane are removed by centrifugation (Clewell and Helinski, 1969; Freifelder, 1971) or by selective precipitation in the presence of sodium dodecylsulphate and a high concentration of sodium chloride (Hirt, 1967; Guerry et al, 1973). In repeated attempts, we were unable to isolate plasmid DNA from these strains as CCC molecules in a CsCl-ethidium bromide density gradient after a clearing spin had been used to remove chromosomal DNA. In these experiments a number of parameters were varied systematically, including lysis procedure, and the length and speed of clearing spin, but none of these methods yielded plasmid DNA. However the R plasmid RP1 could be isolated routinely from P.putida using this type of method. It is possible that degradative plasmids cannot be isolated in this manner because they are associated with the membrane-chromosomal
DNA complex and are precipitated with it.

Palchaudhuri and Chakraborty (1976) reported that using the method of Sharp et al. (1972) plasmid DNA could be isolated from strains of *Pseudomonas* that carry other degradative functions. Genetic evidence suggested that these functions were plasmid-specified. We have isolated plasmid DNA from the series of strains that carry the Tol function using a modification of this procedure. The method, which is described fully in Section 3.3, involves momentary alkali denaturation and then neutralisation. When the DNA is denatured the two strands of chromosomal DNA and open circular plasmid (OC) DNA separate whereas the two strands of CCC plasmid DNA remain topologically linked. Therefore on neutralisation the CCC plasmid DNA renatures and the chromosomal DNA and OC plasmid DNA remain single stranded. Single stranded DNA is removed from the mixture by adsorption to nitrocellulose and CCC plasmid DNA is then concentrated in a CsCl-ethidium bromide equilibrium density gradient.

4.3 Characterisation of plasmid DNA

After application of the isolation procedure, each of the strains gave a discrete band of presumptive plasmid DNA in a CsCl-ethidium bromide density gradient. We have used two criteria to characterise and assess relatedness between these plasmids. One was the banding pattern yielded after electrophoresis on agarose gels of DNA fragments generated by digestion with the endonuclease EcoRI. The results obtained are presented in Figs. 4.1 and 4.2. These
Fig. 4.1  Agarose gel electrophoresis of fragments generated by EcoRI digestion of plasmid DNA from the two groups of strains that carry closely related plasmids. The track marked A is of plasmid DNA from a transformant clone (see Section 4.5), and a λ digest is included to calibrate the sizes of the fragments.
Fig. 4.2 Agarose gel electrophoresis of EcoRI digests of unique species of plasmid DNA from Tol⁺ strains, together with those of λ and PaW 1 plasmid DNA.
experiments were carried out principally by C.J. Duggleby. The other was the determination of the size of circular molecules by electron microscopy. In most cases CoIE1 was used as the internal standard, but for those preparations which, on preliminary inspection were found to contain molecules which were similar in size to CoIE1, RP1 was used. Six molecules of each plasmid species were measured. The size class(es) of plasmid(s) present in each preparation are shown in Table 4.1.

The plasmid preparations from all strains except MT 1, MT 3 and MT 14 contained only a single size class of circular molecules. Strains MT 1 and MT 3 yielded plasmids which fell into two size classes. An electron micrograph showing representative molecules of the two size classes carried by strain MT 1, is presented in Fig. 4.3.

The EcoRI digests of plasmid preparations from strains MT 14, MT 15 and MT 19 gave a relatively large number of clearly defined bands after agarose gel electrophoresis, suggesting that either there were a number of different plasmids present, or a single very large one. Electron microscopy showed that strains MT 15 and MT 19 contained a single size class of very large molecules. However, the plasmid preparation from strain MT 14 contained a number of different sizes of circular molecules, some of which were very large. The distribution of circular molecules into the various size classes is shown in Table 4.2. Further experiments with this strain are described in Chapter 6.
Fig. 4.3  Electron micrograph showing the relative sizes of the two plasmid species carried by strain MT 1.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid size (Md ± standard error)</th>
<th>Name assigned to plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.putida (arvilla) mt-2</td>
<td>78.1 ± 1.1</td>
<td>pWW0 (TOL)</td>
</tr>
<tr>
<td>MT 16</td>
<td>81.3 ± 2.5</td>
<td>pWW16</td>
</tr>
<tr>
<td>MT 17</td>
<td>75.0 ± 0.6</td>
<td>pWW17</td>
</tr>
<tr>
<td>MT 18</td>
<td>75.6 ± 0.9</td>
<td>pWW18</td>
</tr>
<tr>
<td>MT 21</td>
<td>74.4 ± 0.8</td>
<td>pWW21</td>
</tr>
<tr>
<td>MT 1</td>
<td>52.0 ± 0.4</td>
<td>pWW2</td>
</tr>
<tr>
<td></td>
<td>4.8 ± 0.1</td>
<td>pWW4</td>
</tr>
<tr>
<td>MT 3</td>
<td>79.9 ± 0.8</td>
<td>pWW3</td>
</tr>
<tr>
<td></td>
<td>4.0 ± 0.1</td>
<td>pWW4</td>
</tr>
<tr>
<td>MT 5</td>
<td>103.3 ± 4.9</td>
<td>pWW5</td>
</tr>
<tr>
<td>MT 12</td>
<td>75.1 ± 1.4</td>
<td>pWW12</td>
</tr>
<tr>
<td>MT 13</td>
<td>75.7 ± 1.4</td>
<td>pWW13</td>
</tr>
<tr>
<td>MT 14</td>
<td>25 - 202</td>
<td></td>
</tr>
<tr>
<td>MT 15</td>
<td>169.1 ± 2.6</td>
<td>pWW15</td>
</tr>
<tr>
<td>MT 19</td>
<td>151.5 ± 2.7</td>
<td>pWW19</td>
</tr>
<tr>
<td>MT 20</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

ND = No Data.
Md = Million daltons.

Grids were first made without any internal standard. Then new grids were made including ColE1 (taken as 4.2 Md (Bazaral and Helinski, 1968)) or RP1 (determined using ColE1 as 38.9 ± 0.5 Md) as internal standards. For the determination of size of a molecule the sizes of three molecules of ColE1, or one molecule of RP1, that were within the same field of view were determined. With the exception of the size classes present in the plasmid preparation from strain MT 14 (see Table 4.2), six molecules of each size class were measured.
Table 4.2  Size distribution of plasmid molecules from strain MT 14

<table>
<thead>
<tr>
<th>Size class (Md ± standard error)</th>
<th>No. of molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.2 ± 0.5</td>
<td>6</td>
</tr>
<tr>
<td>39.5 ± 0.8</td>
<td>8</td>
</tr>
<tr>
<td>60</td>
<td>3</td>
</tr>
<tr>
<td>68</td>
<td>3</td>
</tr>
<tr>
<td>76</td>
<td>1</td>
</tr>
<tr>
<td>202.5 ± 1.5</td>
<td>(a)</td>
</tr>
</tbody>
</table>

All circular molecules on those grids examined were photographed and measured. ColE1 was used as an internal standard.

(a) A total of 20 very large molecules were identified. The value given is for the seven, chosen at random, that were measured.
So far it has not been possible to obtain circular molecules or banding patterns for the presumptive plasmid DNA preparation from strain MT 20. However, extremely large linear molecules of DNA were observed by electron microscopy. Since visualisation of circular molecules of the 200 Md size class of MT 14 was extremely difficult, it is possible that the circular plasmid molecules isolated from MT 20 were even larger and consequently too fragile to be characterised by these techniques.

Densitometry traces of the gels showed that the EcoRI digest of plasmid DNA isolated from \textit{P. putida} mt-2 gave about 29 bands, assuming that peaks containing twice the unit amount of DNA represent coincident bands; alternatively some may represent duplications of particular regions within the plasmid (Fig. 4.4). Comparison of the mobilities of these fragments with those of known size generated by EcoRI digestion of phage \lambda DNA indicated that the sum of the sizes of the fragments was about 75 Md. This was in agreement with the size of plasmid DNA as determined by electron microscopy, and showed that there was a single plasmid species rather than, for instance, two plasmids of similar size. Similar arguments apply to the other plasmid preparations containing a single size class.

4.4 Relationships between the plasmid species

Similar patterns of banding of endonuclease-generated DNA fragments after electrophoresis on agarose gels is evidence of relatedness between the plasmids (Thompson \textit{et al}, 1974). The most striking result from the comparison of gel patterns after digestion with the endonuclease EcoRI.
Fig. 4.4  Densitometry trace of the electrophoresis pattern of EcoRI digested plasmid DNA from strain *P. putida* mt-2.
was that the bands given by four of the plasmid preparations were indistinguishable from those of TOL, the plasmid carried by P. putida mt-2 (Fig. 4.1). Digestion of these plasmids with another endonuclease, Xma I, also gave indistinguishable gel patterns. Examination of the plasmid preparations by electron microscopy had already shown that each strain contained a single size class of plasmid, and these were very similar in size (Table 4.1). Figure 4.5 shows an electron micrograph of a plasmid molecule isolated from P. putida mt-2 as a representative of this group. We conclude that this type of plasmid is widely distributed in nature.

Plasmid DNA from strains MT 12 and MT 13 was similar in size to the plasmid from strain P. putida mt-2. However, plasmid DNA from strains MT 12 and MT 13 gave gel patterns after EcoRI treatment which were indistinguishable from each other, but different from that given by TOL. Strains MT 12 and MT 13 were distinguishable morphologically, therefore a given plasmid may be present in a number of biotypes.

Strains MT 12 and MT 13 were isolated from the same enrichment culture, and since both plasmids are transfer proficient, it is possible that the plasmid carried by one strain transferred to the other during isolation.

Plasmid DNA from the other strains yielded unique patterns, as expected from their size determinations. However, there are similarities between the patterns given by MT 1, MT 3, MT 5, MT 19 and TOL, and certain bands may be common to several of the patterns (Fig. 4.2). This suggests the possibility that these plasmids are related.
Fig. 4.5  Electron micrograph of a representative molecule of the plasmid species carried by strain P. putida mt-2. The small molecules are the internal standard, ColE1, which has a size of 4.2 Md.
4.5 Correlation of plasmid species with Tol$^+$ phenotype

Transformation provides the most rigorous demonstration that a given plasmid species is responsible for a given phenotype. C.J. Duggleby has transformed plasmid DNA from \textit{P. putida} mt-2 into a genetically marked reportedly plasmid-free strain, ac$^{34}$, using the method of Chakrabarty \textit{et al} (1975). We found that plasmid DNA isolated from three independent Tol$^+$ transformants gave endonuclease fragment patterns which were indistinguishable from that of the plasmid DNA from \textit{P. putida} mt-2 (Fig. 4.1). The size of these plasmids, as determined by electron microscopy, was also indistinguishable from that of the parental strain. Attempts to isolate plasmid DNA from the recipient strain, ac$^{34}$, were carried out simultaneously. After several attempted isolations no plasmid bands were detectable in CsCl-ethidium bromide density gradients indicating that ac$^{34}$ was indeed plasmid-free. Transformation experiments with plasmid DNA from strain MT 17 gave similar results. These experiments show unequivocally that the group of plasmids typified by that carried by \textit{P. putida} mt-2 are indeed TOL plasmids.

We have not been able to transform plasmid DNA from the strains that do not fit into the \textit{P. putida} mt-2 group. However, strains MT 3-B1, MT 15-B1, MT 16-B1, MT 19-B1, MT 20-B1 and MT 21-B1, which are Tol$^-$ segregants (Williams and Worsey, 1976), were found to be plasmid-free, suggesting that loss of the Tol phenotype can be associated with loss of the plasmid. Cured derivatives of the remaining eight strains still contained plasmid DNA.
However, where only one plasmid species is present, the Tol function can be assigned to these plasmids on genetic criteria. A detailed analysis of the molecular events involved in curing of strain *P. putida* mt-2 is presented in Chapter 5.

### 4.6 Discussion

The results of the transformation experiments strongly suggest that the degradation of toluene and the xylenes is plasmid-specified in at least some of the soil pseudomonads we have studied. In some strains loss of the plasmid is correlated with loss of the Tol function. Also, genetic evidence indicates that the Tol function is plasmid-specified in all of the cases examined. In each strain degradation proceeds via the meta pathway, but there is more than one type of plasmid involved; the results of Williams and Worsey (1976) indicate that these specify analogous rather than homologous enzymes.

It is interesting that the TOL plasmids from a number of independently isolated strains (*P. putida* mt-2, MT 16, MT 17, MT 18 and MT 21) are closely related. These strains were also grouped together by Williams and Worsey (1976) on genetic and physiological criteria. Therefore, a given plasmid may be very widely distributed in nature, and this implies a remarkable conservation in the genetic structure of the plasmid.

A given plasmid may be present in different *Pseudomonas* species, as is exemplified by the plasmid present in strains MT 12 and MT 13. However, since the isolation procedure
involves selective enrichment, and at least some of the plasmids are transmissible, it is not clear whether any of the isolates are the natural hosts for the plasmids they carry.

The remaining plasmids are different, though in some cases molecular relationships are suggested by similarities in endonuclease fragment patterns. The possibility that there are common DNA sequences, whether in the catabolic, transfer or replication functions of the plasmids, was not investigated. Strains MT 1 and MT 3 each carried two plasmid species. We have not determined whether both of the plasmids in these strains are involved in the Tol phenotype.
CHAPTER 5

Two modes of loss of the Tol function
from Pseudomonas putida mt-2

5.1 Introduction

One of the criteria by which the degradation of m- and p-toluate by P.putida mt-2 was originally judged to be plasmid-specified was the loss of the function at frequencies higher than those normally found for mutations (Williams and Worsey, 1974). As described in Chapter 4, while characterising plasmid DNA from this strain and a number of independently isolated toluate-utilising Pseudomonas strains, we examined a Tol derivative of each strain for the presence of plasmid DNA. Some of the strains (MT 16, MT 17, MT 18 and MT 21) carried plasmids which were very closely related to the TOL plasmid of P.putida mt-2, and among this group strains MT 16-B1 and MT 21-B1, which are Tol- segregants of strains MT 16 and MT 21 respectively, were found to be plasmid-free. However, Tol- derivatives of strains P.putida mt-2, MT 17 and MT 18 still carried plasmid DNA. A study of the molecular basis of curing in these strains is described in this Chapter, and has been published (Bayley et al, 1977).

5.2 Isolation of Tol- derivatives

In cells carrying a TOL plasmid, degradation of the toluates and benzoate proceeds via meta cleavage of catechol. In Tol- clones when growth is on benzoate, catechol is metabolised through the (chromosomally-encoded) ortho pathway (Section 1.4). Although both pathways are
present in Tol+ strains the meta pathway predominates since it is substrate-induced whereas the ortho pathway is product-induced. Spontaneously "cured" cells have a selective advantage when grown on benzoate since cells utilising the ortho pathway grow faster than those utilising the meta pathway, and consequently Tol− clones overgrow Tol+ clones and can readily be isolated (Williams and Murray, 1974).

5.3 Characterisation of plasmid DNA from Tol− derivatives

Plasmid DNA isolated from strain PaW 8, a previously described "cured" derivative of P.putida mt-2 derived by mitomycin C treatment (Williams and Murray, 1974), and from a benzoate "cured" Tol− derivative of P.putida mt-2, PaW 80, was examined by electron microscopy. Each plasmid DNA preparation contained a single size class of circular molecules of 48.6 Md and 48.0 Md respectively (Table 5.1). Since the parental plasmid had a size of 78 Md (Chapter 4) it was possible that loss of the Tol function may have occurred by excision of a specific region of the TOL plasmid.

To test this hypothesis ten Tol− clones were isolated independently from P.putida mt-2 by Worsey and Williams (personal communication). Of these, six contained plasmid DNA (Table 5.1). C.J. Duggleby submitted this plasmid DNA and plasmid DNA isolated from strains PaW 8, PaW 80, MT 17-B1 and MT 18-B1 to electrophoresis on agarose gels after digestion with the endonuclease EcoRI. The patterns were indistinguishable; therefore at this level of
Table 5.1  Independent Tol\textsuperscript{−} derivatives of strains containing TOL plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent</th>
<th>Mode of curing</th>
<th>Plasmid</th>
<th>Plasmid sizes with standard errors</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaW 1</td>
<td>-</td>
<td></td>
<td>pWWO</td>
<td>78.1 ± 1.1</td>
</tr>
<tr>
<td>PaW 8</td>
<td>PaW 1</td>
<td>mitomycin C</td>
<td>pWW0-1</td>
<td>48.6 ± 1.0</td>
</tr>
<tr>
<td>MT 16-B1</td>
<td>MT 16</td>
<td>benzoate</td>
<td>no plasmid</td>
<td>-</td>
</tr>
<tr>
<td>MT 17-B1</td>
<td>MT 17</td>
<td>benzoate</td>
<td>pWW17-1</td>
<td>ND</td>
</tr>
<tr>
<td>MT 18-B1</td>
<td>MT 18</td>
<td>benzoate</td>
<td>pWW18-1</td>
<td>ND</td>
</tr>
<tr>
<td>MT 21-B1</td>
<td>MT 21</td>
<td>benzoate</td>
<td>no plasmid</td>
<td>-</td>
</tr>
<tr>
<td>PaW 80</td>
<td>PaW 1</td>
<td>benzoate</td>
<td>pWW0-2</td>
<td>48.0 ± 1.1</td>
</tr>
<tr>
<td>Paw 81,83,84,87,88,89</td>
<td>PaW 1</td>
<td>benzoate</td>
<td>pWW0-81,83,84,87,88,89</td>
<td>ND</td>
</tr>
<tr>
<td>PaW 82,85,86,90</td>
<td>PaW 1</td>
<td>benzoate</td>
<td>no plasmid</td>
<td>-</td>
</tr>
</tbody>
</table>

ND = No data.
resolution the plasmids had all lost the same region(s) (Fig. 5.1). Comparison of the densitometry traces of the gel patterns given by the deleted plasmid and the parental TOL plasmid shows that nine fragments have been lost (Fig. 5.2). No new bands were observed. The apparent absence of new bands suggests that the deletion is of a single segment with ends less than about 0.2 Md from EcoRI cleavage sites. Alternatively, it is possible that new fragments were generated with mobilities which happen to be coincident with those of fragments lost from the parent strain.

Comparison of the mobilities of the nine fragments with the mobilities of fragments of known size derived from EcoRI digestion of phage λ indicate that the sum of the molecular weights of the fragments was about 27 Md. This calculated loss of DNA corresponds adequately with that inferred by electron microscopy of two representative shortened plasmids (Table 5.1). Therefore we conclude that there are specific regions for the excision of a segment that codes for at least part of the TOL pathway.
Fig. 5.1  Agarose gel electrophoresis of EcoRI digests of plasmid DNA from PaW 1 and ten Tol^- derivatives of this strain.
Fig. 5.2  Comparison of densitometry traces of electrophoresis patterns of EcoRI digested plasmid DNA from strains PaW 1 (upper trace) and PaW 8 (lower trace). The arrows indicate the positions of the nine fragments present in the digest of plasmid pWW0 but not of plasmid pWW0-1.
CHAPTER 6

Effect of growth substrate on the molecular structure of the plasmid carried by strain MT 14

6.1 Introduction

In Chapter 4 the isolation and characterisation of TOL plasmid DNA from thirteen independently isolated strains of *Pseudomonas* is described. When this plasmid DNA was examined by electron microscopy, the preparations from most of the strains were found to contain only a single size class of circular molecules. However, circular molecules of plasmid DNA from strain MT 14 fell into a number of different size classes ranging from 25 Md to 202 Md (Table 4.2). Each size class could represent an unrelated plasmid species. However, the number of circular molecules in the 202 Md size class greatly exceeds the number in any other size class. Since large molecules are very fragile, in the true size distribution of plasmid molecules within the cell an even higher proportion would be of the 202 Md size class. Therefore, if the plasmids of the various size classes are unrelated to each other, and if each cell carries every plasmid, the copy number per cell of the 202 Md plasmid species would be large. This seems unlikely for a plasmid of this size.

R plasmids have been shown by both genetic (Watanabe, 1963; Watanabe, 1967) and physical (Nisioka et al, 1969; Cohen and Miller, 1970; Rownd et al, 1971) criteria to consist of two distinguishable components: the resistance
transfer factor (RTF) which is involved in the infectious transfer of the R plasmid, and the resistance determinant component which carries most of the drug resistance genes.

Perlman and Rownd (1975) have found that when Proteus mirabilis carrying the R plasmid NR1 was cultured for many generations in drug-free medium, the plasmid was present as a single size class of molecule which consisted of the RTF and one copy of the r-determinant. However, when the strain was cultured in medium containing chloramphenicol there was an increase in the proportion of plasmid DNA relative to the chromosome, the NR1 DNA was much higher in molecular weight and it was heterogeneous in size. These changes were due to the formation of poly-r-determinant R plasmids and autonomous poly-r-determinants. Cells carrying these types of molecules have a selective advantage over cells carrying monomeric plasmids, since they have several copies of the drug-destructive genes and can therefore inactivate the drug more efficiently. This "transition" was reversed when the cells were once again grown in drug free medium.

Since strain MT 14 was grown on m-toluate medium prior to plasmid isolation, it is possible that a range of size classes of plasmid molecules was observed because growth on m-toluate resulted in a transition type of effect. The experiments described in this chapter were designed to test this hypothesis, and some of the results have been published (Broda et al, 1978).
6.2 Size of plasmid DNA carried by strain MT 14 after growth for 80 generations on L-broth

To determine whether the heterogeneity of the plasmid preparation in strain MT 14 could be due to the formation of molecules carrying several copies of all or part(s) of the plasmid as a response to growth on m-toluate, a previously m-toluate grown culture was serially subcultured in L-broth. The m-toluate grown stationary phase culture was diluted $10^4$ fold into L-broth. After the culture had reached a cell density of approximately $1 \times 10^9$ cells/ml, the $10^4$ fold dilution was repeated. Each subculturing represents about 10 generations, and in total the cells were grown for 80 generations on L-broth. After each dilution single colonies were isolated and their phenotypes determined. Besides the Tol function, the plasmid carried by strain MT 14 carries genes for phenylacetic acid utilisation and mercury resistance. None of these markers were lost during the subculturing.

Plasmid DNA was isolated from 500 ml of cells which had been grown on L-broth for 80 generations using the alkaline denaturation/nitrocellulose method. The plasmid preparation was examined by electron microscopy. In the absence of an internal standard only one size class of circular molecules was observed. Based on the measurement of 12 molecules, and using ColE1 as the internal standard, the size of the plasmid species was 48 Md (Table 6.1). An electron micrograph of a representative molecule of this plasmid species is presented in Fig. 6.1. This result supports the hypothesis that when strain MT 14 is grown
Fig. 6.1  Electron micrograph of a representative molecule of the plasmid species carried by strain MT 14 after serial subculture on L-broth. This plasmid species has a size of 48 Md. A molecule of the internal standard, ColE1, which has a size of 4.2 Md, is also included.
Table 6.1  Effect of growth substrate on the size distribution of circular plasmid molecules from strain MT 14

<table>
<thead>
<tr>
<th>Size Class (Md)</th>
<th>Distribution of circular molecules into size classes in plasmid preparations from:</th>
<th>Preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) m-toluate</td>
<td>(2) cells grown for 80 generations on L-broth</td>
<td>(1) m-toluate</td>
</tr>
<tr>
<td>(2) cells grown cells</td>
<td>(3) cells of a single colony from (2) grown for 10 generations on L-broth</td>
<td>(2) m-toluate</td>
</tr>
<tr>
<td>(3) cells of the same single colony from (2) grown for 80 generations on m-toluate</td>
<td>(3) cells of the same single colony from (2) grown for 80 generations on m-toluate</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Size (Nd)</th>
<th>Preparation (1)</th>
<th>Preparation (2)</th>
<th>Preparation (3)</th>
<th>Preparation (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td></td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>8</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td></td>
<td>12</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>60</td>
<td>3</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>68</td>
<td>3</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>76</td>
<td>1</td>
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<td></td>
<td>3</td>
</tr>
<tr>
<td>104</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>202</td>
<td>20</td>
<td></td>
<td></td>
<td>20</td>
</tr>
</tbody>
</table>

Preparation (1) is the original MT 14 plasmid DNA preparation (see Section 6.1).
Preparation (2) is described fully in Section 6.2.
Preparations (3) and (4) are described fully in Section 6.3.
on m-toluate, the plasmid it carries undergoes a reversible "transition".

6.3 Size of plasmid DNA after growth on m-toluate of cells of strain MT 14 carrying only the 48 Md size class

The change in size of plasmid DNA from strain MT 14 which is described in Section 6.2 would, according to the model presented, correspond to the formation of plasmid molecules containing only one copy of each of the regions of the plasmid. An alternative explanation is that regions of the plasmid which code for unknown phenotypes were lost in a similar manner to the loss of the Tol function from the plasmid carried by _P.putida_ mt-2 (Chapter 5). The changes would only be reversible if the former model is correct.

A single clone was isolated from the culture of strain MT 14 which had been grown for 80 generations on L-broth (Section 6.2). Cells from this clone were diluted into 500 ml of L-broth and grown to early stationary phase. Plasmid DNA was isolated from this culture and examined by electron microscopy. Of twelve molecules measured, eleven were of the 48 Md size class and one was 39 Md. The smaller plasmid may represent a molecule of the 48 Md size class from which a specific region of DNA has been lost (Table 6.1).

Cells from the same clone were also diluted into m-toluate medium and grown to a cell density of approximately $1 \times 10^9$ cells/ml. This culture was diluted $10^4$ fold in fresh m-toluate medium. In this manner cells
were cultured for 80 generations on m-toluate. At each dilution step single colonies were isolated and tested for mercury resistance and the ability to utilise phenylacetic acid. After growth for 80 generations on m-toluate, all the colonies that were tested carried these markers. Plasmid DNA was isolated from 500 ml of this culture and examined by electron microscopy. A number of different size classes ranging from 25 Md to 200 Md were observed. As in the determination of the size distribution of circular molecules in the original plasmid preparation from strain MT 14 (Chapter 3 and Section 6.1), all circular molecules observed on a given grid were measured. There is a striking similarity in the size distributions of circular plasmid molecules in the two preparations (Table 6.1). However, several plasmid sizes were observed in this preparation that were not represented in the size distribution of the original MT 14 plasmid DNA preparation. An electron micrograph of a molecule of the 200 Md size class is presented in Fig. 6.2.

These experiments are consistent with the hypothesis that when strain MT 14 is grown on non-selective medium it carries only a single size class of plasmid, but when the strain is grown on m-toluate medium, all or part of the plasmid is duplicated to give a range of size classes of circular molecules, some of which are very large. This change is reversed when the cells are once more grown on non-selective medium.
Fig. 6.2  Electron micrograph of a representative molecule of the plasmid species carried by strain MT 14 after serial subculture of a Tol+ clone from a previously broth-grown culture on m-toluate. The size of this plasmid species is 200 Md. Molecules of ColE1, which has a size of 4.2 Md, are also present.
6.4 Gel electrophoresis of endonuclease digest of plasmid DNA from strain MT 14

The evidence presented in Sections 6.2 and 6.3 strongly suggests that after growth on L-broth strain MT 14 carries a 48 Md plasmid which probably consists of a single copy of each of the genes, but when the strain is grown on m-toluate, region(s) of the plasmid are duplicated to give plasmids with a range of sizes. However, these data gives no indication as to how much of the plasmid is duplicated. Comparison of the densitometry traces of the gel patterns of endonuclease digestion fragments given by the plasmid in its two states, should yield information on this point. If the change involves the formation of dimers and trimers etc., then the fragments would be present in the same proportions as in the plasmid from the broth grown cells. However, if only specific regions of the plasmid were duplicated, then the endonuclease fragments from these regions would be present in a higher proportion in the plasmid from the m-toluate grown cells.

Plasmid DNA from each of the preparations described in Sections 6.2 and 6.3 was digested with the endonuclease EcoRI. After electrophoresis no DNA was observed in the gel; since the preparations were quite old it was probable that the DNA had already been degraded. Samples of each of the cultures from which this plasmid DNA had been isolated were stored in the deep freeze. Consequently, all subsequent experiments were carried out using plasmid DNA from cells in the same state as those used in the
electro-microscopy experiments. Therefore it is possible to compare directly the results from different experiments. Cells from the deep frozen stock were grown to early stationary phase on the appropriate medium. Plasmid DNA was isolated from these cultures. Again on electrophoresis of EcoRI digests on agarose gels no DNA could be visualised. Since the plasmid bands in CsCl-ethidium bromide density gradients were very faint even on ultra violet illumination, and the concentration of DNA appeared to be very low on examination by electron microscopy, it was possible that the plasmid DNA yield from strain MT 14 was too low for endonuclease analysis. These experiments were carried out on plasmid DNA isolated from 500 ml cultures. In an attempt to increase the yield plasmid DNA was isolated from 3 litres of each culture. Initially, the plasmid DNA was banded in six CsCl-ethidium bromide density gradients. The material from the plasmid bands was pooled and recentrifuged in an attempt to concentrate the DNA. However on ultraviolet illumination of the second density gradient, no plasmid band was observed. This may be because the DNA was nicked either by the ultra-violet light, or by passage through the syringe needle. Therefore, in subsequent experiments the plasmid DNA was dried down in vacuo over concentrated H₂SO₄ until the required concentration was reached.

Agarose gel electrophoresis of the EcoRI digests of this plasmid DNA gave no bands, but a smear of very small fragments of DNA was observed at the bottom of the gel. In repeated attempts, the endonuclease digests of several
preparations of this plasmid DNA gave only this characteristic smear on agarose gel electrophoresis. To ensure that no exonucleases were present, the preparations were extracted with phenol immediately after isolation. The plasmid DNA was incubated with different preparations of reaction mixture and digested with the endonucleases Xho and Sal as well as with different preparations of EcoRI. In almost every case, on agarose gel electrophoresis only the smear of small fragments was observed, although parallel digestion of plasmid DNA from strain P. putida mt-2 gave good banding patterns. One preparation of plasmid DNA was halved; one half was incubated with EcoRI and the reaction mixture, the other was incubated with reaction mixture alone. On agarose gels, when EcoRI was present in the incubation mixture, only the characteristic smear was observed; however, when the plasmid DNA was only incubated with reaction mixture, discrete bands of plasmid DNA were observed. Since parallel digestions with other plasmid species gave good banding patterns, it seems improbable that a good banding pattern of endonuclease-generated fragments of plasmid DNA from strain MT 14 could be obtained. The reasons for this are not clear.

However, in one experiment faint banding patterns were obtained of EcoRI digestion fragments of plasmid DNA which had been isolated from both broth-grown cells and m-toluate-grown cells. The reasons for the success of this attempt are not known. The patterns, which are presented in Fig. 6.3, are of poor quality, but some
Fig. 6.3  Agarose gel electrophoresis of EcoRI digests of plasmid DNA from: A, cells of strain MT 14 after serial subculture on L-broth; B, cells of strain MT 14 after serial subculture of a Tol$^+$ clone from a previously broth-grown culture on m-toluate. Also included is a $\lambda$ digest for calibration of the sizes of the fragments.
information can be extracted from them. The same four fragments predominate in each pattern and there is a background of many very faint bands. However, in the pattern given by plasmid DNA from broth-grown cells the predominating bands appear to be more intense with respect to the background than they are in the pattern given by plasmid DNA from cells grown on m-toluolate. Comparison of the mobilities of the four predominating fragments, with those of fragments of known size derived from EcoRI digestion of phage λ DNA, indicated that the sum of the sizes of the fragments was about 47 Md. These fragments are present in the same proportions in both plasmid preparations.

6.5 Discussion

The electron microscopy data support the hypothesis that when strain MT 14 is grown on m-toluolate, all or part of the plasmid is duplicated to give rise to a range of size classes of plasmid molecules. However, comparison of the gel patterns of EcoRI fragments given by the plasmid in its monomeric and complex forms suggests that the molecular basis of the change is not analogous to the transitioning of R plasmid NR1 during growth on medium containing chloramphenicol (Perlman and Rownd, 1975). In this model only a specific region of the plasmid is duplicated, so that in the pattern given by transitioned DNA the digestion fragments from the duplicated region would be present in more copies than the other fragments. In the gel pattern given by plasmid DNA from m-toluolate
grown cells of strain MT 14, the four predominating fragments are present in the same number of copies. This result suggests that during growth on m-toluate dimers and trimers etc., are found.

The electron microscopy data also support this hypothesis. According to the transition model of Perlman and Rownd (1975) the plasmid molecules would be distributed into a range of sizes at regular intervals from the unit size upwards, and no size class would predominate. In the size distribution of duplicated plasmid DNA from strain MT 14, the 200 Md size class predominates (Table 6.1). Since large molecules are very fragile, within the cell there is probably an even higher proportion of molecules in this size class. This species could be a tetramer, and the 100 Md plasmid a dimer, of the 48 Md plasmid. It is possible that there are much larger molecules than the 200 Md species which contain many copies of the plasmid, but these would be too fragile to be seen by electron microscopy.

The intermediate size classes could result from the excision and insertion of specific regions of DNA in these plasmid species in a similar manner to the loss of the Tol function from P. putida mt-2 (Chapter 5). There are interesting relationships between the sizes of these plasmids; for instance, for each plasmid species which is larger than monomeric size, there is a corresponding type of molecule which is less than 50 Md by about the same amount. There would be different digestion fragments in these molecules which would be represented in the gel
pattern as faint background bands. The remainder of the background bands could result from the formation of multimers. If fusion could occur between different sites on the plasmid, new fragments would result.

Background bands would not be expected in the gel pattern given by plasmid DNA of the 48 Md size class. However, this experiment was carried out using a bulk culture after growth for 80 generations on L-broth, rather than on cells derived from a single colony. In this pattern the background bands are less intense with respect to the predominating bands than they are in the pattern given by plasmid DNA from m-toluate grown cells. Therefore, it is possible that the background is due to the presence of some residual multimers even after 80 generations on L-broth. Also, since on examination by electron microscopy a molecule of 39 Md was observed in the plasmid DNA prepared from an L-broth grown single colony, excision and insertion of specific regions of DNA appears to take place in the monomer.

The evidence presented in this Chapter supports the hypothesis that when strain MT 14 is grown on m-toluate multimers of the plasmid it carries are formed.

Circular molecules of plasmid DNA from strain MT 20 could not be visualised by electron microscopy (Section 4.3). It was possible that when the strain was grown on m-toluate, the plasmid it carried formed multimers which were too large to be examined by this technique. To test this hypothesis cells of strain MT 20 were subcultured for 80 generations on L-broth using the method described
in Section 6.2. Plasmid DNA was isolated from these cells and examined by electron microscopy. No circular molecules were observed. Therefore if the plasmid carried by strain MT 20 is very large, its size appears to be unaffected by growth substrate.
7.1 Introduction

One of the criteria used in the classification of plasmids is their compatibility relationships (Sections 1.7 and 1.8). There are at least ten incompatibility (Inc) groups in Pseudomonas (Jacoby, 1977; Sagai et al, 1976). The plasmids of a particular incompatibility group often have common properties including the range of drug resistances, plasmid size, transfer proficiency and resistance or susceptibility of the plasmid host to specific bacteriophages.

The molecular relationships between members of a single incompatibility group, and between plasmids of different groups, have been determined in relatively few cases. Plasmids can be compared using a number of molecular techniques. A great deal of information on sequence relationships can be acquired from heteroduplexes (Sharp et al, 1973); however the molecular characterisation of Pseudomonas plasmids is very limited, and derivatives of these plasmids with mapped insertions and deletions for reference points are not available. Interpretation of the data would therefore be very complex. Heteroduplex analysis is most useful for detailed study of the regions of homology between a small number of plasmids. However, the technique is too time consuming to be suitable for studies in which many plasmids are compared. As discussed in Chapter 4, relatedness between plasmids can also be assessed by comparing the fragmentation pattern on agarose gels of endonuclease
digests of plasmid DNA. Related plasmids give similar patterns of fragments (Thompson et al, 1974; Duggleby et al, 1977). However, if unrelated fragments have similar mobilities, the results are ambiguous. This technique is most useful in determining whether related plasmids are identical. The degree of homology between two plasmids can also be determined by DNA-DNA hybridisation (Guerry and Falkow, 1971), however this gives no indication of the region of the plasmid in which there is homology. This technique is much simpler than the others and is suitable for looking at the general relationships between many plasmids.

In this chapter the use of in situ hybridisation of endonuclease-generated fragments of plasmids, both of the same, and of different incompatibility groups, is described. This technique involves the hybridisation of single stranded radioactively labelled DNA probe to an endonuclease-generated fragmentation pattern bound to a nitrocellulose filter (Heinaru et al, 1978). This technique combines the advantages of hybridisation experiments with those of endonuclease digest patterns.

7.2 Preparation of bacterial strains

The plasmids which were used are described in Table 7.1. In most cases the original host of the plasmids was P.aeruginosa. However, NAH and TOL originated in P.putida, and R527 was originally isolated in Serratia marcescens although it can be transferred readily to P.aeruginosa. The R plasmids were provided by G.A. Jacoby in strain PU21 which is an Ilv\(^-\) Leu\(^-\) Str\(^+\) Rif\(^+\) F\(^-\) derivative of the PAO
Table 7.1  Characteristics of Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Incompatibility Group</th>
<th>Size (Md)</th>
<th>Country of origin</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP1</td>
<td>P-1</td>
<td>39</td>
<td>U.K.</td>
<td>Cb Km Tc</td>
</tr>
<tr>
<td>R527</td>
<td>P-1</td>
<td>50</td>
<td>Spain</td>
<td>Cb Cm Gm Km</td>
</tr>
<tr>
<td>R679</td>
<td>P-4</td>
<td>5.6</td>
<td>Canada</td>
<td>Sm Su</td>
</tr>
<tr>
<td>Rms148</td>
<td>P-7</td>
<td>95</td>
<td>Germany</td>
<td>Sm</td>
</tr>
<tr>
<td>FP2</td>
<td>P-8</td>
<td>59</td>
<td>Australia</td>
<td>Hg Pmr</td>
</tr>
<tr>
<td>R2</td>
<td>P-9</td>
<td>49</td>
<td>Japan</td>
<td>Cb Sm Su</td>
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<td>pMG18</td>
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<td>67</td>
<td>Japan</td>
<td>Cb Gm Km Sm</td>
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<td>Nah⁺</td>
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<tr>
<td>TOL</td>
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<td>78</td>
<td>Japan</td>
<td>Tol⁺</td>
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<tr>
<td>R91</td>
<td>P-10</td>
<td>35</td>
<td>U.K.</td>
<td>Cb</td>
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</tbody>
</table>

Abbreviations; Cb, carbenicillin resistance; Cm, chloramphenicol resistance; Gm, gentamicin resistance; Km, kanamycin resistance; Sm, streptomycin resistance; Su, sulphonamide resistance; Tc, tetracycline resistance; Hg, HgCl₂ resistance; Pmr, phenylmercuric acetate resistance; Tol, the ability to metabolise m-xylene, p-xylene, toluene, m-toluate, and p-toluate; Nah, the ability to metabolise naphthalene and salicylate.
line. However, this strain carries cryptic plasmids of molecular weight 1.7 to 9.5 Md (Pemberton and Clark, 1973), which would give bands of homology on hybridisation if both plasmids were isolated from this strain. Therefore, the plasmids were transferred to the plasmid-free strain of P.putida, ac 34. I am very grateful to Dr Jacoby for transferring the plasmids R679, FP2 and pMG18 into this strain. The R plasmid R91 would not go into ac 34, so it was transferred to the plasmid-free P.aeruginosa strain PAC. I transferred the remaining plasmids to strain ac 34 using the method described in Section 3.8.

7.3 Isolation of plasmid DNA

R plasmids can be readily isolated from P.aeruginosa by the method of Guerry et al (1973), in which the plasmid DNA in a conventional cleared lysate is concentrated by CsCl-ethidium bromide density gradient centrifugation. However, with the exceptions of the R plasmids RP1 and R2, repeated attempts to isolate these plasmids from strain ac 34 by this method were unsuccessful, although plasmid DNA could be isolated from these strains when the alkaline denaturation/nitrocellulose method was used. The similarity of this result to our observations on the isolation of degradative plasmid DNA (Section 4.2) suggests that the failure of clearing spin methods in the isolation of both types of plasmid from P.putida is at least partly due to the properties of the host strain.
7.4 Gel electrophoresis of endonuclease digests of plasmid DNA

Most of the plasmids were digested with endonuclease EcoRI. However, the R plasmid RP1 has only one cleavage site for many endonucleases, including EcoRI. Since much more information is obtained from this type of experiment if homology can be assigned to particular fragments of the plasmid, RP1 was digested with the endonuclease Pst I, which gives six fragments. R527 has four EcoRI cleavage sites. However, it is of the same incompatibility group (IncP-1) as RP1, and was included in the study so that the relationships between two different plasmids of this group could be determined. Consequently, R527 was digested with the endonuclease Pst I, so that if R527 and RP1 have any common regions these may give rise to common bands in the fragment patterns. R679 also has only one EcoRI cleavage site; however in this case digestion into more fragments was not carried out since it has a size of only 5.6 Md, and is therefore much smaller than many of the digestion fragments of the other plasmids.

Electrophoresis of endonuclease digests of plasmid DNA was carried out on vertical slab gels with sample slots which extended across the full thickness of the gel (Section 3.9). This ensured that after electrophoresis the fragments were distributed across the full thickness of the gel. This made the subsequent transfer of DNA to the nitrocellulose filter more efficient. The gel patterns of endonuclease digests of plasmid DNA are presented in Fig. 7.1 and analysed in Table 7.2.
Fig. 7.1  Agarose gel electrophoresis of fragments generated by endonuclease digestion of plasmid DNA. Plasmids RP1 and R527 were digested with PstI; the remaining plasmids were digested with EcoRI. An EcoRI digest of λ DNA is included to calibrate the sizes of the fragments.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Endonuclease</th>
<th>Number of bands apparent by fluorescence</th>
<th>Number of bands corresponding to fragments with a size of at least 1 Md</th>
<th>Total number of fragments apparent from densitometry traces</th>
<th>Plasmid size (the sum of the fragment sizes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP1</td>
<td>PstI</td>
<td>5</td>
<td>3</td>
<td>6</td>
<td>39</td>
</tr>
<tr>
<td>R527</td>
<td>PstI</td>
<td>8</td>
<td>5</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>R679</td>
<td>EcoRI</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>5.6</td>
</tr>
<tr>
<td>Rms148</td>
<td>EcoRI</td>
<td>13</td>
<td>13</td>
<td>18</td>
<td>95</td>
</tr>
<tr>
<td>R2</td>
<td>EcoRI</td>
<td>11</td>
<td>8</td>
<td>11</td>
<td>49</td>
</tr>
<tr>
<td>pMG18</td>
<td>EcoRI</td>
<td>14</td>
<td>11</td>
<td>15</td>
<td>67</td>
</tr>
<tr>
<td>NAH</td>
<td>EcoRI</td>
<td>20</td>
<td>12</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>TOL</td>
<td>EcoRI</td>
<td>23</td>
<td>17</td>
<td>31</td>
<td>78</td>
</tr>
<tr>
<td>R91</td>
<td>EcoRI</td>
<td>10</td>
<td>8</td>
<td>10</td>
<td>35</td>
</tr>
</tbody>
</table>
Table 7.2 (contd.)

(a) In this column the total number of bands that could be visualised by fluorescence is recorded.

(b) In the hybridisation experiments only homology in bands corresponding to fragments with a size of at least 1 Md was recorded (see Section 7.5). In this column the number of such bands in each pattern is given.

(c) The total number of fragments given by digestion of a plasmid species is often greater than the number of fluorescent bands, since some bands apparently represent two or more fragments of common mobility (see Section 7.4). In this column the total number of fragments is given.
The total number of fragments given by digestion of a plasmid species is usually greater than the number of fluorescent bands observed after electrophoresis, since some bands apparently represent a number of fragment species of common mobility. The total number of fragments was determined from densitometry traces of the gels, assuming that peaks corresponding to twice the unit amount of DNA represent coincident bands. The sum of the sizes of the fragments, which were determined by the comparison of the mobilities of the fragments with those of fragments of known size derived from EcoRI digestion of phage λ DNA, gave the plasmid size. The yield of FP2 DNA was too low for analysis of its banding pattern by densitometry. The value given for its molecular weight is that determined by Pemberton and Clark (1973).

7.5 *In situ* hybridisation of radioactively-labelled plasmid DNA probes against endonuclease-derived fragments of plasmid DNA

The hybridisation experiments were carried out by the method of Heinaru et al (1978), which is fully described in section 3.9. After electrophoresis, the endonuclease-generated fragments were denatured in the gel and then transferred to a nitrocellulose filter. Radioactively-labelled plasmid DNA with a high specific activity was prepared in *vitro* by 'nick translation'. The plasmid DNA probe was then hybridised to the fragments which were bound to the nitrocellulose filter. Bands in the pattern which represented fragments which had homology with the probe were visualised by autoradiography.
The relationships between plasmids of six different incompatibility groups were determined by this technique. Four IncP-9 and two IncP-1 plasmids were also included in the study so that plasmids of the same incompatibility group could be compared. Experiments were designed in which the relationships between every pair of plasmids were determined. One plasmid of each pair, as probe, was hybridised to the endonuclease-generated fragments of the other, which were bound to a nitrocellulose filter. With the exception of RP1, functions which were carried by the plasmids have not been assigned to specific fragments, and the order of the fragments within the plasmid has not been determined. Therefore, with the exception of the IncP-9 plasmids, in most cases the reciprocal hybridisations were not carried out. The plasmid yield of FP2 was very low and consequently it was not possible to compare it with all the other plasmids. Rms148 plasmid DNA banded very closely to the chromosomal DNA in CsCl-ethidium bromide density gradients, and on collection of the plasmid DNA it always became heavily contaminated with chromosomal DNA. When this DNA was used as probe there was considerable binding of DNA that was not due to homology between the plasmids. A high background was observed after autoradiography which obscured the bands of homology. Consequently, this plasmid has not been compared with certain of the other plasmids.

Radioactively-labelled probes were made from all the plasmid DNA preparations. These were hybridised against the endonuclease-generated fragmentation patterns of three
to nine plasmid species which were transferred from the same gel to a nitrocellulose filter. In all cases a digest of the DNA preparation from which the probe was made was included on the gel as a control. The results of the hybridisation were only considered valid if all bands in this control track showed homology with the probe. Hybridisation to fragments that were smaller than 1 Md was very inefficient, and in some experiments such fragments did not hybridise in the controls. Therefore, since a negative result for these fragments was ambiguous, hybridisation was only recorded in fragments that were larger than 1 Md (Table 7.2). When a hybridising band represented several fragments, it was not possible to determine which of these species had homology with the probe. Therefore, the number of bands, rather than the number of fragments which hybridised with the probe, was recorded.

7.6 Relationships between plasmids of the IncP-1 group

The relationships between two IncP-1 plasmids, RP1 and R527, were studied. The bands in the Pst I-generated fragmentation patterns of each of these plasmids were given numbers according to their mobilities, the band of lowest mobility being band 1, etc.

All five bands in the RP1 pattern showed some homology with the R527 probe (Fig. 7.2). Two of these bands represented fragments which were smaller than 1 Md and were not recorded in Tables 7.5 and 7.6 because, even when there was 100% homology, hybridisation to fragments of this size was not always successful. However, in this experiment,
Fig. 7.2  Autoradiograph of Pst I digests of plasmids RP1 and R527 after electrophoresis on an agarose gel, denaturation, transfer to a nitrocellulose filter and hybridisation with $^{32}$P-labelled R527 plasmid DNA probe.
the hybridisation of probe to all the RP1 bands emphasises the point that a large proportion of the RP1 sequences must be present in R527. Unfortunately, the percentage of RP1 that has homology with R527 cannot be determined by this technique, because although it shows which fragments have homology, it gives no indication of the proportion of the fragment that is homologous.

Jacoby et al (1976) have prepared recombinants between plasmids of incompatibility groups P-1 and P-2. Some of these have very similar properties to R527 and it has been suggested that R527 may be a natural IncP-1 : IncP-2 recombinant. It carries the same markers as RP1 and several additional resistance markers (Table 7.1). R527 is larger than RP1 (they have sizes of 50 Md and 39 Md respectively). Comparison of the Pst I-generated fragmentation patterns of the two plasmids shows that:

1) In the R527 pattern there are bands of the same mobility as all of the RP1 bands except one (band 3).

2) The two Pst I-generated fragments which comprise band 1 of RP1 have slightly different mobilities and can be distinguished by densitometry of gels (but not of autoradiographs). In contrast, the two fragments comprising band 1 of R527 are indistinguishable, so that one of the fragments that are represented by band 1 of RP1 does not have a size analogue in the pattern of R527.

3) There are four bands in the R527 pattern for which there are no bands with the same mobility in the RP1 pattern. Since two fragments from RP1 do not have size analogues in R527, R527 cannot have arisen simply by the insertion
into RP1 of a piece of DNA carrying the additional resistance markers; such an insertion would only have changed a single fragment. However, if the two fragments are adjacent to each other in RP1, it is possible that R527 consists of RP1 from which a piece of DNA has been deleted and a piece inserted in the same region.

7.7 Relationships between plasmids of the IncP-9 group

Hybridisation experiments were carried out between four plasmids of the IncP-9 group. The bands in each EcoRI-generated fragmentation pattern were given numbers following the procedure described in Section 7.6. Using this nomenclature, the bands in each pattern which had homology with the various probes are recorded in Table 7.4. Different periods of autoradiographic exposure (18 hours, 3 days, and 7 days) were used. The longer periods were necessary to reveal hybridisation with smaller fragments since less radioactively-labelled probe was bound to these.

The plasmids studied were R2, pMG18, NAH and TOL, which have molecular weights of 49, 67, 48 and 78 Md respectively. R2 and pMG18 are R plasmids; pMG18 carries the same resistance genes as R2 plus several additional ones (Table 7.1). NAH and TOL encode the degradation of naphthalene and toluene respectively, and both pathways involve the meta cleavage of catechol. These pairs of plasmids carry related functions, so that they might be expected to show some homology. However, by this criterion the R plasmids would not be expected to show homology with the degradative plasmids. The EcoRI-generated fragmentation
patterns (Fig. 7.1) of R2 and pMG18 have a significant number of fragments that correspond in size, but other than this the four plasmids yield quite different patterns.

The results of the hybridisation experiments show that there is a considerable amount of homology between the four plasmids (Tables 7.3 and 7.4). In each of the cases examined, most of the bands in the fragmentation pattern hybridised with each of the probes (Table 7.3), and in general the same bands in a particular pattern hybridised with the probes of the other plasmid species (Table 7.4).

The homology of the R2 fragments with the other probes can be summarised as follows:

1) All eight bands in the EcoRI-generated fragmentation pattern of R2 had some homology with both pMG18 and NAH.
2) All bands of R2 except that which represented the largest fragment had some homology with TOL.

However, often only parts of fragments would be homologous with the probe, and this must be the case where NAH showed homology with all the fragments of R2, since R2 carries drug resistance genes which are not carried by NAH. Therefore, generally, the sum of the sizes of the fragments which show some homology will be considerably higher than the overall homology between the plasmids but will indicate the maximum amount of homology that may exist.

Hybridisation of the various probes to the EcoRI-generated fragmentation pattern of pMG18 showed that:

1) Nine of the eleven pMG18 bands had some homology with NAH.
2) The same bands, except for the largest, also had some homology with TOL.
Table 7.3  Number of bands in the EcoRI-generated fragmentation patterns of IncP-9 plasmids which show homology with IncP-9 plasmid probes

<table>
<thead>
<tr>
<th>Plasmid DNA probe</th>
<th>Number of bands hybridising with probe in the EcoRI generated fragmentation patterns of plasmids:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R2</td>
</tr>
<tr>
<td>R2</td>
<td>(8)</td>
</tr>
<tr>
<td>pMG18</td>
<td>8</td>
</tr>
<tr>
<td>NAH</td>
<td>8</td>
</tr>
<tr>
<td>TOL</td>
<td>7</td>
</tr>
</tbody>
</table>

- No data.

( ) Numbers in brackets are the controls of probes hybridised with their own fragmentation patterns and represent the total number of bands corresponding to fragments with a size of more than 1 Md.

Hybridisation was only recorded for bands which represented fragments with a size of more than 1 Md (see Section 7.5).
Table 7.4  Table showing which bands in the IncP-9 plasmid DNA fragmentation patterns 
hybridise with the various IncP-9 plasmid probes

<table>
<thead>
<tr>
<th>Plasmid DNA probe</th>
<th>Band numbers of bands hybridising with probe in the fragmentation patterns of plasmids:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>R2</td>
<td>1, 2, 3, 4, 5, 6, 7, 8.</td>
<td></td>
</tr>
<tr>
<td>pMG18</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11.</td>
<td></td>
</tr>
<tr>
<td>NAH</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15.</td>
<td></td>
</tr>
<tr>
<td>TOL</td>
<td>2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17.</td>
<td></td>
</tr>
</tbody>
</table>

- No data.

The bands in each pattern were given numbers according to their mobilities. The band of lowest mobility was band 1, etc.
It is interesting that the same bands of pMG18 should have homology with both degradative plasmids even though the three plasmids carry different functions.

Seven of the eight R2 bands have size analogues in the pMG18 fragmentation pattern, and these showed the same homologies with NAH and TOL probes. This is shown clearly in the autoradiograph presented in Fig. 7.3. NAH probe hybridised to the seven bands of common mobility in both patterns, and in addition hybridised to one other band in the R2 pattern and two more bands in the pMG18 pattern. This suggests that pMG18 may have resulted from the insertion into R2 of a piece of DNA carrying the additional resistance markers, since an insertion event would involve one fragment being lost and two new ones formed. According to this model:-

1) The fragments represented by bands 1, 2, 4, 5, 6, 7 and 8 of R2 would be the same as those represented by bands 1, 3, 5, 6, 8, 10 and 11 of pMG18 respectively.

2) The insertion of the extra piece of DNA into fragment 3 of R2 would then result in the formation of fragments 2 and 4 of pMG18, which would consist partly of R2 DNA and partly of inserted DNA.

3) The other two bands in the pMG18 fragmentation pattern would consist totally of inserted DNA. This would be consistent with the observation that these fragments did not show homology with any of the IncP-9 plasmid probes. The results of the remaining hybridisations can be summarised as follows:-

1) Nine of the twelve NAH bands showed homology with pMG18 and eight showed homology with TOL. Of these, seven showed homology with both probes.
Fig. 7.3 Autoradiograph of EcoRI digests of IncP-9 plasmids after electrophoresis on an agarose gel, denaturation, transfer to a nitrocellulose filter and hybridisation with $^{32}$P-labelled NAH plasmid DNA probe.
2) Of the 17 TOL bands, the same nine showed homology with both pMG18 and NAH, two more also showed homology with pMG18 and one more with NAH.

When the excision of a specific region of the TOL plasmid results in the loss of the Tol function (Chapter 5) nine EcoRI fragments are lost. Two of these fragments share bands with fragments that are not lost so their individual hybridisations cannot be determined, and three fragments are smaller than 1 Md so hybridisation with these cannot be detected reliably. Of the remaining four fragments two band in the same position so that three bands that represent fragments which are larger than 1 Md are lost from the fragmentation pattern after the excision event. These are bands 1, 4 and 13. None of these bands had homology with pMG18 which indicates, as expected, that pMG18 does not have homology with the probable degradative region of TOL. Of these three bands, only band 4 had homology with NAH; this could represent genes which are involved in the meta cleavage of catechol which is common to both pathways.

These experiments show that within the IncP-9 group, in all of the cases examined, R plasmids are related to degradative plasmids. This result is very interesting since the two types of plasmids encode unrelated functions, and originate from bacteria which were isolated from different habitats. However, although there is considerable homology between the IncP-9 plasmids there cannot be any very long continuous stretches of complete homology, since there are relatively few fragments of the same mobility
in different patterns (with the exception of those in the patterns of R2 and pMG18); therefore most of the fragments are unique. Since the same bands in a particular fragmentation pattern often hybridised with all the IncP-9 probes, it is possible that some sequences are common to all four plasmids. Three of these plasmids originated from Japan (Table 7.1); however NAH originated in the UK, and four strains of *Pseudomonas* carrying plasmids which were indistinguishable from this TOL plasmid were isolated in the UK (Chapter 4). Therefore, these common sequences are not restricted to a small geographical location.

7.8 **Relationships between plasmids of different incompatibility groups**

To determine whether there are similarities between plasmids of different incompatibility groups, nine plasmids from six different groups were compared. Their characteristics are described in Table 7.1. RP1 and R527 were digested with *Pst I* and the other plasmids were digested with *EcoRI*. The *EcoRI* digests gave banding patterns on agarose gels which were dissimilar (Fig. 7.1), although some digests contained a few fragments which corresponded in size to fragments in other digests. The results of these experiments were recorded in a similar manner to those of hybridisations between IncP-9 plasmids (see Tables 7.5 and 7.6).

There is much less homology between plasmids of different incompatibility groups than there is between plasmids of the same group. Generally, if two plasmids from different incompatibility groups had at least one function in common
Table 7.5  Number of bands in plasmid DNA fragmentation patterns which show homology with plasmid probes of different incompatibility groups

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Number of bands hybridising with probe in the endonuclease generated DNA fragmentation patterns of plasmids:-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasmid</td>
</tr>
<tr>
<td></td>
<td>RP1</td>
</tr>
<tr>
<td></td>
<td>IncP-1</td>
</tr>
<tr>
<td>RP1</td>
<td>(3)</td>
</tr>
<tr>
<td>R527</td>
<td>3</td>
</tr>
<tr>
<td>R679</td>
<td>0</td>
</tr>
<tr>
<td>FP2</td>
<td>0</td>
</tr>
<tr>
<td>R2</td>
<td>2</td>
</tr>
<tr>
<td>pMG18</td>
<td>2</td>
</tr>
<tr>
<td>NAH</td>
<td>2</td>
</tr>
<tr>
<td>TOL</td>
<td>0</td>
</tr>
<tr>
<td>R91</td>
<td>2</td>
</tr>
</tbody>
</table>

- No data.
0 No homology.
() Numbers in brackets are the controls of probes hybridised with their own fragmentation patterns and represent the total number of bands corresponding to fragments with a size of more than 1 Md.

Hybridisation was only recorded for bands which represented fragments with a size of more than 1 Md.
Table 7.6  Table showing which bands in plasmid DNA fragmentation patterns hybridise with plasmid probes of different incompatibility groups

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Band numbers of bands hybridising with probe in the fragmentation patterns of plasmids:</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>RP1</td>
</tr>
<tr>
<td>probe</td>
<td>IncP-1</td>
</tr>
<tr>
<td>RP1</td>
<td>+</td>
</tr>
<tr>
<td>R527</td>
<td>1,2,3.</td>
</tr>
<tr>
<td>R679</td>
<td>0</td>
</tr>
<tr>
<td>FP2</td>
<td>0</td>
</tr>
<tr>
<td>R2</td>
<td>1,3.</td>
</tr>
<tr>
<td>pMG18</td>
<td>1,3.</td>
</tr>
<tr>
<td>NAH</td>
<td>1,2.</td>
</tr>
<tr>
<td>TOL</td>
<td>0</td>
</tr>
<tr>
<td>R91</td>
<td>1,3.</td>
</tr>
</tbody>
</table>

- No data.
0 No homology.
+ All bands show homology when the same plasmid species was used for the preparation of both fragmentation pattern and probe.

The bands in each pattern were given numbers according to their mobilities. The band of lowest mobility was band 1, etc. For simplicity, the results of the hybridisations between pairs of IncP-9 plasmids are omitted from this table, but are presented in Table 7.4.
they showed some homology, whereas if their known functions were unrelated no homology was observed. Twenty eight pairs of plasmids from different incompatibility groups were compared. Eleven pairs had no homology and, of these, ten had no common function (Tables 7.5 and 7.1). The exception was R527 with R679, which both encode resistance to streptomycin and sulphonamide. Therefore these plasmids must carry unrelated genes which encode the same functions. With the exception of R679, all the plasmids studied are transfer proficient and the pairs of plasmids which have no homology must have unrelated transfer and replication functions. The only resistance marker carried by FP2 is resistance to mercury, therefore it does not have any markers in common with the plasmids to which it was hybridised. Of the plasmids tested, FP2 only showed homology with R91; therefore with this exception, FP2 is quite different to other Pseudomonas plasmids.

The other seventeen pairs of plasmids showed some homology, but in most cases the homology was only in one or two bands and was located in the largest three fragments (Table 7.6). Although there is variation between different digests, between 40% and 98% of the plasmid is present in the three largest fragments so that this result is not surprising. Most pairs carry common functions, but six do not. These are: NAH, RP1; NAH, R527; NAH, Rms148; R91, FP2; R91, R679 and TOL, Rms148. There was only a slight amount of homology between the last two pairs. This is illustrated in Fig. 7.4, which shows the results of the hybridisation with R91 probe. This autoradiograph had been
Fig. 7.4  Autoradiograph of endonuclease digests of plasmids from different incompatibility groups after electrophoresis on agarose gels, denaturation, transfer to nitrocellulose filters and hybridisation with $^{32}$P-labelled R91 plasmid DNA probe. Plasmid RP1 was digested with Pst I; the other plasmids were digested with EcoRI.
exposed for three days but the homology between R91 and R679 is barely detectable although hybridisation of R91 probe to the other plasmids is quite clear. Longer exposure showed clearly that hybridisation between R91 and R679 was genuine. Those plasmids which show homology but do not carry common functions may have related transfer or replication functions, or the homology may lie in sequences of DNA that encode unknown functions.

The results suggest that there may be relationships between plasmids of the IncP-1, IncP-7 and IncP-9 groups. For instance:

1) In four of the cases where homology was shown between plasmids that did not carry a common function an IncP-9 plasmid was paired with an IncP-1 plasmid or an IncP-7 plasmid.

2) Band 3 of Rms148 showed some homology with all IncP-9 plasmid probes that were tested.

3) TOL was the only IncP-9 plasmid that had no homology with the IncP-1 plasmids.

4) All the IncP-1 and IncP-9 plasmids, except TOL, had homology with band 1 of R2. It is possible that at least part of the sequence carried by fragment 1 of R2 is also carried by the IncP-1 plasmids and the IncP-9 plasmids other than TOL. Since RP1 only showed homology with this R2 band, no other sequence can be common to these plasmids.

The Transposon 1 (Tn1) element of RP1, which carries the carbenicillin gene (Hedges and Jacob, 1974), contains three Pst I sites, and digestion gives internal fragments
of 1.85 and 0.45 Md (Grinsted et al, 1977). The latter was too small for reliable in situ hybridisation experiments, but the former which was represented by band 3 in the RP1 fragmentation pattern could be examined. All of the plasmids which encode carbenicillin resistance had some homology with this fragment, so that their carbenicillin genes must be related. These plasmids also had homology with band 1 of RP1; since Tn1 extends into one of the fragments which comprise band 1, this could represent homology in Tn1 or could also represent homology in other functions. The autoradiograph presented in Fig. 7.5 clearly shows the hybridisation of R2 probe to bands 1 and 3 of RP1.

All plasmids which carry carbenicillin resistance hybridised only with band 3 of R91, which suggests that the gene for carbenicillin resistance is located on the R91 fragment that is represented by this band. Similarly, all such plasmids hybridised with band 1 of R2. The hybridisation of RP1 probe to the R2 fragmentation pattern is shown in Fig. 7.5. RP1 only hybridised with band 1 of R2, so that the gene coding for carbenicillin resistance is possibly located on this fragment. R527 and R91 also hybridise with fragment 2 of R2. Besides carbenicillin resistance, R527 also has streptomycin and sulphonamide resistance in common with R2, and hybridisation with fragment 2 of R2 may represent homology in these genes. R2 and R91 both inhibit the fertility of FP2 (Jacoby, 1977) so homology between these plasmids may represent sequences involved in this characteristic.
Fig. 7.5  Autoradiograph of a Pst I digest of plasmid RP1 and EcoRI digests of plasmids R679 and R2 after electrophoresis on agarose gels, denaturation, transfer to nitrocellulose filters and hybridisation with $^{32}$P-labelled RP1 and R2 plasmid DNA probes.
7.9 Discussion

The results described in this Chapter show quite clearly that plasmids of the same incompatibility group are related to each other even if they encode entirely different functions. However, generally, plasmids of different incompatibility groups only show some homology with each other if they encode the same function(s). Homology is restricted to a few fragments and may be only in the genes which code for the common function, the remainder of the plasmid being unrelated. However, some IncP-9 plasmids show some homology with IncP-1 and IncP-7 plasmids even though they carry different functions. Therefore, in some cases plasmids of different incompatibility groups may have similarities in their replication or transfer genes, or in genes which code for functions which have not yet been assigned to the plasmid. Grindley et al (1973) have determined the proportion of homology between a number of plasmids from various E.coli incompatibility groups. In the general discussion the results presented in this thesis are compared with their work (Section 8.2).
CHAPTER 8
Discussion

8.1 Introduction

The individual experimental results that are presented in this thesis have already been discussed in the experimental sections. In this Chapter the results are discussed in a more general way and are compared with the work of other groups. The implications of this work on our understanding of the molecular structure and organisation of plasmids, and on the models suggested for plasmid evolution, are also discussed.

8.2 The molecular structure and relationships of TOL plasmids

The study of the degradative pathways of the saprophytic pseudomonads is important because of their vital role in the recycling of organic matter back to carbon dioxide in the carbon cycle. It has been suggested that degradative plasmids have made a significant contribution to the nutritional versatility of soil Pseudomonas and it is probable that, in addition to the few reported cases, many other catabolic functions are plasmid-specified. However, from the limited data available at present, it is difficult to assess the importance of plasmid-specified catabolism in the environment. Indeed, such plasmids have only been isolated in a narrow range of micro-organisms including fluorescent P.putida strains and non-fluorescent Pseudomonas species that have very similar nutritional profiles to P.putida.

It is probable that in Pseudomonas the genes for
particular catabolic pathways are frequently located on a plasmid. Certainly, the Tol function was plasmid-mediated in each of the thirteen independently isolated *Pseudomonas* strains examined in Chapter 4. It is interesting that five of these strains carried plasmids which are very similar, since one of these strains, *P. putida* mt-2, was isolated in Japan (Nôzaki et al., 1963), whereas the others, MT 16, MT 17, MT 18 and MT 21, were isolated in Wales. The analogy with the R plasmids R1, R6 and R100 is striking. These R plasmids originated in hospital isolates from England, Germany and Japan respectively, but are clearly related (Sharp et al., 1973). The ubiquity of particular types of R plasmids could result from dissemination of their host strains as human commensals. However, such an explanation seems unlikely for degradative plasmids of soil bacteria. Two other strains carried plasmids that were indistinguishable from each other but different to the first group. The remaining strains carried unique plasmids, although there appeared to be some similarities in their EcoRI-generated fragmentation patterns. Heinaru et al. (1978) have recently shown that the plasmids carried by strains MT 19 and *P. putida* mt-2 do, indeed, have some homology with other TOL plasmids. Radioactive probes of plasmids pWW19 and TOL were hybridised to the EcoRI-generated fragmentation patterns of a number of degradative plasmids. Plasmid pWW19 had homology not only with the plasmids from strains MT 1, MT 3, MT 5 and MT 19 (as suggested in Chapter 4), but also with pWW13, TOL, SAL, NAH and OCT. TOL had homology with each of these except OCT. The
inter-relationships between these plasmids appear to be complex.

The plasmids varied considerably in size; the one carried by strain MT 15 had a size of 170 Md and was the largest. The most generous estimates suggest that the transfer function and the degradative pathway could not account for more than 50 Md, so that it is possible that the plasmids may encode other functions. When one strain, MT 14, was cultured on m-toluate medium it carried plasmids that ranged in size from 25 Md to 200 Md. However, after extensive growth on L-broth only one size class of 48 Md was observed. The larger molecules are thought to be multimers of this size class (Chapter 6). The change is reversible. In this manner, when m-toluate is available, the bacterium carries several copies of the degradative genes which may enable it to grow more rapidly than strains carrying monomeric plasmids, and therefore give it a selective advantage. However, when no suitable carbon source is available the plasmid is observed to revert to its monomeric state.

The deletion of a specific region of about 27 Md from TOL, the plasmid carried by P.putida mt-2, results in the loss of the Tol function (Chapter 5). Downing et al (personal communication) have determined the Xho and HindIII maps of TOL and the deleted plasmid pWWO-1. These show that, when pWWO-1 is formed, one continuous stretch of DNA is deleted from TOL. The sex factor, factor K, is capable of mobilising the non-transmissible plasmid XYL (Friello et al, 1976) which encodes the Tol function. Factor K and
XYL may be analogous to the two components of the TOL plasmid. The contour lengths of XYL and factor K are 5 μm and 40 μm respectively. XYL\textsuperscript{+}K\textsuperscript{+} cells carry plasmid molecules that have contour lengths of 5 μm, 10 μm, 40 μm, 45 μm and 50 μm. It is therefore probable that XYL is able to integrate into, and excise from, factor K.

Recently, Chakrabarty et al (1978) have shown that, in P. aeruginosa PAO, the plasmid TOL dissociates to give a nonconjugative plasmid, TOL\textsuperscript{a}, which encodes the degradative function and a transfer plasmid, TOL\textsuperscript{Δ}. Their sizes are 28 Md and 48 Md respectively. These observations suggest that the Tol function of plasmid pWWO may be located on a transposon. This possibility is discussed in Section 8.4.

### 8.3 Incompatibility and plasmid relationships

The results presented in Chapter 7 show that, generally, plasmids of the same incompatibility group have a significant proportion of their polynucleotide sequences in common. It is striking that, while most fragments in an IncP-9 plasmid digest show homology with other IncP-9 plasmid probes, there are few fragments of common mobility after electrophoresis of the digests on agarose gels. This suggests that there are only relatively small segments of common sequences and that these are scattered around the plasmids. Alternatively, since single base changes can produce or destroy a cleavage site, there may be long continuous stretches of related sequences which differ slightly and consequently have quite different fragmentation patterns. From the results presented these models cannot
be resolved since this technique gives no indication of the proportion of a fragment that has homology. The two IncP-1 plasmids studied have four fragments of common mobility and these could represent a 22 Md continuous stretch of DNA that is common to the two plasmids.

Plasmids of different groups show little or no homology. In fact, ten of the sixteen pairs of plasmids from different incompatibility groups that did not encode a common function, showed no homology. The other six pairs did show some homology; four of these involved an IncP-9 plasmid paired with an IncP-1 or an IncP-7 plasmid and these three groups appear to be related. In each case only a few fragments showed homology. Some of these are large, but it is possible that only a small proportion of each fragment is homologous and that the total homology between the plasmids is only slight. Of the twelve pairs of plasmids that carry common functions, all but one showed some homology.

These observations are in agreement with the work of Grindley et al (1973) who determined the extent of homology between a series of E. coli plasmids. DNA reassociation experiments between fifteen plasmids from six incompatibility groups were described. All of the plasmids examined originated in Enterobacteriaceae. These experiments indicated that there is usually close homology (greater than 70%) between plasmids of the same group, and little or no homology between plasmids of different groups. There was one exception to the correlation between incompatibility and high degree of DNA relatedness. Four plasmids of the \textit{H} incompatibility group were included in the study. Three
of these showed a high degree of homology with each other but minimal homology with the fourth. However, this result is not surprising on consideration of the work of Smith et al. (1973) which showed that the plasmids of this group can be split into two subgroups. Members of the same subgroup are strongly incompatible, whereas members of different groups exhibit weaker interactions (Taylor and Grant, 1977).

Grindley et al. (1973) found that the sizes of plasmids of a particular group fell within a relatively small range. However, I found that the plasmids of the IncP-9 group of Pseudomonas varied considerably in size; those studied ranged from 48 Md to 78 Md.

Therefore, both the results presented in this thesis, and those of Grindley et al. (1973), show that the incompatibility groups are distinct from each other but that different members of the same group are similar. This suggests that incompatibility is usually correlated with a close phylogenetic relationship. This is particularly interesting in the case of the IncP-9 plasmids of Pseudomonas. Some of the plasmids of this group encode unrelated functions, they originated in different species of Pseudomonas (P. putida and P. aeruginosa), that were isolated from dissimilar habitats (they are soil and clinical isolates) in different geographical locations (U.K. and Japan) and yet they are related.
8.4 Transposable genetic elements

The origin of R plasmids and their antibiotic resistance determinants has long been a subject of speculation. Generally, when an antibiotic becomes used extensively at therapeutic levels within a bacterial population, R plasmids that encode resistance to this antibiotic rapidly emerge. In many cases the new antibiotic resistance determinants are found to be carried by well known plasmid types of an established incompatibility group. The carbenicillin gene of the IncP-1 plasmid RP1 has recently been shown to reside on a genetic element, transposon 1 (Tn1), which is able to undergo translocation from one replicon to another (Hedges and Jacob, 1974). Transposition can take place in the absence of significant genetic homology. Tn1 has a size of about 2 Md, and is therefore large enough to code for the protein responsible for carbenicillin resistance, TEM β-lactamase, and several other proteins (Hedges et al, 1974). Tn1 is unable to replicate autonomously but can insert at many sites on a recipient replicon.

The TEM β-lactamases of diverse R plasmids are found to be very similar, regardless of the geographical source or species of origin of the plasmid (Hedges et al, 1974). Heffron et al (1975) have shown that a similar translocatable element is present in a wide variety of naturally occurring R plasmids. This observation is supported by my hybridisation experiments (Chapter 7). Tn1 has no internal EcoRI cleavage sites, and only one band in the EcoRI fragmentation patterns of each of the carbenicillin
resistance plasmids showed homology with all of the other carbenicillin resistance plasmids. Therefore, these may be unrelated plasmids that have received the same or similar transposons.

The complex inter-relationships that appear to exist between TOL plasmids (Chapter 4; Heinäru et al., 1978) and their unusual mode of curing (Chapter 5) suggest that the Tol function may be carried on a transposon. Jacoby et al. (1978) have transposed the tol genes from TOL to RP4. The recombinant, RP4-tol, was incompatible with IncP-1 plasmids and, in addition to the Tol function, carried all the RP1 markers except tetracycline resistance. The recombinant, in turn, could translocate the tol genes to another plasmid, pMG5. Integration of the genes that encode the Tol function into RP4 allows this specialised Pseudomonas pathway to be expressed in *E. coli*. However, the enzymes of the pathway were present at a lower level in *E. coli* than in *Pseudomonas*, and *E. coli* strains could only use toluene, m- and p-xylene as carbon source. Growth on the intermediates may in part have been unsuccessful because of their toxicity for *E. coli*. It would be interesting to determine whether the expression of the Tol function is limited in *E. coli* because some of the enzymes of the pathway require species-specific membrane sites, or because a component of the degradative pathway is encoded by the *Pseudomonas* chromosome, or other factors. Nakazawa et al. (1978) and Chakrabarty et al. (1978) have also reported recombination between TOL and RP4. However, all of these recombinants have a size of about 76 Md which implies an
insertion into RP4 of about 38 Md. This is a different type of recombination to that observed between TOL* and TOLΔ although the TOL* genes are included in the 38 Md segment. Growth on benzoate of P. putida cells that harbour the recombinant plasmid results in the specific excision of the TOL* segment and the residual RP4 derivative still carries a 10 Md TOL plasmid segment. These genetic elements are far larger than any of the drug resistance transposons that have been identified so far.

8.5 Evolution of plasmids

Many different types of plasmids have been found in Pseudomonas and possibly other types of plasmids specifying other functions will be described in the near future. The genus Pseudomonas appears to be a reservoir of plasmids. An understanding of the evolution of these plasmids may give an important insight into the evolution and organisation of the genome as a whole.

Degradative plasmids cannot have arisen by the accumulation of genes from the end of the pathway backwards, since many biochemical intermediates are either rare, unstable or unable to cross the cell membrane. Such plasmids only confer an advantage if they carry most or all of the degradative pathway. Wheelis (1975) has suggested that degradative plasmids are derived by recombinational excision of clustered degradative genes from the chromosome. This idea is supported by the observation that naphthalene dissimilation may be either chromosomally or plasmid-encoded in different strains (Dunn and Gunsalus, 1973) and that
the degradative plasmid CAM is able to integrate into and mobilise the chromosome (Shaham et al, 1973). However, our results (Chapter 4) indicate that generally the Tol function is plasmid-specified.

Genetic exchange between bacterial strains is important in the evolution of a bacterial population. Plasmids may play a vital role in this process. In the short term, the infectious spread of plasmids through a microbial population may affect the efficiency with which it can respond to an environmental change, whether availability of a new carbon source or presence of a toxic substance. Over longer periods, recombination between different plasmids, and between plasmids and the chromosome can produce new plasmid types, and mobilisation of the chromosome by certain plasmids enables chromosomal recombination to take place between different strains. Consequently, a number of different plasmid types that specify the same function may result, as is illustrated by the range of TOL plasmids described in Chapter 4. Also, plasmids encoding new metabolic capacities may evolve in this manner.

It has been suggested that a number of sequences, and in particular transposons, are widespread in nature (Chapter 4; Chapter 7; Heinaru et al, 1978; Heffron et al, 1975). Heffron et al (1975) have suggested that the diverse range of R plasmids that carry very similar carbenicillin transposons have arisen as a result of the transposition of this segment from plasmid to plasmid (Section 8.4). Transposons that carry genes for other resistances have also been identified. These observations
could explain the facts that plasmids of different incompatibility groups often carry related resistance determinants, and that plasmids of the same group, although they are closely related to each other, often carry different functions (Chapter 7).

Degradative plasmids may have arisen in a similar manner. Jacoby et al (1978) have reported that the Tol function is located on a transposon. It has been shown that, while TOL plasmids are heterogeneous, they appear to be related to one another (Chapter 4; Heinariu et al, 1978). Therefore, it is possible that some of these may represent unique plasmids which have received a TOL transposon. It seems probable that transposons are important in the evolution of plasmids.

A deeper understanding of the evolution of plasmids, and of the genetic and biochemical processes that are involved in degradation, may enable strains of Pseudomonas to be constructed that could produce protein (for example, for animal foodstuffs) from unusual carbon sources or aid in the control of industrial and agrochemical pollution.
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