The Transfer and Stability of the Dehalogenase I Gene of *Pseudomonas putida* PP3

by

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Declaration

This thesis has not been submitted to any other University for any other degree, and the work described is my own, except where otherwise stated.

Summary

An R-prime, pUU2, derived from the broad host-range plasmid R68.44 was generated carrying the dehalogenase I gene from Pseudomonas putida PP3. This R-prime enabled its host to use 2-monochloropropionic acid (2MCPA) as sole carbon and energy source. The R-prime was studied by restriction endonuclease analysis. The process of R-prime formation was examined in detail and was shown to have suffered from interference from an insertion sequence originating from the PP3 chromosome. The dehalogenase I PP3 chromosomal insert was shown to interfere with the plasmid transfer functions and to be capable of translocation within the plasmid.

A Pseudomonad containing the R-prime pUU2 was grown under phosphate limitation on 2MCPA in a chemostat. During 2600h. a range of mutants were isolated in which the plasmid was shown to have undergone several modifications including the acquisition of 10kb of novel DNA, the transfer of the dehalogenase I gene to the chromosome, and in some cases the concomitant loss of the plasmid.

A new range of 2Mcpa^{+} R-prime plasmids were generated using a chemostat-derived plasmid-minus strain and R68.45. These demonstrated that though the insertion sequence IS21 of R68.45 may be involved in R-prime formation it need not be intimately associated with the chromosomal insert into the plasmid, at least in the case of the dehalogenase I gene. This, together with the frequency of R-prime formation, lends support to the view that the dehalogenase I gene may be on a transposable element.

Restriction fragments from the R-prime pUU2 were cloned into pAT153 and pKT231. Clones were isolated capable of growth of 2MCPA and their plasmids were analysed by restriction digestion.

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ABBREVIATIONS

Ap	Ampicillin
ATP	Adenosine triphosphate
bp	Base pairs
Cm	Chloramphenicol
Cma	Chromosome mobilizing ability
DCA	Dichloroacetic acid
22DCPA	2'-2-dichloropropionic acid
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Enhanced chromosome mobilization
EDTA	Na_2ethylene diamine tetra acetic acid
IS	Insertion sequence
kb	Kilobase pairs
Km	Kanamycin
MCA	Monochloro acetic acid
2MCPA	2'-monochloropropionic acid
Mu	Bacteriophage Mu
Nal	Naladixic acid
R'	R-prime
RNA	Ribonucleic acid
SDS	Sodium d decyl sulphate
Sm	Streptomycin
Tc	Tetracycline
TEMED	N,N,N,N',-tetramethyl-1,2-diaminoethane
Tn	Transposon
λ	Bacteriophage Lambda

Restriction fragment nomenclature.

These numbers and letters enable any particular restriction fragment to be identified in terms of the enzyme or enzymes required to produce it, whether it is common to a fragment in a parental plasmid, whether it is a novel fragment and what its relative position in the digest is. Most of the plasmids discussed in this thesis derive in some way from R68, R68.44 or R68.45. The nomenclature of Nayudu and Holloway (1981) has been followed for these plasmids; the first letter identifies the enzyme and the second the restriction fragment. For example, PA, is the largest restriction fragment produced by PstI, while SB is the second largest fragment produced by SmaI.

In the case of derivative plasmids a number prefixes the symbols so as to identify the plasmid; for example '1' for fragments from pUU1, or '3' for fragments from pUU3. If the restriction fragment is common to one in the original R68 the original letters naming the R68 fragment are also kept. For example, 1PA, refers to a fragment in pUU1 which is identical to fragment PA in R68. If however a fragment in a derivative plasmid is not identical to one in R68 an 'X', for extra, preceeds the letter identifying the enzyme used and a number follows. For example 2XS3 identifies a fragment as the third largest extra fragment produced by SmaI in pUU2. If a fragment is produced by two different enzymes a slash separates the letters identifying the enzymes; for example, 2XE/H1, is the largest extra fragment in pUU2 produced by a double digestion with EcoRI and HindIII.

In the diagrams of restriction digests double bands are identified by '*'.

Nomenclature of inserts.

Several of the plasmids derived from R68, R68.44 and R68.45 carry inserts of DNA. These are identified by the letter 'I' for insert, followed by a number. This number refers to the plasmid in which the insert is found.

The inserts are: I1, found in pUU1.

I2, the insert carrying the dehalogenase I gene found in pUU2 and in some plasmids derived from pUU2.

I3, found in pUU7.

I5, found in pUU5. I5 is very similar to I3.

CHAPTER 1

INTRODUCTION

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INTRODUCTION

1.1 Microbial degradation of xenobiotics and halogenated organic compounds

This century, especially the last forty years, has seen the addition of vast quantities of synthetic compounds to the biosphere. This addition has been as a result, directly or indirectly, of Man's exploitation of the environment and resources of this planet; and has occurred via coincidental pollution due to industry and agriculture or through the deliberate addition of compounds such as pesticides in agriculture, general pest control and war. These environmental changes are concomitant with a net increase on an unprecedented scale in material wealth worldwide, especially in the Northern hemisphere, whose sustainability is questionable.

Many of these synthetic compounds are novel to the environment, known as xenobiotics, and their breakdown must therefore pose new metabolic problems to microorganisms. As an illustration of the magnitude of this introduction of novel compounds to the environment, Greaves et al. (1976) calculated that the herbicidal additions to Britain's agricultural land in 1973 amounted to 30mg kg^{-1} of top soil assuming the herbicides were mixed evenly through the top 0.5cm of the soil. 57 million litres of Agent Orange (2,4,5-trichlorophenoxyacetic acid) containing an estimated 170 kilos of dioxin were sprayed in the South as a defoliant by the USA during the Vietnam war (Ruby, 1984). The challenge presented by these novel compounds to microorganisms has led to the evolution and presumed spread of new metabolic pathways and enzymes. While most xenobiotics are capable of being mineralized in time by bacteria, certain compounds, such as DDT and the chlorinated biphenyls, have proved remarkably recalcitrant to bacterial

metabolism (Clarke, 1981). What is surprising is not that these compounds have proved recalcitrant to biodegradation, but that enzyme pathways have evolved in such a short period of time to metabolize the bulk of the xenobiotic additions to our environment.

The range of compounds which are mineralised by micro-organisms is vast. For example amongst the compounds that the Pseudomonads can metabolise are alkanes, mono- and polycyclic hydrocarbons, alicyclics, heterocycles, phenolics, aliphatic and aromatic halogenated compounds, terpenes and flavenoids (Ribbons and Williams, 1981). In addition to the direct mineralisation of a particular chemical many of the enz-ymes involved exhibit a broad specificity which enables other related compounds to be partially mineralised, a phenomenon known as co-oxidation (Perry, 1979) or co-metabolism (Slater and Bull, 1982), thereby rendering these metabolites available to biodegradation by other bacteria.

The phenomenon of co-metabolism suggests that in the soil the interactions between more than one bacterial species may be more important in the metabolism of novel compounds than the pure cultures normally studied in the laboratory. Certainly studies with mixed bacterial populations in both closed (batch) and open (continuous) culture conditions have revealed synergistic metabolic interactions between more than one microbial species (Table 1.1). Such microbial communities probably play an important biodegradative role in the soil, and it is likely that it is within more general microbial populations that the enzymes and pathways required for the degradation of novel metabolites evolve. However classical enrichment techniques have led to the isolation of pure bacterial strains capable of degrading a wide range of chemicals. Most of the bacterial strains isolated from such enrichment techniques prove to be Pseudomonas species. Stanier et al. (1966) described 267 strains of Pseudomonas which could utilize a range of 146 compounds as growth substrates.

TABLE 1.1

COMPOUNDS DEGRADED BY THE SYNERGISTIC METABOLIC INTERACTION OF MORE THAN ONE BACTERIAL SPECIES (Adapted from Slater and Bull (1982))

Trichloroaceticacid
2,2-dichloropropionic acid (Dalapon)
3,6-dichloropicolinic acid (Lontrel)
3,4-dichloropropionanilide
2-(2-methyl 4-chloro) phenoxypropionic acid
chlorobenzoate
4-chlorobiphenyl
4,4'-dichlorobiphenyl
polychlorinated biphenyls
0,0-diethyl 0-2-isopropyl 4-methyl 6-pyrimidinyl thiophosphate (Diazinon)
0,0-diethyl 0-p-nitrophenol (Parathion)
1,5-di-(2,4-dimethyl-phenyl) 3 methyl 1,3,5-triazapenta 1,4-diene (Amitraz)
isopropylphenylcarbamate
cyclohexane
cycloalkanes
dodecylcyclohexane
n-hexadecane
n-alkanes and other hydrocarbons
crude oil
3-methyl heptane
benzoate
phenol
3,5-dihydroxyphenol
styrene
ligno aromatic compounds
linear alkybenzene sulphonates
alkylphenol ethyoxylates
2-ethyldecyl 1-decathoxylate
nitrosamines

1.1.1 Halogenated aromatic compounds

Some of these biodegradative enzymes and pathways have been found to be chromosomally coded while many are plasmid coded (Table 1.2). A degradative plasmid whose genetics and biochemistry have been extensively studied is the TOL plasmid pWW0, of Pseudomonas putida mt 2 (Williams and Murray, 1974; Williams and Worsey, 1975; Benson and Shapiro, 1978; Worsey et al., 1978; Downing and Broda, 1979; Inouye et al., 1981a, b; Franklin et al., 1981; Franklin et al., 1983; Lehrbach et al., 1983). TOL codes for the enzymes required for the catabolism of toluene, m- and p-toluate (Williams and Murray, 1974; Worsey and Williams, 1975) and also for the catabolism for pseudocumene and 3-ethyltoluene (Kurz and Chapman, 1981). These compounds are degraded via the meta pathway (Figure 1.1) when P. putida mt-2 contains the TOL plasmid. However in cured (plasmid-minus) strains benzoate is metabolized via the ortho pathway. The enzymes for the latter pathway are chromosomally coded for, but if TOL is present the enzymes for the ortho pathway are not induced. Benzoate induces benzoate oxidase I in the cured strain; this enzyme does not act upon m-toluate. However in the TOL containing strain two benzoate oxidases, I and II, are induced by benzoate; benzoate oxidase II is also induced by m-toluate. This latter enzyme is most active towards m-toluate, though it also shows activity towards other benzoates. P. putida mt-2 has a higher growth rate on benzoate via the ortho than the meta pathway. Therefore growth in this substrate will allow selection of spontaneous TOL^- strains.

1.1.2 Halogenated alkanoic acids

Halogenated alkanoic acids are found widely in the environment either from natural sources or as xenobiotics through their use as herbicides and pesticides (Suida and De Bernardis, 1973; Audus, 1976). These compounds are

Table 1.2

BIODEGRADATIVE PLASMIDS

Pathway for degradation of	Reference
Octane	Chakrabarty et al. (1973)
Camphor	Rheinwald et al. (1973)
Naphthalene	Dunn and Gunsalus (1973)
Salicylate	Chakrabarty (1972)
2,5-xylenol	Hopper and Kemp (1980)
Toluene/xylene	Williams and Murray (1974)
2,6-dichlorotoluene	Vanderbergh et al. (1981)
3-chlorobenzoate	Chatterjee et al. (1981)
	Reineke et al. (1982)
4-chlorobenzoate	Chatterjee and Chakrabarty (1982)
and 3,5-dichlorobenzoate	Chatterjee et al. (1981)
p-hydroxybenzoic acid	Salkinoja et al. (1979)
p-cymene	Ribbons and Wigmore (1977)
Alkyl benzene sulphonate	Cain et al. (1977)
5-amino 4-chloro 2-phenyl 3-pyridazmine	Kreis et al. (1981)
6-aminohexanoic acid cyclic dimer	Negoro et al. (1980)
Nicotine/nicotinate	Thacker et al. (1978)
2,4-dichlorophenoxyacetic acid	
and 2-methyl phenoxyacetic acid	Pemberton and Fisher (1977)
2-methyl, 4-chlorophenoxyacetic acid	Franklin et al. (1981)
p-cresol	Hewetson et al. (1978)
Di-nitro ortho cresol	Franklin et al. (1981)
Phenanthrene biphenyl	Kiyohara et al. (1983)
p-chlorobiphenyl	Kamp and Chakrabarty (1979)
Nylon oligomers	Negoro et al. (1983)
Monofluoroacetic acid	Kawasaki et al. (1981a, b, c)
and monochloroacetic acid	

FIGURE 1.1

METABOLISM OF TOLUENE AND XYLENE BY *PSEUDOMONAS PUTIDA* mt-2 VIA THE *ORTHO* AND *META* PATHWAYS[1]

1. Based on Williams and Murray (1974) and Franklin *et al.* (1983)

R, R' = H: toluene

R = H, R' = CH_3: *m*-xylene

A: toluene (xylene)

B: benzyl alcohol

C: benzaldehyde

D: benzoate (toluate)

E: catechol

F: 2OH muconic semialdehyde

G: 4 oxalocrotonate (enol)

H: 4 oxalocrotonate (keto)

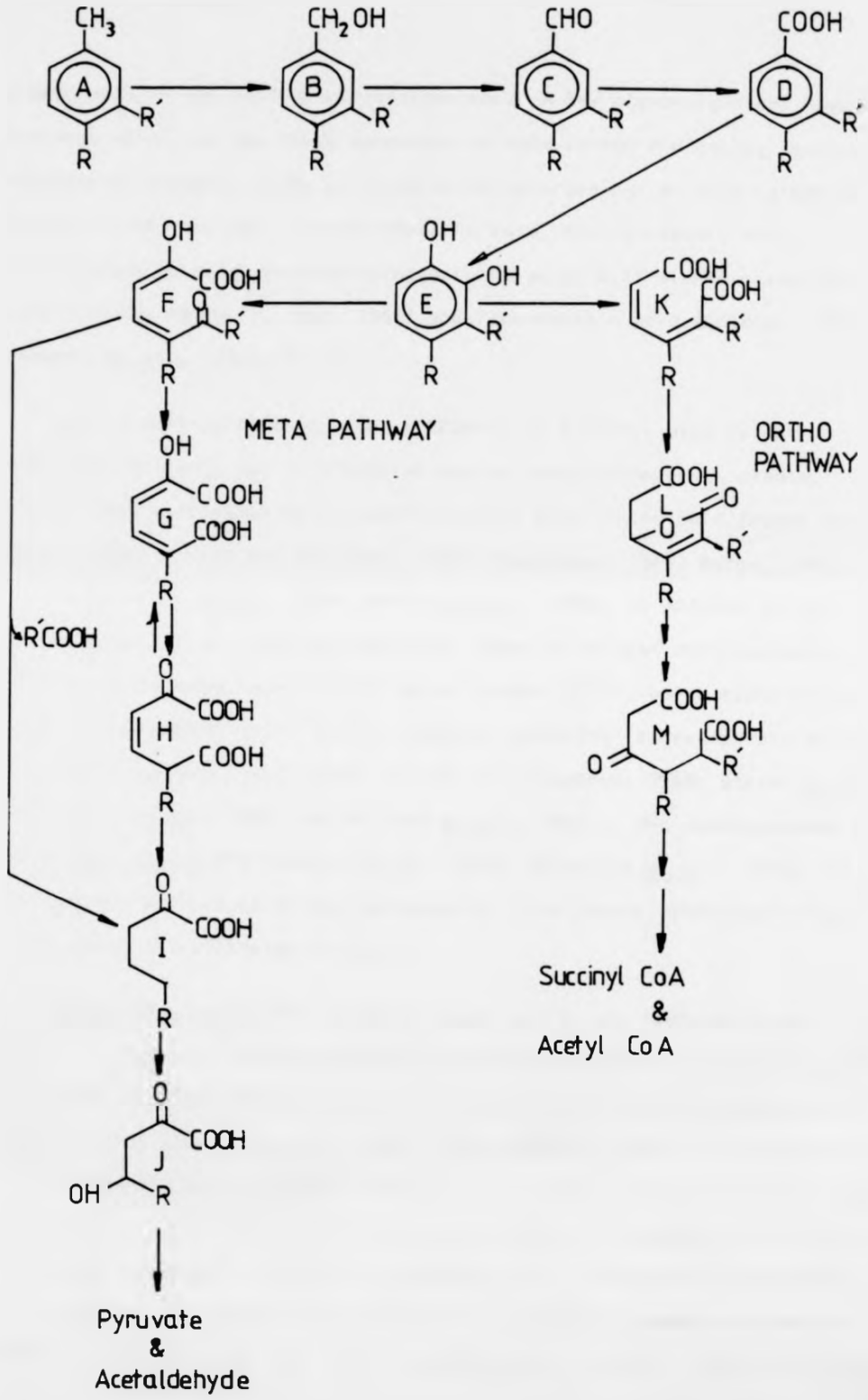
I: 2 oxopentenoate

J: 4OH 2 oxovalerate

K: cis, cis-muconate

L: muconolactone

M: β-ketoadipate


CH3
CH2OH
CHO
COOH
A
B
C
D
R
R'
OH
F
E
K
META PATHWAY
ORTHO PATHWAY
G
H
I
J
L
M
R'COOH
Succinyl CoA
&
Acetyl CoA
Pyruvate
&
Acetaldehyde

broken down in the soil by micro-organisms. A few micro-organisms have been isolated which can use these compounds as sole carbon and energy sources. Halogenated alkanoic acids utilized by micro-organisms as sole carbon or energy sources include: monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, 2-monochloropropionic acid, 2,2'-dichloropropionic acid (Jensen, 1957a, b, 1959, 1960) and fluoroacetic acid (Goldman, 1965; Kawasaki et al., 1981a, b, c).

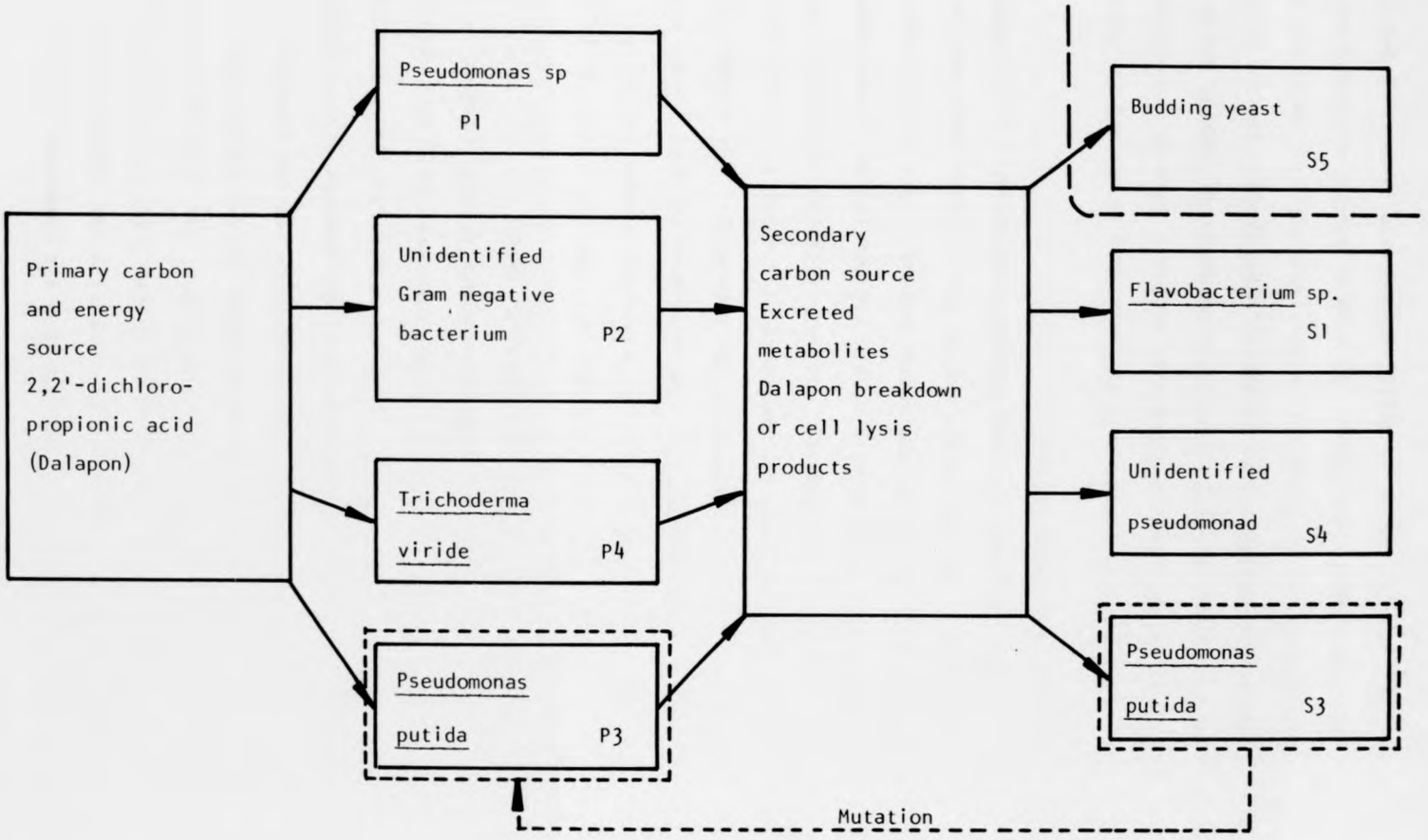
2,2'-dichloropropionic acid (22DCPA) is a widely used selective herbicide (Dalapon) and is effective against monocotyledonous plants. 22DCPA has been shown to be readily broken down in the soil (Magee and Colmer, 1959; Hirsch and Alexander, 1960; Macgregor, 1963; Burge, 1969; Foy, 1975; Berry et al., 1976; Senior et al., 1976a, b; Allison et al., 1983). Some of these micro-organisms have been shown to possess dehalogenases, also known as halidohydrolases (Little and Williams, 1971), which hydrolytically remove the halogens from 22DCPA, thereby rendering it susceptible to further degradation (Jensen, 1957, 1960; Hirsch and Alexander, 1960; Slater et al., 1979; Berry et al., 1979; and Allison et al., 1983). The dehalogenases of Pseudomonas putida PP3 (Senior et al., 1976; Weightman et al., 1979) are a good example and are of direct relevance to this thesis, therefore they will be discussed in considerable detail.

Pseudomonas putida PP3, formally known as P3, was isolated from a chemostat community growing on 22DCPA (Dalapon) through its acquired ability to grow on 22DCPA and 2-monochloropropionic acid (2MCPA) as sole carbon and energy sources (Senior et al., 1976). The chemostat community contained six or seven members which could be divided into primary and secondary utilizers (Figure 1.2). Initially three primary utilizers were isolated, P1, P2 and P4, capable of growth on 22DCPA in pure culture. The community contained four secondary utilizers, S1, S3, S4 and S5, which were unable to grow on 22DCPA in pure culture. S5, a pink budding yeast, was only loosely attached

FIGURE 1.2

THE STRUCTURE OF A MICROBIAL COMMUNITY GROWING ON 2,2'-DICHLOROPROPIONIC ACID[1]

1. From Senior *et al.* (1976a)


Primary carbon and energy source 2,2'-dichloro-propionic acid (Dalapon)
Pseudomonas sp P1
Unidentified Gram negative bacterium P2
Trichoderma viride P4
Pseudomonas putida P3
Secondary carbon source Excreted metabolites Dalapon breakdown or cell lysis products
Budding yeast S5
Flavobacterium sp. S1
Unidentified pseudomonad S4
Pseudomonas putida S3
Mutation

to the community and was lost when the chemostat dilution rate was increased above $0.2h^{-1}$. The resultant community was stable, except for one change, for over 13,500h of continuous growth. P2 was the dominant primary utilizer at dilution rates of less than $0.25h^{-1}$, at dilution rates greater than $0.45h^{-1}$ P1 was dominant. The kinetic theory of enrichment cultures predicts that the organism with the highest affinity for the substrate or the highest maximum specific growth rate will out compete others (Powell, 1958; Slater and Bull, 1978). This did not occur with the Dalapon community; presumably the interactions between so many organisms were more complex and unspecifiable than those in the two-membered systems usually studied in competition experiments (Veldkamp and Jannasch, 1972).

After 2,900h a new primary utilizer was isolated, P. putida P3. This organism was identical in all respects, except in its ability to grow on 22DCPA, to P. putida S3. The appearance of this fourth primary utilizer did not upset the balance of the community. The suggestion that P3 had evolved by mutation from S3 was confirmed by growing S3 in pure culture on propionate and 22DCPA in a chemostat. S3 initially was not able to utilize 22DCPA but after 1,200h a derivative strain had evolved which was capable of utilizing 22DCPA as sole carbon and energy source. This strain displaced S3 from the chemostat. The new strain was in all respects identical to P3. In addition to 22DCPA P3 also utilized 2-monochloropropionic acid (2MCPA) as its sole carbon and energy source.

Assays with crude cell free extracts of S3 and P3 showed that they both exhibited inducible dehalogenase activity towards monochloroacetate (MCA), dichloroacetate (DCA), 2-monochloropropionate (2MCPA) and 2,2'-dichloropropionate (22DCPA). P3 contained dehalogenase activities towards these substrates ten to forty-fold higher than S3. S3 could not grow on any of the chlorinated substrates. P3 grew on 22DCPA and 2MCPA but could not grow on MCA or DCA, though it could dehalogenate these last two compounds if they

were present in the growth media (Slater et al., 1979).

Pseudomonas putida P3, now known as PP3, was shown by polyacrylamide gel electrophoresis to possess two dehalogenases, known as fraction I and fraction II (Weightman et al., 1979). Partial purification of the two dehalogenases revealed that they possessed different activity profiles to the four major substrates (Table 1.3). Over 95% of the DCA activity was present in fraction II dehalogenase, while the bulk of the activity towards 22DCPA and 2MCPA was in fraction I dehalogenase. The two dehalogenases were equally active towards MCA. Fraction I dehalogenase possessed the major part of the activity towards 2-monochlorobutyric acid, while fraction II possessed 80% of the activity towards trichloroacetic acid. Goldman isolated two dehalogenases from a bacterium which were induced separately by MCA and DCA and showed no activity towards 22DCPA (Goldman, 1965; Goldman and Milne, 1966; Goldman et al., 1968). The dehalogenases of PP3 were not inducible separately and their substrate profiles indicate that they were different from those of Goldman.

Weightman and Slater (1980) grew PP3 in continuous culture on 2MCPA and isolated two mutant strains, PP309 and PP310, which showed elevated dehalogenase activity compared to that of the parent PP3. This was due to elevated fraction I dehalogenase activity. The level of dehalogenase fraction II remained at the parental level. Fraction I dehalogenase possessed the bulk of the activity towards 2MCPA. Therefore a mutant strain with elevated fraction I dehalogenase activity would possess a selective advantage under these growth conditions. The nature of the mutation was not determined.

The stereospecificity of fraction I and II dehalogenases was examined in order to determine the relationship between the two enzymes (Weightman et al., 1981). PP3 grew on D,L-2MCPA dechlorinating both isomers. Both fraction I and II dehalogenases were active towards both isomers of 2MCPA, though in both cases the activity towards L-2MCPA was 80% of that towards

Table 1.3

SUBSTRATE ACTIVITY PROFILES OF CRUDE CELL FREE EXTRACTS OF PP3 AND PARTIALLY PURIFIED FRACTION I AND II DEHALOGENASES[1]

	Dehalogenase specific activities [μmol substrate min^{-1} (mg protein)$^{-1}$][2]			
	MCA	DCA	2MCPA	22DCPA
PP3 crude extract	0.45	0.63	0.33	0.08
	(1)	(1.29)	(0.42)	(0.23)
Fraction I	0.62	0.07	0.40	0.19
	(1)	(0.11)	(0.65)	(0.31)
Fraction II	1.05	1.83	0.26	0.13
	(1)	(1.74)	(0.25)	(0.12)

1. From Weightman et al. (1979).
2. The figures in brackets are dehalogenase substrate activity ratios with respect to MCA.

D-2MCPA. The major difference between the two enzymes was that the product of the dechlorination of 2MCPA by fraction I dehalogenase, lactate, retained the optical configuration of its precursor, while fraction II dechlorination produced lactate with the opposite optical configuration to its precursor. PP3 can use D-lactate as a growth substrate, possibly because of the presence of a lactate racemase. These results, and the dehalogenases' different responses to inhibition by sulphydryl blocking agents, led Weightman et al. (1981) to postulate two different mechanisms for the action of fraction I and II dehalogenases. They suggest therefore that these two enzymes probably did not evolve from a common ancestral dehalogenase gene, though they may share common control mechanisms.

Other micro-organisms capable of growth on halogenated alkanoic acids have been shown to contain one or more dehalogenases (Davies and Evans, 1962; Goldman et al., 1968; Hardman and Slater, 1981; Kawasaki et al., 1981a, b, c, 1982; Motosugi et al., 1982; Allison et al., 1983). None of these possessed identical substrate activity profiles to those of fraction I and II dehalogenases from P. putida PP3. Hardman and Slater (1981) examined dehalogenases from sixteen bacterial isolates enriched for by growth on 2MCPA or MCA as sole carbon and energy source. The isolates proved to contain between one and four dehalogenases which could be separated on the basis of their electrophoretic mobility. Dehalogenases of the same mobilities were found in different isolates, though they did not always have the same substrate activity profiles. Fraction I dehalogenase from PP3 has the same electrophoretic mobility as one of the enzymes in these other bacterial isolates. No enzyme had the same mobility as fraction II dehalogenase from PP3. All the new dehalogenases from these new isolates were inducible. These results indicate the wide range of dehalogenases present in soil bacteria and their variety of substrate affinities.

Plasmid coded dehalogenases have been reported (Kawasaki et al.,

1981a, b, c, 1982). Moraxella sp. strain B and Pseudomonas sp. strain C were isolated by enrichment upon fluoroacetate. They were found to possess very similar conjugative plasmids coding for two dehalogenases, H-1 and H-2, and mercury resistance. In Pseudomonas C. H-1 and H-2 were consitutive, while in Moraxella B H-1 was inducible while H-2 was consitutive. Transfer of the plasmids to the other host indicated that the regulation of expression of the dehalogenase genes was host dependent.

The variety of dehalogenases present in soil bacteria is again brought out by an examination of their stereospecificity. The results of Weightman et al. (1981) have already been discussed above. The different dehalogenases examined by Goldman et al. (1958) and Little and Williams (1971) act solely on L-halogenated alkanoic acids. Motosugi et al. (1982) isolated a Pseudomonas strain which dehalogenated both D and L halogenated alkanoic acids. This strain proved to possess one dehalogenase which converted 2MCPA to the opposite optical isomer of lactate. In this respect it resembles the activity of dehalogenase fraction II from P. putida PP3.

1.2 Gene Transfer

Gene transfer has proved to be a powerful tool in the study of the genetics and biochemistry of bacteria. Gene transfer has also been suggested as an important mechanism in bacterial evolution, especially in the spread of novel genes through bacterial populations (Anderson, 1966, 1968; Reanney, 1976, 1977, 1978; Bennett and Richmond, 1978). This section will deal briefly with genetic recombination, mechanisms of gene transfer and barriers to gene transfer before going on to discuss in more detail chromosome mobilization and prime formation by broad host-range plasmids.

1.2.1 Recombination

Genetic recombination can be defined as the reassortment of nucleotides

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1.2.1 Recombination

Genetic recombination can be defined as the reassortment of nucleotides

along nucleic acid molecules (Clarke, 1971). These reassortments can occur within a molecule to produce deletions, duplications, inversions or transpositions; or between two parental molecules to produce one or two molecules derived in part from the parental molecules.

Where extensive homology exists between DNA molecules exchange of DNA in E. coli is catalysed by the general recombination system in which the rec A gene product is very important. Non-homologous recombination does not require the rec A gene product and may be either site specific, in which an exchange of DNA occurs only at specific regions in one or both of the parental DNA molecules; or illegitimate, which includes other rec A-independent events.

General recombination occurs between DNA molecules with extended homology. It generally occurs at high frequency. The cross over point between the two molecules is usually random. In the general recombination system the rec A gene produced plays an essential key role. The rec A protein is a DNA dependent ATPase which unwinds DNA in the presence of ATP. It forms DNA duplexes by binding homologous single strands to form displacement or D-loops (Glass, 1982). The mechanism of general recombination thus requires the breaking of phosphodiester bonds and their subsequent rejoining to form a heteroduplex hybrid product in which one single strand becomes covalently linked to another, both derived from distinct parental DNAs. Examples in which general recombination plays an important role are homologous Hfr x F^- crosses, transduction and chromosome mobilization due to secondary F-prime formation (Pittard and Adelberg, 1964).

Site specific recombination does not require the rec A product and occurs at specific sites in one or both parental DNAs. It generally involves the insertion of foreign DNA into the chromosome. The insertion elements involved are bacteriophage, plasmids, insertion sequences and transposons. Unlike the case of general recombination, in which portions of DNA from one

parent may be lost due to the reciprocal nature of the event, site specific recombination is an integrative event in which the entire genetic content of both elements involved are recombined.

The integration of bacteriophage λ is double site specific in that it requires a specific site in the E. coli chromosome, $att^{\lambda}B$, between the gal or bio operons, and $att^{\lambda}P$ on λ itself. In the absence of $att^{\lambda}B$ λ will integrate into other sites in the E. coli chromosome but at a greatly reduced frequency (Glass, 1982). Similarly F insertion to form certain Hfrs is also double site specific (Davidson et al., 1975).

Insertion sequences (IS) are short pieces of DNA of about 700-1500 base pairs (bp), they usually have terminal inverse repeats. They code for no identified gene product. Transposons (Tn) are larger between about 4-21 kilobase pairs (kb) in length. They consist of coding regions flanked by insertion sequences (IS). The coding regions usually code for a resistance gene, for example to kanamycin in Tn 5 (Reznikoff et al., 1981) or to mercury in Tn 501 (Bennett et al., 1978), though a few transposons are known which code for other functions, for example Tn 951 which carries a lactose operon (Cornelis et al., 1978). In addition transposons code for the enzymes required for transposition of the repressor protein required to regulate the expression of the transposase (Berg and Berg, 1981). Transposition appears to lead to the duplication of a pre-existing nucleotide sequence in the target DNA, five or nine base pairs long. Transposition, i.e. insertion of IS or Tn, is non-specific but not entirely at random. There do appear to be preferred sites of insertion. Transposition can be seen to be of variable site-specificity.

Illegitimate recombination includes all other non-homologous, non-rec A gene product dependent recombination events. These include deletions and duplications. Little is known about their generation. Duplications are more common than deletions but tend not to be stable unless they occur in a rec A

background (Glass, 1982). The formation of certain F-primes or specialised transducing phages are included amongst illegitimate recombinations (Davidson et al., 1975; Kayajanian and Cambell, 1966; Weisberg and Adhya, 1977). As is the fusion of non-homologous ends of DNA (Murray and Murray, 1974).

1.2.2 Mechanisms of Gene Transfer

Mechanisms for gene transfer in bacteria include transformation, transduction and conjugation.

Transformation is the transfer of naked DNA into the bacterial cell. Transformation systems fall into two groups. In pneumococcus and Bacillus subtilis growth in certain media can render the cells physiologically competent (i.e. capable of DNA uptake). The DNA is cut into short pieces at the surface of the cell and is taken into the cell as single stranded DNA. Within the cell the DNA forms a heteroduplex with the chromosomal DNA and recombination occurs (Notani and Setlow, 1974). It is not certain whether plasmid transformation follows the same pattern (Low and Porter, 1978). Gram negative bacteria with the exception of Haemophilus require artificial treatments to make the cell permeable to DNA, usually with $CaCl_2$. Such artificial systems exist for E. coli and Pseudomonas putida and aeruginosa (Cohen et al., 1972; Chakrabarty et al., 1975). The uptake of plasmid DNA requires no known recipient cell recombination function (Oishi and Irbe, 1977). The transformation of chromosomal DNA occurs at very low frequencies, and its mechanism is unknown, and occurs only in certain mutants of E. coli (Oishi and Cosloy, 1972).

In transduction bacterial DNA becomes packaged along with or in place of bacteriophage DNA in the viral capsid and may therefore be transferred on infection to a new bacterial cell. In generalized transduction DNA derived

at random from the bacterial chromosome replaces the viral DNA in the capsid (Ozeki and Ikedi, 1968). Specialized transduction, on the other hand, occurs when a lysogenic phage is inaccurately excised from its attachment site in such a way that it consists of phage and chromosomal DNA. This is then packaged into the viral capsid, released, and can infect a new host. Phage possess specific attachment sites in their hosts, for example λ inserts between gal and bio in E. coli, therefore the genes transferred in specialized transduction tend not to be randomally distributed around the chromosome (Franklin, 1971). In addition, specialized transducing phages may acquire genes through transposition (Cohen, 1976). Generalized transduction occurs at a much lower frequency than specialized transduction.

During conjugation plasmid DNA is transferred in vivo from one bacterial cell to another. A plasmid is an autonomous, extrachromosomal, self replicating, circular DNA molecule. Plasmid sizes vary from a few kilobase pairs (kb) to several hundred kb. The number of plasmids per cell can be between one and several hundred depending on the plasmid concerned. The presence of a plasmid is usually not essential to a bacterium under normal growth conditions. In addition to self replication plasmid DNA codes for other functions. In the case of cryptic plasmids these functions are not known. Sex factors are plasmids which code for their conjugal transfer between bacteria and are capable of mobilizing the bacterial chromosome, they code for no other known functions; F is a much studied sex factor in E. coli (Glass, 1982) and FP2 in Pseudomonas aeruginosa(Pemberton and Holloway, 1973). R plasmids code for resistance to antibiotics. In addition some R-plasmids are self transmissible and may be able to act as sex factors, for example RP4 (Towner, 1978). Many R plasmids also provide resistance to heavy metals, for example mercury (Hedges et al., 1974). Catabolic plasmids have already been mentioned (Table 1.2). These plasmids enable their hosts to grown on a wide range of substrates, some of which are exotic or xenobiotic. Predominantly catabolic plasmids are conjugative. Plasmids are classified according to

their incompatability group (Datta, 1975). This refers to plasmids which are unable to co-exist within the same host cell. It is probable that incompatibility is due to interference between common replication mechanisms on the different plasmids (Broda, 1979).

Conjugal plasmid transfer is a complex procedure. This is brought out by the proportion of a plasmid devoted to conjugation; plasmids which share a similar conjugation system possess 40-80% homology while those that do not possess less than 10% (Falkow et al., 1974). In F (94.5 kb) about 33kb determine the conjugation system, within which about 20 genes involved in conjugation have been identified (Willetts and Skurray, 1980). An E. coli cell possessing F (i.e. F^+) produces thin proteinaceous structures known as F-pili. These F-pili interact with an F-minus strain (lacking F, therefore possessing no pili) to produce a conjugation bridge, through which F is transferred to the F^- strain. It is not known whether F is transferred 'naked' or whether there is a 'pilot' protein bound to the 5' terminus (Glass, 1982). Normally plasmid replication is initiated at oriV (the origin of vegetative replication). However during conjugation a process analogous to replication occurs initiated at the origin of transfer (oriT). One DNA strand is nicked at oriT and transferred by rolling circle replication, 5' end first, into the F^- cell. The complementary strand is left behind. Both strands are immediately used as templates for double stranded DNA synthesis by DNA polymerase III in a discontinuous fashion. The plasmid re-circularizes in the recipient (Glass, 1982).

F is capable of mediating transfer of E. coli chromosomal DNA by means of either Hfr x F^- or F-prime x F^- matings. F can insert in a linear form into the E. coli chromosome. This occurs at a fixed number of sites and in a specific orientation. A functional rec A product is required for this process. The resultant Hfr can subsequently transfer a large part of the E. coli chromosome and part of F into an F^- recipient. Transfer is initiated

from oriT and the 5' end enters the recipient first. A partial diploid is thus formed in the recipient. Homologous recombination occurs for which a functional rec A is required and F is lost. Imprecise excision of F from the host chromosome leads to the formation of an F-prime which contains some chromosomal DNA with or without the concomitant loss of some F DNA. Conjugal transfer of the F-prime to an F^- strain can lead to homologous recombination between the chromosomal DNAs or to the insertion of the F prime into the recipients' chromosome. If the recipient is recombinationally deficient the F-prime may remain as such (Glass, 1982). In recombinationally proficient E. coli strains F-primes are capable of mobilizing the chromosome at high frequency to F^- strains. This is due to a high rate of recombination between the chromosome and the homologous region on the plasmid leading to the integration of the F-prime into the chromosome and the subsequent transfer of chromosomal markers to the F^- recipient (Pittard et al., 1963; Pittard and Adelberg, 1963; Low, 1972). Many conjugative plasmids, though not F, are capable of transfer between species (broad host-range plasmids). Some of these are also capable of chromosome mobilization and plasmid prime formation (Holloway, 1979).

Plasmids lacking transfer genes can generally be mobilized by other plasmids possessing these genes, this is a rec A independent phenomenon. This is probably due to the mobilized plasmid possessing ori T from which conjugal transfer can be initiated (Willetts and Crowther, 1981). Plasmids lacking ori T can also be mobilized if they can be covalently linked to a functional ori T in the mobilizing plasmid. This situation is analogous to chromosome mobilization which usually requires a certain amount of homology due to common IS sequences in both replicons (Davidson et al., 1975). If no such homology exists mobilization may also occur due to cointegrate formation during transposition of a transposable DNA element from one of the plasmids to the other (Leemans et al., 1981; Willetts et al., 1981b; Riess et al., 1983).

Insertion sequences (IS) and transposons (Tn) have been introduced in the discussion of recombination. Both are capable of rec A independent transfer within and between replicons, albeit within the confines of the bacterial cell. Insertion sequences code for no known function, and though they may not be able to transfer functional genes they can profoundly affect the behaviour and interaction of other genetic material. Several of these properties of ISs have been discussed above. Transposons transfer in an analogous manner to ISs, but carry known functional genes. Allied with conjugal transfer Tns permit the transfer of genes between chromosome and plasmid, plasmid and plasmid and, indirectly between chromosome and chromosome.

1.3 Barriers to gene transfer

The uncontrolled influx of foreign genetic material may be disadvantageous to an organism. Therefore all organisms possess barriers to foreign DNA. Bacteria, especially soil bacteria exhibit a range of phenotypic diversity. The limited acquisition of new phenotypes or metabolic capabilities may convey a selective advantage to a bacterium. Perhaps for this reason barriers to DNA transfer do not totally block DNA uptake by bacteria. DNA may be prevented from entering the cell, or it may be prevented from forming a functional replicon or recombining within the cell, or the DNA may be prevented from replicating.

Extracellular DNAases may be present which can physically disrupt the DNA outside the cell (Rogers, 1961), or degrade it at the surface of the cell (Seto et al., 1975). To enter the cell the DNA has to cross the cell wall and membrane. These present a considerable barrier which it is surprising any DNA can penetrate (this has been mentioned above in the discussion on transformation). If a cell already contains a plasmid of the same incompatibility group as a plasmid which can normally enter the cell by conjugation the passage of the second plasmid across the cell wall will be

prevented by surface exclusion (Novick, 1969; LeBlanc and Falkow, 1973). This phenomenon is distinct from incompatibility and is due to the presence of a plasmid determined mucopeptide layer (Beard and Bishop, 1975). Surface exclusion is not an absolute barrier, a small amount of leakage does occur.

Once within the cell the endogenous restriction system of the host may destroy the incoming DNA. A wide range of restriction endonucleases are produced by bacteria which recognise and clear foreign DNA (Nathans and Smith, 1977; Roberts, 1979). The host's own DNA is protected against restriction by modification enzymes which usually methylate certain bases. Only similarly modified DNA will survive. Closely related DNAs to that of the host may be similarly modified. Only modification of incoming plasmid DNA will enable it to overcome the restriction system. Restriction systems can decrease transformation frequencies by $\times 10^{-5}$ (Nagahari and Sakaguchi, 1978). Incoming DNA may also be inactivated by other mechanisms, for example SP82G DNA suffered about 40 lesions when transfected into Bacillus subtilis (Green et al., 1968).

Inside the cell the incoming DNA must become a replicon and replicate or recombine to be perpetuated. The DNA may fail to replicate or replicate at an inadequate rate to keep pace with cell division (Stocker, 1956). If the cell already contains a plasmid of the same incompatibility group one of the plasmids is excluded (Datta, 1975). This is probably due to interference between similar plasmid replication control systems. Incompatibility may favour either the incoming of the resident plasmid (Falkow, 1975). Genetic material from one bacterial species may not be able to replicate adequately in another. For example, the E. coli plasmids pSC101 and RSF1010 will not replicate in Bacillus subtilis (Sakaguchi, 1980). Differences in transcription and translation systems between bacterial species may prevent the expression of genes on incoming genetic material. Franklin et al. (1981b) observed that Pseudomonas genes were poorly expressed in E. coli and suggested that

Pseudomonas promoters function less efficiently with *E. coli*, than with native *Pseudomonas* RNA polymerase. *E. coli* messenger RNA (mRNA) would not translate in *Bacillus brevis* or *Caulobacter crescentus* *in vitro* translation systems (Szer and Leffler, 1974).

The previous section stressed mechanisms of gene transfer while this has stressed the barriers. Both mechanisms and barriers exist. Gene transfer certainly occurs in the laboratory and presumably in the environment. The mechanisms and barriers probably determine that it occurs in a regulated rather than in an uncontrolled way.

1.2.4 *Gene transfer by R68 and its relatives*

Chromosome mobilization ability (Cma) and plasmid-prime formation, though extensively studied in *E. coli* and F, is by no means confined to these replicons. In fact both appear to be common properties among plasmids and to occur in a wide variety of bacterial species (Holloway, 1979). Many of these Cma plasmids are resistance (R) plasmids from the incompatibility group Inc P-1 (Datta, 1975). In addition to Cma and R-prime (R') formation some of these R-plasmids are capable of interspecific transfer, thereby enabling gene transfer to occur across the species barrier (Holloway, 1979). Interspecific gene transfer has been suggested as being a mechanism in bacterial evolution (Reanney, 1976, 1977, 1978; Bennett and Richmond, 1978; Clarke, 1978, 1981b).

An R-plasmid whose Cma and R' activities have been much studied is R68 and its close derivatives and relatives RP1, RP4, RK2, and R18 (Holloway and Richmond, 1973; Beringer, 1974; Haas and Holloway, 1976). R68, RP1, RP4, RK2 and R18 have been shown to be extremely similar, if not identical, by heteroduplex and restriction endonuclease analyses (Burkhardt *et al.*, 1979; Currier and Morgan, 1981; Stokes *et al.*, 1981). These plasmids have

been studied by restriction endonuclease digestion (DePicker et al., 1977; Grinsted et al., 1977; Meyer et al., 1977c; Riess et al., 1980a, b; Currier and Morgan, 1981; Nayudu and Holloway, 1981; Stokes et al., 1981), heteroduplex analysis (Burkardt et al., 1979; Leemans et al., 1980), insertional mutagenesis (Barth and Grinter, 1977; Meyer et al., 1977a; Barth et al., 1978; Barth, 1979; Priefer et al., 1980; Simon and Puhler, 1980; Thomas et al., 1980; Harayama et al., 1981; Lanka and Barth, 1981; Al-Doori et al., 1982; Cowan and Krishnapillai, 1982), the construction of mini-replicons from which plasmid DNA non essential for maintenance has been deleted (Figurski et al., 1976; Hedges, 1976; Barth and Grinter, 1977; Meyer et al., 1977a; Sakanyan et al., 1978; Haque, 1979; Thomas et al., 1979, 1980, 1981, 1982; Ditta et al., 1980), deletion analysis (Figurski et al., 1976; Thomas et al., 1980), molecular cloning of plasmid fragments (Guiney and Helinski, 1979; Watson et al., 1980; Meyer and Hinds, 1982; Lanka et al., 1983; Pohlman et al., 1983a, b; Schmidhauser et al., 1983; Kornacki et al., 1984), and DNA sequencing of portions of the plasmid (Stalker et al., 1981; Bechhofer and Figurski, 1983; Guiney and Yakobson, 1983, Waters et al., 1983). This body of work enables a composite picture of the plasmid to be built up (Figure 1.3).

R68 is a plasmid of about 57.3kb in size which codes for resistance to the antibiotics ampicillin (Ap), kanamycin (Km) and tetracycline (Tc). Ampicillin resistance is carried on the transposon Tn1 or TnA. The three antibiotic resistance genes are not clustered together but are distributed around the plasmid. Similarly the trf (regions specifying trans-acting replication and maintenance functions) and the tra (regions required for conjugal transfer) genes are not clustered, but are scattered around the plasmid separated by the antibiotic resistance genes. This feature has made the construction of mini-replicons derived from R68 difficult. In F the genes required for conjugal transfer were all clustered together (Willetts and

FIGURE 1.3

Map of R68[1]

1. Based on Guiney and Helinski (1979), Riess et al. (1980a,b), Thomas (1981), Lanka and Barth (1981), Nayudu and Holloway (1981) and Al-Doori et al. (1982)

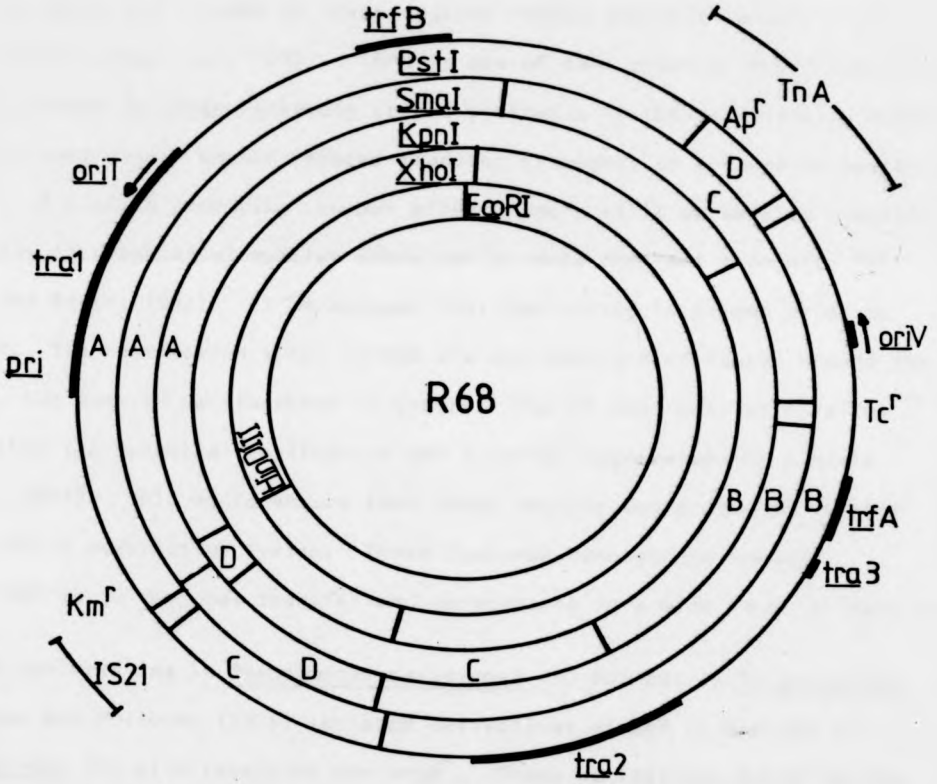
oriV: origin of unidirectional vegetative plasmid DNA replication

oriT: origin of transfer

tra: regions required for conjugal transfer

trf: regions specifying trans-acting replication or maintenance functions

pri: region specifying primase


trfB
PstI
SmaI
KpnI
XhoI
EcoRI
TnA
Ap^r
oriT
tra1
pri
A
A
A
D
C
oriV
R68
Tc^r
HindIII
B
B
B
trfA
tra3
D
Km^r
IS21
C
D
C
tra2

Skurray, 1980). Thomas (1981) suggests that the essential regions of the plasmid RK2 may have been clustered but have become separated by antibiotic resistance transposons. RK2, and presumably R68, possesses considerably more replicating genes than narrow host range plasmids. This has been suggested as giving the plasmid greater independence of the host replication machinery and therefore a greater degree of promiscuity (Thomas, 1981). Certainly Tn7 insertion mutations in some of these regions reduce the host range of R18 (Cowan and Krishnapillai, 1982). RP4 is one of few plasmids which specifies its own primase (primases initiate strand synthesis in the recipient). Mutants in the primase region showed reduced transfer frequency to a range of bacterial species. A plasmid producing its own primase would still be able to transfer conjugally to a bacterial species whose own primase does not recognize RP4 (Lanka and Barth, 1981). It is assumed that the strand is primed prior to transfer. The restriction sites in R68 are not evenly distributed around the plasmid, but tend to be clustered in groups. Few of the restriction sites fall within the essential replication and transfer regions of the plasmid (Thomas, 1981). This would ensure that these regions would tend to survive a recipient's restriction system. These features demonstrate how well adapted R68 is to conjugal transfer and maintenance in a wide range of species.

R68 exhibits Cma in Pseudomonas aeruginosa PAT but not in P. aeruginosa PAO. Haas and Holloway (1976) isolated derivatives of R68 in matings in P. aeruginosa PAO with selection for $ArgB^+$. These derivatives exhibited Cma at 10^{-3} - 10^{-5} recombinants per donor cell in PAO, compared to 10^{-8} for R68. R68.44 and the more stable derivative R68.45 possess all the antibiotic resistance characteristics of the parental R68. This increased ability to mobilize chromosomal DNA is known as enhanced chromosome mobilization (ECM). ECM plasmids have been isolated using similar techniques from other Inc-P plasmids including R18 (Holloway and Richmond, 1973) and R906 (Hedges, et al., 1974). Apart from its increased stability R68.45 and R68.44 are identical as

shown by heteroduplex analysis (Leemans et al., 1980) and restriction digestion (Willetts et al., 1981).

Jacob et al. (1977) showed that R68.45 had acquired a small piece of DNA and was slightly larger than R68. This 2.1 kb of DNA was inserted near the kanamycin resistance gene and has been shown to be a tandem duplication of a portion of R68 (Riess et al., 1980a, b). The insert has been known variously as ISP (Riess et al., 1980b), IS8 (Leemans et al., 1980) and IS21 (Willetts et al., 1981). Leemans et al. (1980) suggested the presence of 0.3 kb of foreign DNA between the copies of IS8. However Willetts et al. (1981) did not detect this. They found that IS21 could transpose at high frequencies to other molecules. They suggest that R68-45 had been formed from R68 by a transition-like event (Rownd et al., 1975). This would produce a duplication of IS21 without any intervening R68 DNA between the two copies. This was first suggested by Riess et al. (1980a). They suggest that the formation of R68.45 from R68 was entirely an R68 dependent event. However this does not explain why R68.45-type plasmids were produced at higher frequencies in certain matings than in others (Haas and Holloway, 1976). It is the tandem duplication of IS21 to produce R68.45 from R68 which is supposed to account for the ECM characteristics of R68-45 (Riess et al., 1980a, b; Leemans et al., 1980; Willetts et al., 1981). Other ECM plasmids have been isolated from nature and shown to be identical to R68.45; for example pM060 (Jacoby and Matthews, 1979). R68.45 was not stable and occasionally reverted to the R68 form, sometimes with the additional loss of Km^r; this instability can be frustrating in experimental work, but can be simply checked for (Haas and Holloway, 1976). A variant of R68.45, R68.45 $sup_{dnaG}15$ was isolated in E. coli which exhibited 72-170 fold higher Cma frequencies than R68.45. This variant mobilized chromosomal genes in Rhizobium meliloti (Ludwig and Johansen, 1980).

In addition to Cma R68 and R68.45 were capable of R-prime formation. R-prime formation occurred at low frequency (10^{-8} per donor cell) with rec A recipients

or in interspecific crosses where there was a lack of homology between the transferred DNA and the recipients' chromosome (Holloway, 1978; Holloway et al., 1980). R68 or its derivatives and relatives have been reported to mobilize the chromosome and generate R-primes in a wide range of species (Table 1.4).

R68.45 mobilized chromosomal DNA of different sizes depending on the donor species; from small (10 minutes of the chromosome) in Pseudomonas aeruginosa PAO (Haas and Holloway, 1976) to large in Rhizobium leguminosarum (Beringer et al., 1978; Johnston et al., 1978). R68.45 generally showed non-polarized chromosomal transfer from multiple sites in the donor chromosome; R68, on the other hand, has only one origin site in P. aeruginosa PAT (Holloway, 1979). Only a small percentage of the recombinant clones inherited the intact plasmid, R68.45 became unstable and the plasmid marker was lost, or the whole plasmid was lost.

The chromosomal DNA inserted into R68.45 in the formation of R-primes (R's) ranged in size from 2.3 kb of Rhizobium meliloti chromosome (Kiss et al., 1980) to about 142 kb of P. aeruginosa PAC chromosome (Hedges and Jacob, 1977a). This latter value is over twice the size of the original R68.45 itself. Some of the R's were unstable (Hedges et al., 1977; Holloway et al., 1978) whilst others have been reported to be stable (Johnston et al., 1978a; Morgan, 1982).

The stability of Inc-P plasmids in P. aeruginosa was strain specific. (Chandler and Krishnapillai, 1974b). This instability usually involved the loss of resistance markers and may be progressive, rather than involving the loss of the entire plasmid (Holloway, 1979). Haas and Holloway (1976) observed that R68.45 often lost its ECM characteristics, this loss was often associated with sensitivity to kanamycin (Km^R). Currier and Morgan (1982) observed structural instability of R68.45 in Erwinia caratovora. The deletions all had one end associated with IS21 and could proceed in either

TABLE 1.4

BACTERIAL SPECIES IN WHICH R68, ITS DERIVATIVES AND RELATIVES EXHIBIT CHROMOSOME MOBILIZATION AND R-PRIME FORMATION

These include:

(a) Chromosome mobilization

Species	Plasmid	Reference
Acinetobacter calcoaceticus	RP4	Towner (1978)
		Towner and Vivian (1976, 1977)
Agrobacterium tumefaciens	R68/R68.45	Hamada *et al.*.(1979)
		Hooykaas *et al.* (1982)
Azospirillum brazilense	R68.45	Franche *et al.* (1981)
A. lipoferum	R68.45	Elmerich and Franche (1981)
Caulobacter crescentus	RP4	Barret *et al.* (1982)
Erwinia chrysanthemi	R68.45	Chatterjee (1980)
Escherichia coli	R68/R68.45	Beringer and Hopwood (1976)
		Riess *et al.* (1980a, b)
		Nayudu and Holloway (1981)
Methylophilus methylotrophus	R68.45	Holloway (1981)
Pseudomonas aeruginosa	R68/R68.45	Stanisich and Holloway (1971)
		Holloway (1975)
		Haas and Holloway (1976, 1978)
		Watson and Holloway (1978a, b)
		Holloway *et al.* (1979)
P. fluorescens	R68.45	Lejeune and Mergeay (1980)
		Lejeune *et al* (1983)
P. glycinea	R68/R68.45/RP1	Lacy and Leary (1976)
		Leary (1979)
P. putida		Martinez and Clarke (1975)
		Holloway (1979)

Species	Plasmid	Reference
Rhizobium leguminosarum	R68/R68.45	Beringer and Hopwood (1976)
		Johnston and Beringer (1977)
		Beringer et al. (1978)
		Johnston et al. (1978a, b)
R. meliloti	R68/R68.45/RP4	Kondorosi et al. (1977)
		Meade and Singer (1977)
		Kowalczuk et al. (1981)
R. trifoli	R68/R68.45/ RP1/RP4	Megias et al. (1981, 1982)
Rhodopseudomonas sphaerides	R68.45	Sistrom (1977)
Zymomonas mobilis	R68.45	Holloway (1981)
(b) R-primes		
Acinetobacter calco-acetiens	R68.45	Moore et al. (1983)
Escherichia coli	R68.45/RP1	Olsen and Gonzalez (1974)
		Hollowway et al. (1980)
		Riess et al. (1983)
Klebsiella pneumonia	R68.45/RP4	Dixon et al. (1976)
		Espin et al. (1981)
Methylophilus methylotrophus	R68.45	Holloway (1981)
		Moore et al. (1983)
Pseudomonas aeruginosa	R68.45	Hedges and Jacob (1977a)
		Hedges et al. (1977)
		Holloway (1978)
		Holloway et al. (1979)
		Morgan (1982)
Rhizobium leguminosarum	R68.45	Johnston et al. (1978a, b)
R. meliloti	R68.45	Kiss et al. (1980)
		Kowalczuk et al. (1981)
R. trifoli	R68.45	Kowalczuk et al. (1981)

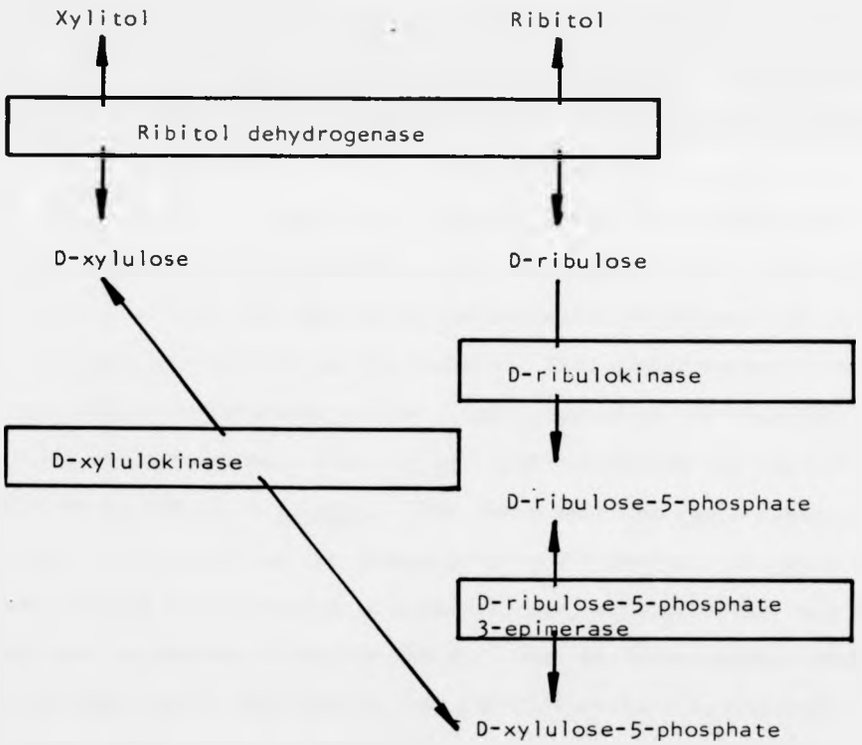
a clockwise or an anti-clockwise direction (Figure 1.4). They suggested that the tandem duplication of IS21 was involved in deletion formation. Deletions associated with phenotypic changes (Km^R, Tra^- or Cma^-) were observed in R68.45 in P. aeruginosa PAO by Haas or Riess (1983). They suggested that deletions of varying lengths occured in a single event in a clockwise direction starting at the lefthand end of the righthand copy of IS21. They did not find any evidence of progressive deletions as occurred in R68 in P. aeruginosa PAT (Godfrey et al., 1980).

Chromosome mobilization in E. coli by F has been discussed above. Holloway (1979) suggested that because of the diversity of plasmids and organisms in which Cma is found, it is probable that there exists more than one mechanism for this phenomenon. Apart from some F-like plasmids there is no evidence that Hfr-formation is the common mechanism for Cma in other plasmids. He suggested that transient integration of the plasmid and chromosome may occur, probably involving rec A^+. However Cma occurred, albeit at a slightly lower frequency, in rec A donors. There is however no evidence for stable integration of R-plasmids, and no demonstration of transient integration. Relatives of R68 have however been made to integrate with the chromosome. Watson and Scaife (1978) cloned λatt into the EcoRI site of RP4, the resultant plasmid promoted low frequency polarized transfer of the E. coli chromosome. Hfr derivatives could be isolated with the plasmid integrated into the E. coli chromosome at attλ. Danilevich et al. (1978) inserted RP4ts (temperature sensitive) into the E. coli chromosome already carrying Tn1 by growing at the non-permissive temperature. Haas et al. (1981) created an Hfr donor of P. aeruginosa PAO by insertion of RPIts into the tryptophan synthase gene. They suggest that the insertion probably involves Tn1. However these artificial examples are the exception rather than the rule. Grinter (1984a, b) isolated an Hfr in E. coli containing RP4 with a fragment of λ cloned into the plasmid inserted into the chromosome. On subsequent excision he found that the plasmid possessed a defective oriV. This suggested

FIGURE 1.4

PATHWAY OF RIBITOL METABOLISM BY KLEBSIELLA AEROGENES[1]

1. Adapted from Clarke (1981)


Xylitol
Ribitol
Ribitol dehydrogenase
D-xylulose
D-ribulose
D-ribulokinase
D-xylulokinase
D-ribulose-5-phosphate
D-ribulose-5-phosphate
3-epimerase
D-xylulose-5-phosphate

that the presence of a second functional origin of replication in the chromosome was deleterious to the host. This could explain why Hfrs of RP4 do not normally occur. In the case of F, its own origin of replication is switched off on integration (Pritchard, 1978).

Hedges and Jacob (1977a) were the first to suggest that the formation of recombinant plasmids by R68.44 involved the interaction with insertion sequences on the plasmid and that this interaction can sometimes lead to the inactivation of the kanamycin resistance gene. Leemans et al. (1980) examined two R's produced by Hedges and Jacob (1977a) by heteroduplex analysis and Southern blotting (Southern, 1979). They found that the chromosomal DNA was integrated into R68.44 at the same position as the duplication in R68.45. In fact the chromosomal DNA insert appeared to be flanked by the direct repeats of R68 DNA. Willetts et al. (1981) identified the tandem repeats as insertion sequences (IS21). They proposed that the formation of a cointegrate during transposition of IS21 from R68.45 to the bacterial chromosome was responsible for chromosome mobilization by the plasmid. This mechanism would explain the low frequency of transfer of the intact plasmid to the recipient when mobilizing the chromosome. Riess et al. (1983) examined the mobilization of pACYC184 by R68.45 in E. coli. They found that the rec A status of the donor had little effect on the frequency of mobilization. In rec A recipients the two plasmids were detected as a cointegrate, with pACYC184 inserted between the two copies of IS21 in R68.45. When the cointegrate resolved they recovered R68, rather than R68.45, and pACYC184 containing one copy of IS21. This copy of IS21 could be inserted in a variety of different sites in pACYC184 but always in the same orientation. In R's with E. coli chromosomal DNA they observed the insert flanked by two copies of IS21 in the same orientation. They suggested that the cointegrate is a model for R' formation. R' formation would therefore occur through the formation of a transient cointegrate with the bacterial chromosome via the two copies of IS21. Insertion

sequence insertion is rec A independent and generally non-site specific (see above). Cma would not require whole plasmid transfer and would therefore occur at a higher frequency than R' formation.

The above model involves the active participation of IS21 in R' formation, and suggests that the chromosomal insert is closely associated with IS21 in the R'. Moore et al. (1983) created R's of pM0170 (identical to R68.45) bearing chromosomal genes from Methylophilus methylotrophus. They examined two of the R's by restriction analysis. In one the chromosomal insert was inserted into one of the copies of IS21. In the second R' they could not identify the location of insertion. In both R's one copy of IS21 was missing. These results were in agreement with the findings of Leemans et al. (1980) on the R's of Hedges and Jacob (1977a), and support the cointegrate model of Riess et al. (1983). However others have located chromosomal inserts in other regions of R's derived from R68.45. Kiss et al. (1980) analysed R's bearing chromosomal genes from Rhizobium meliloti by digestion with Sma I. In one R' they identified the region of insertion as the Sma I-D region of R68.45 (Figure 1.4). This region extends in an anti-clockwise direction from the IS21 region. This plasmid had lost the second copy of IS21. Johnston et al. (1978a) created R's of R68.45 bearing chromosomal genes from Rhizobium leguminosarum. They analysed three R's by Sma I digestion. All three lacked the second copy of IS21, but so had the plasmid which they had digested as R68.45, it was in fact R68. In two of the R's the chromosomal insert was into the largest Sma I band, Sma I-A. This band runs clockwise from the IS21 region. In the third R' the insert was into Sma I-D, the region anti-clockwise from the IS21 region. In all these cases though the site of insertion in the R's of the chromosomal inserts were not precisely located, it is possible that they were all near the region of the tandem duplication of IS21. However the chromosomal insert could equally be towards the other extremities of the Sma I A or D bands, in which case the

association with IS21 would be remote. These results do not confirm the involvement of IS21 in R' formation; more accurate mapping of the location of the chromosomal insert would be required.

R68.45 was isolated in P. aeruginosa PAO from rare matings using R68 and selection for chromosomal markers, particularly arg B (Haas and Holloway, 1976). As discussed above the ECM character of R68.45 was shown to be solely of R68 origin. Attempts have been made to isolate similar ECM derivatives in E. coli by the same methods (Nayudu and Holloway, 1981). They derived two plasmids, pM0163 and pM0168, which showed similar Cma frequencies to R68.45 in E. coli, but not in P. aeruginosa PAO. pM0163 possessed an additional 1.2 kb of DNA near the ampicillin resistance transposon, TnA, while pM0168 had a small insert about 6kb anti-clockwise from the sole EcoRI site of R68 (Figure 1.4). The two inserts had no sequence homology with IS21; however they did show homology with the E. coli chromosome. The restriction patterns of the inserts showed no similarity with those of several known insertion sequences. Nayudu and Holloway (1981) suggest that the inserts were probably transposable elements derived from the E. coli chromosome, and that they were involved in the ECM of the two plasmids. These results suggest the involvement of insertion sequences in Cma. Though why, if the formation of R68.45 from R68 is an R68 mediated event, should R68.45 be isolated only in certain matings in P. aeruginosa PAO is difficult to explain. Transposons and insertion sequences have been introduced into R68 or its relatives and shown to produce Cma and R' formation, for example bacteriophage Mu (Denarie et al., 1977; Murooka et al., 1981; Van-Gijsegem and Toussaint 1982, 1983; Schoonejans and Toussaint, 1983) and Tn 501 (Pemberton and St. G. Bowen, 1981). In these cases Cma was usually polarized from specific origins in the chromosome of Mu lysogens. In R' formation the chromosomal insert was flanked by two copies of Mu (Schoonejans and Toussaint, 1983).

In E. coli F's had been shown to exhibit high Cma compared to F on its

own (Pittard and Adelberg, 1963). Similar Cma had been observed in other plasmid-primes, for example SPC1's in Streptomyces coelicolor (Hopwood and Wright, 1976a, b). In these cases Cma of markers occurred at frequencies dependent on their distance on the bacterial chromosome from the marker carried on the plasmid-prime. Artificial R's have been constructed of R68 or its relatives by cloning chromosomal fragments into the unique Hind III or Eco RI sites (Jacob et al., 1976; Nagahari et al., 1977; Barth, 1979; Juilliot and Boistard, 1979; Beck et al., 1982). The derivative plasmids possessed Cma in the hosts from whose chromosome the inserts were derived. Cma occurred from a particular origin in the chromosome and was dependent on a functional rec A in the donor. Presumably Cma occurred due to homology between the cloned insert and the bacterial chromosome, and it was from this region of homology that Cma occurred. Supporting evidence for this was provided by Grinter (1981) who cloned λ DNA into the Hind III site of RP4. He found that Cma was highest from rec A+ strains lysogenic for λ, non-lysogens and rec A strains showed greatly reduced activity.

Cma or R' formations are therefore dependent on features of the plasmid. If the plasmid contains transposable elements Cma can be random as in the case of R68.45 with IS21 or pM0163 and pM168 with their unidentified insertion sequence; or site specific as for example with the RP4::Mu constructs discussed above. In these cases Cma and R' formation are rec A independent. On the other hand with R' constructs Cma is site specific and rec A dependent.

Such broad host-range Cma plasmids as R68 can transfer genetic material between a wide range of bacterial species. Such a mechanism would allow for the dissemination of genes throughout a natural bacterial population. A gene for a new enzyme of metabolic function would only have to evolve once to be spread to other species, where the new gene could be incorporated into the chromosomal DNA. Alternatively such plasmids allow the bringing together

of different genes evolved in different species perhaps to form a novel pathway.

1.3 Selection in Chemostats

This section will briefly describe the growth of bacteria in chemostats before going on to deal in more detail with the effects of various limiting growth conditions on plasmid stability in chemostat grown plasmid containing bacterial populations.

1.3.1 Microbial growth in the chemostat

A chemostat is a device by which a bacterial culture can be maintained growing at a steady state via external control. It was invented by Novick and Szilard (1950). Growth medium is fed into the culture vessel at a continuous rate while the excess culture is removed by way of some over flow device. The aeration, temperature, pH and rate of influx of fresh medium can be controlled.

Chemostat theory has been dealt with in detail elsewhere (Bull, 1974; Dykuizen and Hartl, 1983). A discussion of the essentials of growth in the chemostat is all that is required here.

The Monod equation (Monod, 1942) describes the basic growth of a bacterial culture

$$\mu = \frac{\mu max\ S}{K_s + S}$$

where μ is the specific growth rate of the micro-organism, μ_{max} is the maximum specific growth rate, S is the growth-limiting substrate concentration, and K_s is the substrate saturation constant (this is numerically equivalent to S at $\mu_{max}/2$).

The rate of influx of fresh medium into the chemostat, the dilution rate

(D, units h^{-1}) is defined as

$$D = \frac{F}{V}$$

where F is the flow through the culture vessel and V is the culture volume.

In the chemostat the rate of change of biomass with respect to time equals the rate of production of biomass by growth minus the rate of removal of biomass from the culture vessel. This can be described mathematically as

$$\frac{dx}{dt} = \mu x - Dx$$

where x is the biomass.

At a steady state $\frac{dx}{dt} = 0$

Therefore $\mu = D$

Thus the growth rate of an organism is determined by the dilution rate, at least at low dilution rates when the substrate concentration is limiting. In other words there is selection amongst a chemostat population for organisms with increased values of μ. μ could be increased due to the increased production of some enzyme essential for the metabolism of the limiting growth substrate, as was observed in P. putida PP3 by Weightman and Slater (1980) or via some increased efficiency of utilization of the growth substrate.

1.3.2 Plasmids in Chemostat Populations

The presence of non-essential plasmids in a chemostat bacterial population is generally considered to constitute a growth disadvantage (Melling et al., 1977; Godwin and Slater, 1980; Adams et al., 1979; Dale and Smith, 1979; Klemperer et al., 1979; Jones and Primrose, 1979; Wouters and Van Andel, 1979; Jones et al., 1980; Wouters et al., 1980; Helling et al., 1981; Noack et al., 1982; Aldrick and Smith, 1983). The presence of a plasmid is considered as placing a significant burden on the metabolic economy of the cell, thereby reducing the cells μ_{max} comparative to plasmid minus

strains, leading to the latter out competing the former and taking over the chemostat.

Reduced growth characteristics due to the presence of a plasmid is demonstrable in many cases though not in all. Zund and Lebek (1980) examined in over 100 R plasmids the effect of the plasmid on the generation time of E. coli. They found that in one quarter of the cases the generation time was increased significantly. However in the case of plasmids larger than 80kb the majority of the R^+ strains showed increased generation times. However these batch growth experiments may not detect the subtle differences in growth rate which may play a crucial role in longer term chemostat studies. Dale and Smith (1979) for example obtained identical growth characteristics for E. coli 114 with or without the plasmid pH121. Yet in mixed batch culture with repeated subculturing, they observed that the plasmid-minus strain had a higher growth rate and outcompeted the plasmid-plus strain. They concluded that the presence of the plasmid affected the organisms ability to compete for nutrients.

Plasmids of a range of sizes show a negative effect on the competitive ability of their hosts against plasmid-minus strains under a wide range of limiting conditions. E. coli strains containing the cloning vector pBR322 were outcompeted by plasmid-minus strains derived from the plasmid-plus strains when grown in chemostats under either phosphate or carbon limitation (Jones and Primrose, 1979; Wouters et al., 1980). On the other hand Melling et al. (1977) found that when E. coli containing the conjugative plasmid RP1 was grown in the chemostat under carbon, magnesium or phosphate limiting conditions the plasmid persisted under all growth conditions. Yet in a chemostat culture containing both R^+ and R^- strains grown under phosphate limitation, the R^- strain took over. However under magnesium and carbon limitation the R^- strain did not. Slater and Godwin (1980) point out, with reference to these experiments, the difficulties involved in the initial establishment of a competition experiment which can influence the outcome. Certainly Wouters

and Van Andel (1979) found in chemostat competition studies with E. coli containing RP1 that the R^- strain outcompeted the R^+ under carbon and nitrogen limitation; however under phosphate limitation in aerobic conditions the reverse was the case. With E. coli and the plasmid R6, on the other hand, they observed that the R^- strain outcompeted the R^+ under all three limiting conditions.

Godwin and Slater (1979) grew E. coli K12 containing TP120 in a chemostat under limiting conditions. Under glucose limitation the plasmid was not eliminated from the population; however a strain containing the plasmid which had lost the tetracycline resistance (Tc^R) marker predominated. Under phosphate limitation a mixed culture was produced containing plasmids which had lost a variety of different markers. A fragmentation of the plasmid appeared to have occurred. TP120A which had lost Tc^R was shown to be 40% smaller than the original TP120 (Slater and Godwin, 1980). In competition studies between strains containing TP120 and TP120A, that containing TP120A won. Therefore the loss of part of a plasmid can confer a selective advantage to its host. A similar result was observed with pBR325 (Noack et al., 1982) where under limiting conditions Tc^R was lost more rapidly than the other plasmid determined resistance markers. However in this case no difference in molecular weight was observed between the Tc^S derivative and the original pBR325.

Characteristics of the plasmid can influence the outcome of competition experiments between strains possessing or lacking a plasmid. Adams et al. (1979) studied the growth in a chemostat of E. coli containing the colicin producing plasmid RSF2124. The relative initial concentrations of the plasmid-plus or -minus strains influenced the outcome of the competition experiments. Though the plasmid-minus strain had a higher growth rate than the plasmid-plus strain and usually outcompeted it, above a certain concentration of the plasmid-plus strain sufficient colicin was produced to

inhibit the growth of the plasmid-minus strain and this strain was outcompeted. In similar experiments using non-colicin producing mutants of the plasmid, the plasmid-minus strain always outcompeted the plasmid-plus strain. In most of these competition experiments the plasmid-plus strain was not entirely eliminated from the culture but was maintained at an extremely low frequency, less than 0.01% in the experiment described above. Helling et al. (1981) grew E. coli containing a colicin-minus mutant of RSF2124 in a chemostat under carbon limitation. The presence of the plasmid lowered the growth rate of its host. They observed an initial decline in the proportion of the population containing the plasmid, followed by oscillations in its relative concentration in the chemostat. RSF2124 contains the transposon Tn3. They conclude that though the plasmid generally conferred a disadvantage, the advantages due to some Tn3 induced mutations periodically outweigh the disadvantages, thereby engendering a cyclical relationship between the plasmid containing and the plasmid lacking strains.

Plasmid-minus strains outcompete plasmid containing strains under a wide range of growth conditions due to the increased μ_{max} of the plasmid-minus strains. The generation of plasmid-minus strains from plasmid-plus strains, segregational loss of the plasmid, is variable and is due to the fidelity of partition of plasmids amongst daughter cells. Jones et al. (1980) found that the plasmids RP1, pDS401 and pDS1109 were maintained without detecting plasmid-minus segregants for at least 120 generations, despite, in the case of pDS1109, the plasmid copy number falling to one fifth of its original value. However if a plasmid-minus inoculum was added this rapidly outcompeted the resident strain. Plasmids pBR322 and pMB9 generated plasmid-free segregants within 30 generations. They concluded that these differences reflect differences in fidelity of partition; they suggested that pBR322 and pMB9 were deficient in this partition function.

Growth conditions in a chemostat can be seen as approximating more to

conditions in the environment than batch culture, especially if they contain a mixed bacterial population (Slater and Bull, 1982). Chemostats can therefore be used as models of environmental events. Conjugal plasmid transfer has been studied in chemostats to determine how important this phenomenon is in the environment. The transfer of R1 and other plasmids has been studied in the chemostat (Levin et al., 1979; Freter et al., 1983). The transfer rates were found to be low but not negligible. Freter et al. (1983) found that the transfer was often secondary, via a transconjugant. All the population did not acquire the plasmid. Rather there was an equilibrium between transconjugants and potential recipients.

The transfer of biodegradative plasmids has also been demonstrated in chemostats. Pseudomonas Sp. B13 capable of growth on 3-chlorobenzoate, acquired the ability to grow on 3,5-dichlorobenzoate through its acquisition of the TOL plasmid from P. putida mt-2 in a chemostat, TOL codes for the required non-specific benzoate dioxygenase (Hartman et al., 1979; Reineke and Knackmus, 1979). Kellogg et al. (1981) grew mixed bacterial populations from toxic waste dumps with organisms containing a variety of biodegradative plasmids in a chemostat on 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and a range of plasmid substrates. The concentration of 2,4,5-T was gradually increased over more than 10 months to 1.5 - 2.0 $mgml^{-1}$, at which point the chemostat culture could be shown to be growing on 2,4,5-T. Whether plasmid transfer had anything to do with this it is difficult to say.

1.4 The Evolution of Enzyme Systems

The raw material for evolution is random point mutations of DNA. The bulk of these mutations are deleterious, but it is only through the gradual accumulation of beneficial mutations that new characteristics emerge. Yet in the last 40 years bacteria have been isolated world wide which possess multiple antibiotic resistance, or the ability to degrade xenobiotics and

other esoteric compounds. The apparent rapid and multiple evolution of these phenotypes would appear to challenge the conventional view of evolution. In this section I will outline some experimental systems in which enzyme evolution has been studied and will deal briefly with possible mechanisms of pathway evolution before going on to deal in more detail with the role of plasmids in bacterial evolution.

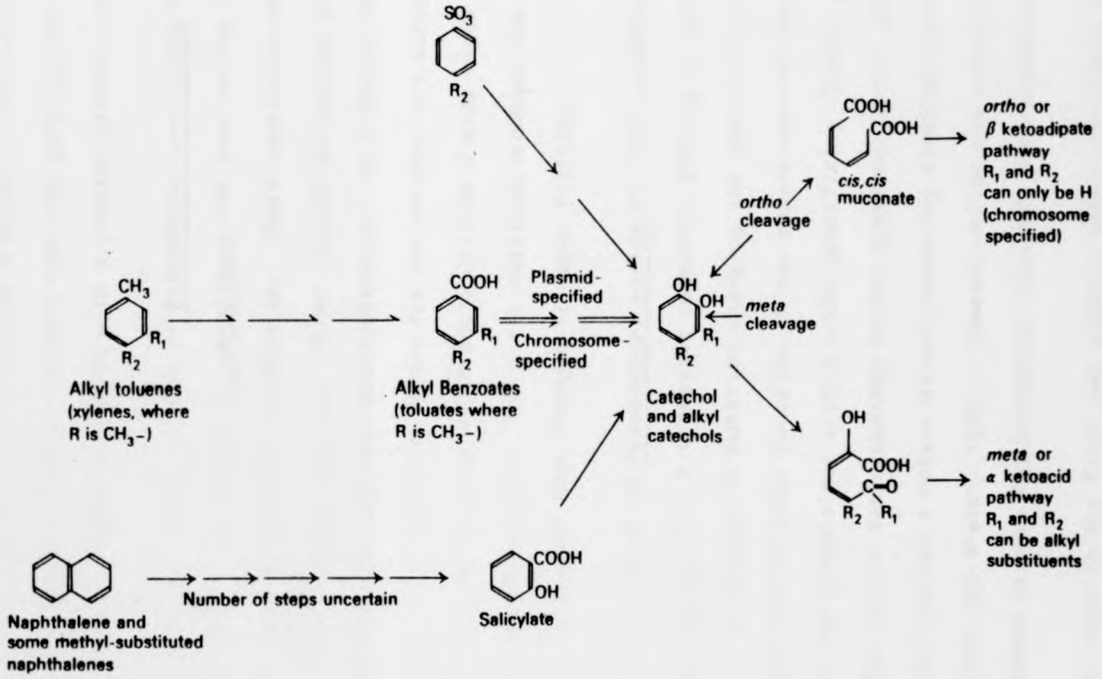
1.4.1 The Evolution of New Enzymes

Many enzymes can alter their specificity through a few simple mutations. Klebsiella aerogenes will grow on many naturally occurring pentoses and pentitols. Other pentoses and pentitols are rare, and though they often can be metabolized they rarely act as inducers for the enzymes required for their metabolisms. However mutants will grow on these substrates; these mutants tend to be constitutive for the required metabolic enzymes (Mortlock, 1976). The following example will illustrate this. Wu et al. (1968) found that Klebsiella aerogenes grew on ribitol by the following pathway (Figure 1.4). D-ribulose induces ribitol dehydrogenases. They isolated a constitutive mutant for ribitol dehydrogenase which was capable of growth on xylitol which is a non-inducing substrate for this enzyme. Two subsequent mutations increased the affinity of the enzyme for xylitol and increased the rate of xylitol uptake. This illustrates the non-absolute specificity of many metabolic enzymes. Three mutations had broadened the range of substrates which Klebsiella aerogenes can utilize. In fact the enzymes mentioned above overlap with a range of other pathways for the metabolism of pentoses and pentitols, so that a mutation in the gene for a single enzyme can have far reaching effects in related pathways (Mortlock, 1976). Further mutations were possible to alter the affinity of ribitol dehydrogenase for xylitol. Hartley (1974) grew Wu et al. (1968)'s ribitol dehydrogenase constitutive mutant in a chemostat on xylitol. From the chemostat he isolated a strain which

FIGURE 1.5

PLASMID MEDIATED DEGRADATIVE PATHWAYS CONVERGING ON CATECHOL[1]

1. Taken from Broda (1979)



***Ps. putida*-borne plasmid-mediated degradative pathways that converge upon catechol.**

produced increased quantities of ribitol dehydrogenase. The enzyme however retained its original poor specific activity towards xylitol. He suggested that this increased production might be due to gene duplication. Hartley et al. (1976) subsequently isolated a mutant strain with a high specific activity towards xylitol. In this case the gene seemed to have undergone mutations to produce an enzyme with a greater affinity towards xylitol.

A similar system is that of Pseudomonas aeruginosa PAC amidase studied by Clarke (1974). Wild type P. aeruginosa PAC grew on the 2- and 3-carbon amides, acetamide and prop-ionamide. These substrates were hydrolysed by an inducible aliphatic amidase. Other related amides had different limited specificities as inducers and substrates. By a series of mutations in the structural and regulatory genes for the amidase she succeeded in obtaining a range of mutant strains which could utilize a wide range of amides with up to 5- and 6-carbon chains. The first mutation rendered the amidase constitutive and the subsequent ones changed its specificities.

The formation of Pseudomonas putida P3 (PP3) capable of growth on 22DCPA from P. putida S3 in a chemostat community growing on Dalapon (Senior et al., 1976) is an example of mutation changing the specificity of an enzyme. In this case the original function of the enzyme was not known, it was assumed that it was a hydrolase with a broad specificity (Slater et al., 1979). As with the ribitol dehydrogenase mutant mentioned above, growth of PP3 in a chemostat yielded a mutant strain producing elevated levels of dehalogenase I (Weightman and Slater, 1980). However unlike the mutant ribitol dehydrogenase and amidase strains PP3 possessed inducible dehalogenases.

At least in the first two examples above the enzyme with altered specifications was derived from an existing functional enzyme. However new enzymes can be derived from genes in silent regions of the chromosome. Hall and Hartl (1974, 1975) studied a strain of E. coli K12 with a deletion in the

lac Z gene. It was unable to grow on lactose as it did not produce β-galactosidase. However they succeeded in isolating mutant strains which could grow on lactose. These mutations mapped in a different region of the E. coli chromosome from the lac operon, in the ebg operon; which in strains of E. coli with a functional lac operon did not direct the synthesis of lactose metabolizing enzymes. These silent regions of the chromosome may be remnants of genes for past metabolic activities of the organism. However they also provide a reservoir of genetic material for future evolution.

Gene duplication in which a copy of the original gene is retained may be an important source of evolutionary change in enzyme activity. The original gene is retained whilst mutations can occur in the copy. Hartley (1974) remarked upon the similarity in sequence and structure of NADH-dependent dehydrogenases. He suggested that these had evolved from an archetypal enzyme with a broad specificity which had been duplicated on many occasions. Modern NADH dependent dehydrogenases have high specificity and high rates of reaction. This type of evolution can be viewed as horizontal evolution.

Horowitz (1945, 1965) suggested that retrograde evolution could account for the evolution of biosynthetic pathways involved in amino acid synthesis. Gene duplication followed by mutation would lead to the evolution of the genes for consecutive enzymes in the pathway. This vertical evolution would require that consecutive steps in the pathway be broadly similar, or at least not radically different. Though this may be the case in certain parts of a pathway it is unlikely to be the case throughout. Clarke (1981) suggested that retrograde evolution is an unlikely method for the evolution of catabolic pathways as not all steps in such pathways are energy providing and many of the intermediates are unstable. She suggests that the horizontal duplication of an entire pathway followed by the mutation of the genes for the individual enzymes within it is a more probable explanation for the evolution of new catabolic pathways.

1.4.2 The Role of Plasmids in Enzyme Evolution

Though mutation probably does give rise to new enzymes in the long term, much mutation constitutes genetic change in a negative sense; it can be viewed as 'noise' (Reanney, 1978). General recombination (discussed above, 1.2) can be viewed as essentially conservative in nature. In other words no loss or gain of genetic information occurs and the order of the genes is not disturbed. General recombination tends to occur between closely related molecules, thereby minimizing variations in the gene pool and tending to equilibrate traits from both parents. Illegitimate recombination, on the other hand, can unite heterologous DNA sequences which have already passed the test of evolution. Such recombination can generate large scale sequence rearrangements, thereby bringing together genetic material from different sources both within the same organisms and between different organisms. Transposons and plasmids can play a crucial role in such macro-genetic shuffling.

Promiscuous plasmids can transfer genetic material between a wide range of bacterial species (Section 1.2). This would enable the spread of a gene which has evolved in one bacterium throughout a bacterial population. Hughes and Datta (1983) and Datta and Hughes (1983) examined the collection of Enterobacteriaceae isolated world wide by E.D.G. Murray during the 'pre-antibiotic' era (1917-1954). 24% of the strains contained transfer proficient plasmids. Amongst these antibiotic resistance was present in only a very small percentage. They examined the incompatibility groups of these 'pre-antibiotic' plasmids and found that 65 out of 84 belonged to the same incompatibility groups as modern antibiotic resistance (R) plasmids. They suggested that these modern plasmids must have acquired their antibiotic resistance genes in the past 25 years through the insertion of new genes into existing plasmids rather than by the spread of previously rare plasmids. This view is borne out by an examination of β-lactamases produced by ampicillin resistant bacteria. Matthew and Hedges (1976) examined TEM-type β-lactamases

produced by plasmids from a wide range of incompatability groups which had been isolated world-wide. They found them to be very closely related. They suggested that lateral gene transfer by promiscuous plasmids would account for this finding.

An examination of the catabolic pathways encoded by the degradative plasmids NAH, SAL and TOL (see Section 1.2) shows that they all converge on catachol (Figure 1.5). Catachol being metabolised via the meta-cleavage pathway. This suggests that parts at least of these pathways share a common evolutionary origin. Supporting evidence for this comes from the finding that these three plasmids share regions of homology (Bayley et al., 1979; Lehrbach et al., 1983). That this homology is not solely due to their transfer genes (they are all in the IncP-9 group) is suggested by heteroduplex mapping of the naphthalene oxidation genes on NAH7 and SAL1 which indicates that these two plasmids have very close homology in this region (Yen et al., 1983). Chatterjee and Chakrabarty (1983) also found very close homology between independently isolated chlorobenzoate-degradative plasmids.

Many degradative plasmids are transfer-proficient (Chakrabarty, 1976). The presence of novel carbon sources in the environment such as 2,4-dichlorophenoxyacetic acid (2,4-D) has been suggested as leading to the spread of 2,4-D degrading plasmids amongst soil bacteria (Pemberton et al., 1979). Such continuous exchange of genetic information on plasmids can accelerate evolution by yielding new recombinants (Reanney, 1976).

Degradative plasmids consist of a large cluster of enzyme structural and regulatory genes and another of transfer genes. Other areas of the plasmids are cryptic. Reactions of degradative plasmids in different hosts and interactions between the plasmids suggest they may be constructed in a modular fashion. An octane utilizing plasmid found in Pseudomonas oleovorans dissociated to form three conjugative plasmids, OCT, MER and K in P. putida

(Chakrabarty and Friello, 1974). The NIC plasmid from P. convexa dissociated to give the sex factor T in P. putida (Thacker and Gunzalus, 1979). The TOL plasmid could dissociate to give TOL (Tra^+, Tol^-) and TOL^* (Tra^-, Tol^+); these two plasmids could reassociate to form a cointegrate (Chakrabarty et al., 1978). TOL^* has been shown to transpose from TOL to other replicons including the broad host-range plasmid RP4 (Nakazawa et al., 1978; Chakrabarty et al., 1978; Jacoby et al., 1978; Ribbons and Williams, 1981). TOL^* can form a transfer proficient cointegrate with K (Chakrabarty et al., 1978). Similarly CAM and OCT can form a cointegrate under certain conditions (Chakrabarty, 1973; Chou et al., 1974). These findings indicate that components of degradative plasmids can disassociate and reintegrate readily to form new genetic permutations. These translocational events suggest that many of these degradative genes are on transposons.

Richmond and Wiedman (1974) have postulated an origin for such modular, transposable, genetic units. They suggested that the acquisition by a bacterium of an IS-type sequence tends to multiple insertions into the bacterial chromosome. Natural selection would eliminate those bacteria in which the inserts inactivate genes. The net effect, therefore, would be to select for inserts at the ends of genes in the chromosome and on plasmids. This modular substructure of the genome could easily lead to the rearrangement of genes, by creating 'hot spots' of recombination, or by enabling virtually any gene to translocate. These genes could then be transferred to other cells or species by promiscuous plasmids.

Above I have stressed the plasticity and interactions of prokaryotic genomes and their extra chromosomal elements. However there also exists a body of evidence which points to the stability and conservatism of bacterial chromosomes. This evidence points out that the macro genominc rearrangements brought about by illegitimate recombination are unlikely to generate competitive phenotypes. Rather the gradual accumulation of small beneficial

mutations tried and tested by selection, coupled with general recombination between closely related molecules will bring about long-term evolutionary change (Sanderson, 1976).

If there were major reshuffling of genes between different groups of bacteria, the existence of clear evolutionary lines would be unlikely. But at the same time major differences in the sequence of genes on the chromosome would be expected even between closely related species. Sanderson (1976) compared the linkage maps of E. coli and Salmonella typhimurium. He found that many of the genes were homologous and that the bulk of the genes were in the same order on both maps. What differences there were could be accounted for by mutation rather than recombination. He compared the amino acid sequences of the enzymes involved in tryptophan synthesis. What differences there were were generally proportional to the taxanomic distance separating the species. Molecular hybridization between the two genomes revealed 80-89% homology (Brenner et al., 1972). Yet these two organisms inhabit the same colon ecosystem, where the high population maximizes the opportunities for cell to cell contact, and both possess potential transfer agents such as prophages and conjugative plasmids. Sanderson (1976) concluded that though gene transfer and integration were possible, there was no evidence for the random shuffling of genes between the chromosomes of different genera as they evolved.

These two views, the potential for genetic plasticity, and the conservatism of the bacterial genome, emphasize what appear to be extreme positions. In fact they may paradoxically not be incompatible. The bulk of the bacterial chromosome contains genetic material for the vast majority of metabolic functions which bacteria encounter in an average range of environmental conditions. Colon flora incidentally inhabit an unusually stable ecosystem. This genetic material has been tried and tested, in other words optimized, by selection throughout the evolutionary past of the bacteria. Therefore this

material would be expected to be particularly well adapted to its function. It is therefore probable that bacteria possess mechanisms to stably conserve this large core of genetic material; any change to it would normally be resisted. Extra chromosomal genetic elements on the other hand are more plastic. They are opportunistic and can engage in rapid and adventurous evolution via the permuting of genes which have previously evolved separately in different species. Bacteria have therefore separate and distinct evolutionary systems. The one dealing with what could be described as the conventional range of environmental and metabolic situations; the other dealing with novel or unusual challenges faced by bacterial populations as wholes, such as heavy metals, antibiotics or xenobiotic compounds. This latter system can cope rapidly with new challenges. Presumably it would only be after considerable exposure to these novel situations that the genes required for coping with them would become stably incorporated into the bacterial chromosome.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2

MATERIALS AND METHODS

2.1 Conditions for culturing bacteria

Bacteria were grown on minimal media or complex media either as solid or liquid.

2.1.1 Minimal media

This was as described by Slater et al. (1979). It contained Basal Medium (Table 2.1) and 0.005 volumes of Trace Elements (Table 2.2); this was sterilized by autoclaving for 15 min. at 10^5 Nm^{-2}. For liquid media, sterile carbon source and other supplements such as antibiotics and amino acids were added to the cold media. For solid media 2% agar (London Analytical and Bacteriological Media Ltd., London) was autoclaved with the media and the carbon source and other supplements were added when the media had cooled to 50°C. The media was then poured into petri dishes and allowed to cool.

TABLE 2.1

Basal Medium

Compound	For 1l.
Dipotassium hydrogen ortho phosphate	1.5g
Potassium dihydrogen orthophosphate	0.5g
Ammonium sulphate	0.5g
Magnesium sulphate	0.2g
Distilled water	To 1l.

TABLE 2.2

Trace Elements

Compound	For 1l.
Na_2 ethylene diamine tetra acetic acid (EDTA)	24g
Na OH	4g
$MgSO_4$ $7H_2O$	2g
$ZnSO_4$ $7H_2O$	0.8g
$MnSO_4$ $7H_2O$	0.8g
$CuSO_4$ $5H_2O$	0.2g
$FeSO_4$ $7H_2O$	4g
H_2SO_4 (concentrated)	1ml
$NaSO_4$	20g
$NaMoO_4$ $2H_2O$	0.2g
Distilled water	To 1l.
	Store in the dark

Carbon sources were used, unless otherwise indicated at 5g carbon per litre. They were made up as 100X stock solutions, sterilized and stored at $4^{\circ}C$. Glucose, sodium lactate and sodium succinate were sterilized by autoclaving for 10 min. at 6.9×10^4 Nm^{-2}. In the case of the chlorinated alkanoic acids: monochloroacetic acid (MCA) (May and Baker, Dagenham, Essex), dichloroacetic acid (DCA) (Koch Light), 2-monochloropropionic acid (2MCPA) (Koch Light) and 2,2-dichloropropionic acid (22 DCPA) (fractional distillate from technical grade material provided by the Dow Chemical Company, Kings Lynn, Norfolk), the pH was adjusted to 7.0 by the addition of NaOH and the stock solutions sterilized by filtration through 0.22µm membrane filters (Sartorius).

Stock solutions of antibiotics were made up as follows: ampicillin, 25mgml^{-1} in water of the sodium salt (Sigma), sterilized by filtration and kept at 4°C; chloramphenicol (Sigma), 34mgml^{-1} in 100% ethanol. stored at -20°C; kanamycin (Sigma), 25mgml^{-1} of kanamycin sulphate in water, sterilized by filtration and stored at -20°C; rifampicin (Sigma), 20mgml^{-1} in 100% methanol and stored at 4°C; streptomycin (Sigma), 100mgml^{-1} streptomycin sulphate in water, sterilized by filtration and stored at -20°C; tetracycline (Sigma), 12.5mgml^{-1} of tetracycline hydrochloride in 50% ethanol (v/v), sterilized by filtration and stored at -20°C. Unless otherwise indicated these antibiotics were used at the following concentrations: ampicillin (Ap), 50µgml^{-1}; chloramphenicol (Cm), 34µgml^{-1}; kanamycin (Km), 50µgml^{-1}; rifampicin (Rif) 250µgml^{-1}; streptomycin (Sm), 750µgml^{-1}; and tetracycline (Tc), 150µgml^{-1}.

Amino acids (Sigma) were used, unless otherwise stated, at concentrations of 25µgml^{-1}. They were made up as 100X stock solutions in water and, with the exception of tryptophan sterilized by autoclaving for 10 min. at 6.9 x 10^{4} Nm^{-2} and stored at 4°C. Tryptophan was sterilized by filtration.

2.1.2 Complex media

For solid media this consisted of nutrient agar made up according to the manufacturer's instructions (Oxoid). This was supplemented as required with antibiotics as above. Liquid complex media consisted of L-broth (Miller, 1972). L-broth contained tryptone (Difco, Michigan, USA), 10gl^{-1}; yeast extract (Difco, Michigan, USA), 5gl^{-1}; NaCl, 10gl^{-1}. The pH was adjusted to 7.5 and the medium sterilized by autoclaving for 15 min. at 10^{5} Nm^{-2}.

Liquid cultures consisted of 10ml in 25ml Universal bottles, 100ml in 250ml conical flasks or 500ml in 2l conical flasks. Liquid cultures were incubated with shaking at ca.100r.p.m. in a Gallenkamp orbital incubator at

30°C for Pseudomonas spp. and 37°C for E. coli. The same temperatures were used for solid media cultures.

2.1.3 King's A and B media

These media were for distinguishing P. aeruginosa and P. putida, and were made up as described by King and Phillips (1978):

A. for pyocyanin	Peptone	$20gl^{-1}$
	Glycerol	$10gl^{-1}$
	K_2SO_4 anhyd.	$10gl^{-1}$
	$MgSO_4$ anhyd.	$1.4gl^{-1}$
	Agar	2% (w/v)
B. for fluorescein	Protease peptone	$20gl^{-1}$
	Glycerol	$10gl^{-1}$
	K_2HPO_4	$1.5gl^{-1}$
	$MgSO_4\ 7H_2O$	$1.5gl^{-1}$
	Agar	2% (w/v)

2.1.4 Storage of bacterial cultures

For short-term storage cultures were kept on agar plates wrapped in Parafilm at 4°C. For longer-term storage cultures were kept on tightly sealed agar slopes containing the appropriate medium at 4°C. In addition 3ml of liquid cultures were mixed with 2ml glycerol 80% (w/v) in basal medium and kept at -20°C. Where possible repeated subculturing of cultures was avoided, and as required fresh plates were inoculated from long-term storage cultures.

2.1.5 Measurement of bacterial growth

A small volume of an overnight culture was inoculated into 100ml of the appropriate liquid medium in a 250ml flask with a side arm, and the flask shaken and incubated. The change in absorbance at 600nm. was followed in a Corning Model 252 calorimeter.

2.1.6 Estimation of bacterial numbers

Serial dilutions were made in phosphate buffer (pH 7.0) (39ml of a $27.8gl^{-1}$ solution of NaH_2PO_4 $7H_2O$, 61ml of a $53.65gl^{-1}$ solution of Na_2HPO_4 $7H_2O$ plus 100ml H_2O). 100µl of appropriate dilutions was spread on an agar plate and the number of colony forming units (c.f.u.) counted after incubation. Alternatively the method of Miles and Mizra (1938) was used in which 20µl spots of diluted culture were allowed to dry on agar plates before incubation.

2.1.7 Continuous culture

This was performed as described by Weightman and Slater (1980). The apparatus consisted of a LHE Series 500 Fermenter Unit (L.H. Engineering, Stoke Poges, Bucks.) The culture volume was 800ml and was agitated at 1000r.p.m. via a L.H. agitation unit and was aerated at a rate of 800ml min^{-1}. Media was pumped into the culture vessel by a Watson-Marlow MHRE 2 flow inducer to give a dilution rate of $D = 0.1h^{-1}$. The media inlet tube included a glass media break to minimize grow back. The media was pumped from a 20 l. glass media vessel. Excess culture was collected in an overflow pipe in a 20 l. container. The chemostat included a port through which the culture could be inoculated and another through which it could be sampled. The tubing was silicone rubber and the joints were held securely together with nylon straps attached by a 'Securatube' tensioning gun (Schuco Scientific Ltd., London). The whole apparatus was sterilized by autoclaving for 20 min.

at $10^5 Nm^{-2}$ before use. The chemostat was run in a warm room at a constant temperature of 30°C.

The medium was that of Hershey (1955):

Tris	12.1g
NaCl	5.4g
KCl	3.0g
NH_4Cl	1.1g
$CaCl_2$	0.11g
$MgCl_2$	0.095g
$NaSO_4$	0.5g
$FeCl_3$	0.00016g
Distilled water to 1 l.	

18 l. were made up in a 20 l. glass container, the pH was adjusted to 7.5 with HCl and the medium was autoclaved for 40 min. at $10^5 Nm^{-2}$. On cooling sterile phosphate as KH_2PO_4 was added at a limiting concentration of $0.005gl^{-1}$ as were the appropriate carbon source and other supplements.

2.2 Isolation of mutants

Antibiotic resistant and auxotrophic mutants were isolated.

2.2.1 Antibiotic resistance

100ml of complex media was inoculated and grown to mid exponential phase. The antibiotic at half the desired concentration was added and incubation continued overnight. 100µl samples of the culture were plated out on nutrient agar plates containing the full strength antibiotic. After incubation individual colonies were picked off.

2.2.2 Auxotrophs

1. A single colony was inoculated into 20ml L-broth and grown overnight.

2. The cells were precipitated by centrifugation at 3000g for 5 min. at 4°C and resuspended in 20ml of minimal medium.
3. After 1h growth 133µl of cycloserine, 15mgml^{-1}, and 1ml of penicillin G, 0.135gml^{-1} were added and incubation continued for 5h.
4. The cells were precipitated at 3000g for 5 min. at 4°C and resuspended in 20ml minimal medium containing the amino acid for which auxotrophy was required and incubated overnight.
5. Steps 2 - 4 were repeated 3 times.
6. The culture was plated out on minimal medium containing the amino acid and colonies were checked for auxotrophy.

2.3 Measurement and detection of dehalogenase activity

Crude cell-free extracts of bacterial cultures were assayed for their dehalogenase activity, and individual dehalogenases identified by electrophoresis.

2.3.1 Measurement of dehalogenase activity

This was performed as described by Weightman et al. (1980) and Beeching et al. (1983). 400ml of an overnight culture grown on minimal medium supplemented with 2MCPA and amino acids as appropriate was pelleted at 5000g for 10 min. at 4°C in a Beckman centrifuge. The pellet was washed and resuspended in 200ml ice cold 0.02M Tris SO_4 (pH 7.9), 1mM dithiothreitol (Sigma). This was repelleted at 5000g for 10 min. at 4°C and resuspended in 7ml of ice cold 0.02M Tris SO_4 (pH 7.9), 1mM dithiothreitol and kept on ice. The cells were disrupted by two passages through a French pressure cell (Amnico International) at 8.3×10^4 Nm^{-2} at 4°C. The cellular debris was removed by centrifugation at 30 000g for 45 min. at 4°C. The supernatant was transferred to a fresh tube and kept on ice. The dehalogenase assays or electrophoresis were performed as soon as possible.

4.2ml 0.2M Tris SO_4 (pH 7.9) and 100µl 0.05 NaCl were incubated in a test tube in a water bath at 30°C for 8 min. 1ml of the cell-free extract was added, the contents of the tube mixed by gently vortexing and the 30°C incubation continued for 2 mins. 200µl of the chlorinated substrate, MCA, DCA, 2MCPA, and 22DCPA, 10% (w/v) (pH 7.0) were added, the contents gently mixed by vortexing and the incubation at 30°C continued. At convenient time points 1ml samples were removed and added to a mixture containing 20ml Base solution (Table 2.3) and 1ml Gelatin-thymol blue solution (Table 2.4). The concentration of chloride ions in this solution was determined by titration against silver ions using a Marius Chlor-O-Counter (F.T. Scientific. Tewkesbury, Glos., U.K. for Labo International, Holland).

TABLE 2.3

Base Solution

Glacial acetic acid	100ml
Nitric acid (concentrated)	8ml
NaCl 0.5M	1ml
H_2O	to 1 l.

TABLE 2.4

Gelatin-thymol blue

White powder gelatin	600mg
Thymol	10mg
Thymol blue pH indicator	10mg
H_2O	to 100ml

The Chlor-O-Counter was calibrated with 50mM NaCl. This standard curve enabled the determination of the dehalogenase activity of the crude cell-free extract. 1 unit of dehalogenase activity was defined as the dehalogenation of 1µmol of substrate per minute.

2.3.2 Discontinuous polyacrylamide gel electrophoresis

This was based on the methods of Laemmli (1970) and Fairbanks et al. (1971) as described by Weightman and Slater (1980) and Hardman and Slater (1981). The electrophoresis was performed on a vertical electrophoresis apparatus (Raven Scientific Ltd., Haverhill, Suffolk). The running gel contained: 0.325M Tris SO_4 buffer (pH 8.8); 1mM dithiothreitol; $80gl^{-1}$ acrylamide (Sigma); $3gl^{-1}$ N,N'-methylene-bis-acrylamide (Sigma); $1gl^{-1}$ $(NH_4)_2S_2O_8$; and $0.5gl^{-1}$ N,N,N',N'-tetramethyl-1,2-diaminoethane (TEMED) (Sigma). The stacking gel contained: 0.125M Tris SO_4 buffer (pH 6.8); $30gl^{-1}$ acrylamide; $1.08gl^{-1}$ N,N'-methylene-bis-acrylamide; $1gl^{-1}$ $(NH_4)_2S_2O_8$; and $0.5gl^{-1}$ TEMED. The electrode buffer contained 0.025M Tris; 0.192M glycine; and 1mM dithiothreitol. 0.1ml 60% (w/v) glycerol, 0.05% (w/v) bromophenol blue, 0.02M Tris SO_4 buffer (pH 7.9) was added to 0.5ml crude cell-free extract. Volumes between 10 and 100µl of this mixture were loaded on to the gel and the gel run at 30mA for 4-5 h. at 4°C using a Shandon Southern, Vokam, powerpack, by which time the bromophenol blue had reached the bottom of the gel.

After electrophoresis the gel was carefully removed from the glass plates and incubated in 500ml of 0.2M Tris SO_4 buffer (pH 7.9), 50mM 2MCPA and 50mM DCA at 30°C for 30 min. The gel was rinsed in 3 changes of distilled water and stained in 100mM $AgNO_2$ at room temperature until dehalogenase bands became visible. The gel was then soaked in 5% acetic acid for 15 min. and then left in distilled water for 16h. The gel was dried down into a piece of Whatman 3MM filter paper and exposed to a UV light source for 15min to enhance the darkness of the bands.

2.3.3 Determination of protein concentration

Protein concentration in the crude cell-free extracts was determined

by the Buiret method (Gornall et al., 1948). 4ml of Buiret solution (Table 2.5) was added to 1ml of crude cell-free extract, mixed and left at room temperature for 30 min. The absorbance at 540nm was measured using a Pye-Unicam SP1700 Spectrophotometer. The concentration of protein was calculated with reference to a standard curve obtained using 1-10mgml^{-1} bovine serum albumin (Sigma).

TABLE 2.5

Biuret solution

$CuSO_4$ $5H_2O$	1.5g
Sodium potassium tartrate	6g
NaOH 10%(w/v), autoclaved 10 min. $6.9 \times 10^4 Nm^{-2}$	300ml
H_2O	to 1 l.

2.4 Plasmid transfer

Plasmids were transferred to new hosts either by conjugation on membrane filters or by transformation.

2.4.1 Membrane matings

This was performed by a modification of the technique of Hedges and Jacob (1977) as described by Beeching et al. (1983). The parent strains were grown either on L-broth or minimal medium supplemented as necessary with antibiotics or amino acids. 5ml of the mid-exponential phase donor strain was mixed with 5ml of the stationary phase recipient strain. The mixture was vacuum-filtered on to the surface of a 0.45µm sterile Millepore filter. 5ml of sterile 0.1M phosphate buffer (pH 7.0) was filtered to wash the cells. The filter was placed on the surface of an L-agar plate and incubated at 30°C for 6 h. The filter was transferred to a sterile Universal bottle and the cells resuspended in 2.5ml 0.1M phosphate buffer (pH 7.0). The suspension was serially diluted and plated out on the appropriate media

to select for the required transconjugants and to determine the numbers of the two parents.

2.4.2 Transformation of plasmid DNA

Two methods were used, one for transformation into E. coli strains and a second for transformation into Pseudomonas strains.

2.4.2.1 Transformation of plasmid DNA into E. Coli

This method was kindly provided by Dr. A.J. Weightman, UWIST, Cardiff. 2ml of an overnight culture of E. coli on L-broth were inoculated into 20ml of L-broth and incubated with shaking at 37°C for 90 mins. The cells were harvested at 3000g for 5 min. at 0°C and resuspended in 10ml of ice cold sterile 10mM $CaCl_2$. The cells were repelleted as above, resuspended in 2ml of ice cold sterile 100mM $CaCl_2$ and kept on ice for 60 min. 200µl of the cell suspension was dispensed into a chilled 1.5ml Sarstedt tube together with 200µl 100mM $CaCl_2$, 1µg plasmid DNA and TE buffer (pH 7.4) to 500µl (TE buffer contained: 10mM Tris Cl, 1mM EDTA). The mixture was kept on ice for 60 min. and then given a heat shock at 42°C for 5 min. 0.5ml L-broth was added and the mixture shaken at 30°C for 90 min. The cells were plated out on appropriate media to select for transformants. A control was always included which had not received plasmid DNA.

2.4.2.2 Transformation of plasmid DNA into Pseudomonas

The method used was that described by Bagdasarian and Timmis (1982). 40ml of L-broth was inoculated with 1ml of an overnight culture grown on L-broth and incubated with shaking at 30°C until mid-exponential phase when the culture density was about 2×10^8 cells ml^{-1}. The culture was cooled on ice and harvested at 3000g for 5 mins. at 0°C. The cells were resuspended

in an equal volume of ice-cold Buffer I. Buffer I contained 10mM 3-[N-morpholino]propanesulphonic acid (MOPS) (Sigma) (pH7.0); 10mM Rb Cl; 100mM $MgCl_2$. The cells were repelleted as above and resuspended in an equal volume of cold Buffer II. Buffer II contained: 100mM MOPS (pH 6.5); 10mM Rb Cl; 100mM $CaCl_2$. The cells were repelleted as above and resuspended in 0.1 volume of ice-cold Buffer II. 200µl portions of the cell suspension were mixed with 1µg of plasmid DNA in a 1.5ml Sarstedt tube and kept on ice for 45 min. The mixture was subjected to a heat shock at 42°C for 1 min. 500µl of L-broth was added and the mixture incubated with shaking at 30°C for 90 min. The cells were plated out on appropriate media to select for transformants. A control containing no plasmid DNA was always included.

2.5 Plasmid purification

A variety of methods were employed depending on whether the mere detection of the presence of a plasmid, the rapid isolation of a plasmid for restriction analysis, or the purification in bulk of the plasmid was required. In the last case the size of the plasmid determined the method of choice.

2.5.1 Plasmid screening

The method of Wheatcroft and Williams (1981) was employed to detect the presence of a plasmid. 1ml of an overnight culture on L-broth was pelleted in a 1.5ml Sarstedt in an Eppendorf microfuge for 1 min. and the supernatant removed by aspiration. The pellet was resuspended in 100µml of Solution A. Solution A contained: 50mM Tris; 50mM EDTA; 0.1mgml^{-1} xylene cyanol FF (BDH, Poole, Dorset); 5% Dow Corning Antifoam RD emulsion (Hopkin and Williams, Chadwell Heath, Essex). 25µl of Solution B were added and mixed by inverting the tube 20 times in 1 min. Solution B contained a saturated solution at 20°C of SDS in 1M NaOH. The tube was then vortexed for 1 min. 20µl samples were then loaded directly onto an agarase gel and electrophoresed. This

method did not always give unambiguous results in my hands. The following method though longer gave much better results.

2.5.2 Rapid method for plasmid isolation

This method is derived from the version of the alkaline lysis method of Birnboim and Doly (1979) given by Maniatis et al. (1982). 1.5ml of an overnight culture on L-broth containing an antibiotic as appropriate was pelleted in a Sarstedt tube for 1 min. in an Eppendorf microfuge. The supernatant was carefully removed by aspiration. The pellet was resuspended in 100µl of ice-cold Lysis Solution by vortexing. Lysis Solution contained: 50mM glucose; 10mM EDTA; 25mM Tris Cl (pH 8.0); and 4mgml^{-1} lysozyme (Sigma). The lysozyme was added just before use. The suspension was kept at room temperature for 5 min. 200µl of freshly prepared ice-cold 0.2M NaOH, 1% (w/v) SDS was added and mixed by inverting the tube sharply 2-3 times. The tube was kept on ice for 5 min. 150µl of ice-cold potassium acetate ($\sim$ pH 4.8) was added, mixed by vortexing the tube gently in an inverted position for 10 sec. and kept on ice for 5 min. The potassium acetate was made from 60ml 5M potassium acetate, 11.5ml glacial acetic acid and 28.5ml H_2O.

The tube was centrifuged in an Eppendorf microfuge for 5 min. at 4°C and the supernatant transferred to a fresh tube. An equal volume of phenol/chloroform (1:1 w/v) was added and the contents mixed by vortexing. The two phases were separated by centrifugation in an Eppendorf microfuge for 2 min. and the upper phase transferred to a fresh tube. 2 volumes of ethanol were added, the contents mixed by vortexing and the tube left to stand at room temperature for 2 min. The tube was centrifuged for 5 min. at room temperature in an Eppendorf microfuge and the supernatant carefully removed by aspiration. 1ml 70% (v/v) ethanol was added, the tube vortexed and centrifuged for 5 minutes at room temperature in an Eppendorf microfuge. The supernatant was carefully removed by aspiration and the pellet briefly dried in a vacuum desiccator.

If the mere detection of the plasmid was required the pellet was dissolved in 10µl TE buffer (pH 8.0), 2µl loading buffer added, before loading onto an agarose gel and electrophoresing. TE buffer is 10mM Tris Cl, 1mM EDTA. Alternatively the plasmid could be digested with a restriction endomelease in which case the pellet was dissolved in a solution containing the enzyme and the appropriate buffer and digested as described below. 10 minutes before the end of the digestion 2µl of $1mgml^{-1}$ DNase-free RNase were added to remove contaminating RNA. DNase-free RNase was obtained by heating a $1mgml^{-1}$ solution of pancreatic RNase (Sigma) at 100°C for 5 min. 5µg of loading buffer were added and the restriction fragments separated by electrophoresis on agarose gel. This method works equally well for small and large plasmids.

2.5.3 Bulk preparation of cloning vectors

This method was based on that of Clewell and Helinski (1969). 10ml of an overnight culture on L-broth and an antibiotic was inoculated into 300ml L-broth and antibiotic and grown to mid-exponential phase. If the plasmid was amplifiable chloramphenicol was added to $170µgml^{-1}$ and shaking incubation continued overnight before harvesting. The culture was harvested by centrifugation at 4000g for 10 min. at 4°C and resuspended in 7.2ml of ice-cold 0.05M Tris Cl (pH 8.0), 25% (w/v) sucrose. 1ml lysozyne solution containing 0.25M Tris Cl (pH 8.0) and $10mgml^{-1}$ lysozyme was added and the mixture gently shaken at 37°C. The mixture was cooled on ice for 5 min. 4.9ml of 0.2M EDTA (pH 8.0) was added, the mixture gently mixed and kept on ice for 8 min. 8.1ml ice-cold solution containing 0.05M Tris Cl (pH 8.0), 0.0625M EDTA, and 2% (v/v) Triton-X-100 (Sigma) was added, gently mixed and the mixture kept on ice for 20 min. The lysate was cleared by centrifugation at 33500g for 15 min. at 4°C. The supernatant was carefully removed and its volume determined. A $2.5mgml^{-1}$ solution of ethidium bromide in water was added to give a final concentration of $500µgml^{-1}$. The cleared lysate was weighed

and CsCl added to give a 48.4% (w/w) solution. The mixture was centrifuged at 100000g for 48 h. at 20°C in an MSE Prepspin 65.

After centrifugation the plasmid band was localized by longwave UV light and removed from above using a 2ml hypodermic syringe fitted with a ## 21 needle. The ethidium bromide was removed by repeated extractions (about 6) with CsCl and water saturated isopropanol. This was prepared by adding 10ml saturated solution of CsCl in water to 200ml isopropanol. 10ml volumes of water were added and mixed in until all the CsCl went back into solution and 2 phases were produced. 2 volumes of sterile distilled water were added to the DNA solution and 6 volumes of ethanol. The tube was kept at -20°C overnight and the DNA precipitated by centrifugation at 12000g for 10 min. at 4°C. The pellet was resuspended in 70% ethanol (v/v) and reprecipitated. The supernatant was carefully removed and the pellet dried in a vacuum desiccator. The pellet was dissolved in a small volume (about 100µl) of TE buffer (pH 8.0) and stored at 4°C. 1µl of the plasmid solution was diluted with water and used to determine the relative concentrations of DNA and protein by measuring the absorbance at 260 and 280nm respectively. 1 OD_{260} unit is equivalent to 50µgml^{-1} DNA. A pure preparation of DNA has an OD_{260}/OD_{280} of 1.8, a smaller value indicates substantial protein contamination.

2.5.4 Bulk preparation of large plasmids

This method is based on those of Bazaral and Helinski (1968) and Barth and Grinter (1974). 300ml of pre-warmed L-broth (+ antibiotic) was inoculated with 10ml of an overnight culture on L-broth (+ antibiotic) and grown until it reached an OD_{600} of slightly less than 0.8. The culture was cooled on ice for 15 min. and the cells pelleted by centrifugation at 4000g for 10 min. at 4°C. The pellet was washed twice in ice-cold TES buffer. TES buffer consisted of: 0.05M Tris HCl (pH 8.0); 0.005M EDTA; and 0.05M NaCl. The

pellet was resuspended in 10ml of spheroplast mix and incubated at 37°C for 10 min. Spheroplast mix was made fresh each time as follows: 5mg RNase (Sigma) were dissolved in 10ml TES buffer and the mixture was heated to 80°C for 15 min.; 1g sucrose was added and dissolved while the mixture was still hot, the solution was cooled and 10mg lysozyme added. The cell suspension was cooled on ice for 5 min. 5ml 2% (v/v) Sarkosyl (Sigma) was added and gently mixed in by pipetting. 10ml TES buffer at room temperature was added. The chromosomal DNA was sheared by passage through a ## 21 syringe needle 20 times. In some cases, especially with the largest plasmids this treatment also sheared the plasmid DNA. In these cases only one passage through needle was required. This treatment produced after centrifugation a mass of chromosomal DNA traversing the plasmid band. This chromosomal DNA, though unaesthetic, could easily be removed with a syringe prior to removing the plasmid band. 25ml of TES buffer was added to the sheared lysate. The volume of the lysate was determined and ethidium bromide at $2.5 mg ml^{-1}$ in TES buffer was added to give a final concentration of $500 \mu g ml^{-1}$. The mixture was weighed and solid CsCl added to give 48.4% (w/w). The mixture was centrifuged at 100000g for 48h. at 20°C. The plasmid band was removed and purified as described above.

Plasmid DNA was routinely stored at 4°C in TE buffer (pH 8.0) in Sarstedt tubes wrapped in Parafilm. All the solutions and equipment used to handle DNA were sterilised and disposable gloves were routinely worn.

2.6.1 Restriction endonuclease digestion of plasmid DNA

0.2 - 1µg plasmid DNA was digested in a volume typically of 20µl. The DNA was mixed with sterile distilled water in a sterile 500µl Sarstedt tube to give a volume of 18µl. 2µl of the appropriate 10x restriction buffer was added and mixed. 1 unit of restriction enzyme was added and the contents of the tube mixed by flicking. The contents of the tube were briefly

centrifuged in an Eppendorf microfuge to concentrate them at the bottom of the tube. The tube was incubated for between 1 and 2 hours. With the exception of digests with SmaI which was incubated at 30°C, all other digests were incubated at 37°C. The restriction enzymes were obtained from BRL, Cambridge, UK, the 10x restriction buffers were either those provided by the manufacturer or were made up according to their instructions. In the case of the plasmids prepared by the rapid method (Section 2.5.2) 25µl of a solution containing water, 10x restriction buffer and the restriction endonuclease in the appropriate concentrations were added directly to the dried down DNA pellet and the pellet dissolved.

After digestion the tube was placed in a water bath at 68°C for 10 min. and then placed on ice. 0.2 volumes of loading buffer was added. Loading buffer consisted of: 60mM EDTA (pH 7.5); 40% Glycerol (w/v); 15% Ficoll (v/v); and one of the following dyes at 0.05% w/v: xylene cyanol FF (BDH) which migrates with 5 kilobase (kb) fragments; bromophenol blue (Sigma) which migrates with 0.5 kb fragments; or orange G (Sigma) which migrates with the gel front. The contents of the tube were loaded on to an agarose gel and electrophoresed.

2.6.2 Electrophoresis of DNA

DNA was electrophoresed in either a large horizontal flat bed apparatus or in a mini-gel apparatus. The large apparatus was constructed from perspex in the workshop of the Department of Genetics, University of Leicester. It measured 30 x 20cm and was capable of accommodating gels cast on glass plates of 16 x 18cm. The gels were cast as follows: the glass plate was wrapped around the edge with masking tape, thereby constructing a wall about 6mm high; 300ml of TBE buffer containing the appropriate percentage of agarose (Type 1: low EEO from Sigma) was heated until the agarose had melted; the agarose was cooled to 50°C and 10mgml^{-1} ethidium bromide was added to give a

concentration of $0.5\mu g ml^{-1}$; the agarose was poured onto the gel plate over which a comb had been clamped 1mm above the surface of the glass and 2cm from one end of the plate to produce the wells; the gel was allowed to set. TBE buffer was made from a 10x stock solution which contained: 0.89M Tris; 0.89M boric acid; and 0.02M EDTA. A range of percentages of agarose were used depending on the features of the digest which were of interest. A 0.5% gel was most frequently used; run at $1Vcm^{-1}$ for 16 h. this would separate restriction fragments between 0.5 - 30 kb. It was possible, after photographing the gel, to continue electrophoresis for up to 60 h. which increased the separation of the larger fragments. Alternatively, to examine restriction fragments less than 1kb in size 1.4% gels were cast which were run at up to $3Vcm^{-1}$ for 5 h.

The comb and tape were removed from the set gel and the gel on its plate placed in the electrophoresis tank. The apparatus was filled with TBE buffer containing $0.5\mu g ml^{-1}$ ethidium bromide to 1mm above the surface of the gel. The digests were carefully loaded into the wells. Also loaded were restriction fragments of known size, these were Hind III, Eco RI/Hind III digests of λ DNA (BRL, Cambridge) or undigested λ. The sizes of these fragments were taken from Southern (1979). The DNA was electrophoresed at the appropriate voltage using a Shandon Southern Vokam power pack.

After electrophoresis the gel was photographed on a UV transilluminator type C61 (Ultra Violet Products Inc., San Gabriel, California, USA) using a Polaroid CU5 camera fitted with a UV and a red filter on Polaroid type 665 film. The migration distances of the restriction fragments were measured from photographic enlargements. The sizes of the unknown fragments were determined from a plot of the natural logarithm of the size against mobility of the restriction digests of λ DNA. Because of this log/linear relationship it was not possible to determine the size of the larger restriction fragments with the same accuracy as the smaller bands. Some bands on the gels were interpreted as being the product of two fragments of the same size. This

interpretation could only be made with any degree of certainty if there were adjacent single fragments with which to compare the putative double bands.

2.6.3 Producing diagrammatic representations of restriction digests

In many cases the data on a restriction digest of a particular plasmid was produced from several gels. It was often convenient, after calculation of the fragments sizes, to produce a diagrammatic representation of the restriction digest for visual comparison with those from other plasmids. These were conveniently produced using a programmable Texas T158 calculator using the program (Figure 2.1). It was assumed that there was a linear relationship between the natural logarithm of fragment size and electrophoretic mobility.

FIGURE 2.1

Program for constructing diagrammatic representations of restriction digests

Step	Key	Comments	Step	Key	Comments
1	2nd Lbl		15	R/S	
2	A	Maximum mobility	16	2nd Lbl	
3	STO		17	D	Maximum kb
4	01		18	STO	
5	R/S		19	04	
6	2nd Lbl		20	R/S	
7	B	Minimum kb	21	2nd Lbl	
8	STO		22	E	kb of fragment
9	02		23	STO	
10	R/S		24	05	
11	2nd Lbl		25	(	
12	C	Minimum mobility	26	(	
13	STO		27	RCL	
14	03		28	03	

Step	Key	Comments	Step	Key	Comments
29	-		52	X	
30	RCL		53	RCL	
31	01		54	04	
32	)		55	lnx	
33	÷		56	)	
34	(		57	STO	
35	RCL		58	07	
36	02		59	(	
37	lnx		60	RCL	
38	-		61	05	
39	RCL		62	lnx	
40	04		63	X	
41	lnx		64	RCL	
42	)		65	06	
43	)		66	)	
44	STO		67	+/-	
45	06		68	+	
46	(		69	RCL	
47	RCL		70	07	
48	03		71	=	
49	+		72	2nd FIX	
50	RCL		73	1	
51	06		74	R/S	Displays mobility

To use the program:

1. Enter maximum mobility. Press 'A'.
2. Enter corresponding kb. Press 'B'.
3. Enter minimum mobility. Press 'C'.

FIGURE 2.6

Bacterial strains and plasmids used or derived in this thesis

a. *Escherichia coli*

Strain	Plasmid	Genotype/phenotype	Derivation/Source
ED8654	-	*sup*E, *sup*F, *hsd*, R^-, M^+, S^+, Met^+, *trp*R	A.J. Weightman
HB101	-	F^-, *hsd*, S20($r_B^- m_B^-$), *rec*A13, *ara*14, *pro*A2, *lac*Y1, *gal*K2, *rps*120 (Sm^R), *xy*l5, *mtl*-1, *sup* E44, λ^-.	P.C. Gowland
	pAT153	Ap^R, Tc^R	Twigg and Sherratt (1980)
	pKT231	Sm^R, Km^R	Bagdasarian *et al* (1981)
CP24	pUU52	As ED8654. $2Mcpa^+$. Tc^R.	*Pst*I fragment of pUU2 in pAT153
CX1	pUU53	As ED8654. $2Mcpa^+$, Sm^R	*Xhs*I fragment of pUU2 in pKT231
CH6	pUU54	"	*Hind* III fragment of pUU2 in KT231
CH7	pUU55	"	"
CH12	pUU56	"	"
CH14	pUU57	"	"
CH15	pUU58	"	"
CH16	pUU59	"	"

b. *Pseudomonas aeruginosa*

PA02	-	Ser^-	A.J. Weightman
PA08	R68	*met*28, *ilv*202, *str*-1, Ap^R, Km^R, Tc^R	B.W. Holloway

Strain	Plasmid	Genotype/phenotype	Derivation/Source
PA025	R68.45	Leu10, argF10, Ap^R, Km^R, Tc^R	A.J. Weightman
PA01162	-	Leu38, R^-, M^+	K.N. Timmis
PAU1	-	As PA01162, Sm^R	Mutant of PA01162
PAU2	-	As PA01162, Rif^R	Mutant of PA01162
PAU8	pUU6	As PA01162, Sm^R Ap^R $Km^R Tc^R$	PPW1 x PAU1
PAU9	pUU28	As PA01162, $2Mcpa^+$, Rif^R,Ap^R,Km^R,Tc^R	PPW2 x PAU2
PAU10	pUU29	"	"
PAU11	pUU40	As PA01162,$2Mcpa^+$, Rif^R,Ap^R,Km^R,Tc^R	PPW37 x PAU2
PAU12	pUU41	"	"
PAU13	pUU42	"	"
PAU14	pUU43	"	"
PAU15	pUU44	"	"
PAU16	pUU45	"	"
PAU17	pUU46	"	"
PAU18	pUU47	"	"
PAU19	pUU48	"	"
PAU20	pUU49	"	"
PAU21	pUU50	"	"
PAU22	pUU51	"	"
PAC174	R68.44	Lys^-,Ap^R,Km^R,Tc^R	P.A. Clarke

c. Pseudomonas putida

Strain	Plasmid	Genotype/phenotype	Derivation/Source
PP3	-	$2Mcpa^+$	Senior et al. (1976)
PaW340	-	Trp^-, Sm^R	P.A. Williams
KT2440	-	Leu^-, R^-, M^-	K.N. Timmis
PPW1	pUU1	$2Mcpa^+, Ap^R, Km^R, Tc^R$	PAC174(R68.44) x PP3
PPW2	pUU2	$Trp^-, 2Mcpa^+, Sm^R, Ap^R, Km^R, Tc^R$	PPW1 x PaW340
PPW2*	pUU2*	$Trp^-, 2Mcpa^+, Sm^R, Ap^R, Km^R, Tc^R$	PPW2 in chemostat
PPW4	-	As KT2440. Nal^R	Mutant of KT2440
PPW5	-	$2Mcpa^+$ His^-	Mutant of PP3
PPW9	pUU3	$Trp^-, 2Mcpa^+, Sm^R, Ap^R, Km^R, Tc^R$	PPW2 in chemostat
PPW10	pUU4	$Trp^-, 2Mcpa^+, Sm^R, Ap^R, Km^R$	PPW2 in chemostat
PPW11	pUU5	$Trp^-, 2Mcpa^+, Sm^R, Ap^R, Km^R$	PPW2 in chemostat
PPW12	-	$Trp^-, 2Mcpa^+, Sm^R$	PPW2 in chemostat
PPW16	pUU7	$Trp^-, Sm^R, Ap^R, Km^R, Tc^R$	PPW1 x PaW340
PPW17	pUU8	$His^-, 2Mcpa^+, Ap^R, Km^R, Tc^R$	PAU8 x PPW5
PPW18	pUU9	"	"
PPW19	pUU10	"	"
PPW20	pUU11	"	"
PPW21	pUU12	"	"
PPW22	pUU13	"	"
PPW23	pUU14	"	"
PPW24	pUU15	"	"
PPW25	pUU16	"	"
PPW26	pUU17	"	"

Strain	Plasmid	Genotype/phenotype	Derivation/Source
PPW27	pUU18	His^-,$2Mcpa^+$,Ap^R,Km^R,Tc^R	PPW16 x PPW5
PPW28	pUU19	"	"
PPW29	pUU20	"	"
PPW30	pUU21	"	"
PPW31	pUU22	"	"
PPW32	pUU23	"	"
PPW33	pUU24	"	"
PPW34	pUU25	$2Mcpa^+$,Ap^R,Km^R;Tc^R	PA08(R68) x PP3
PPW35	pUU26	"	"
PPW36	pUU27	"	"
PPW37	R68.45	Trp^-,$2Mcpa^+$,Cma^+,Sm^R, Ap^R,Km^R,Tc^R	PA025(R68.45) x PPW12
PPW38	pUU30	Leu^-,$2Mcpa^+$,R^-,M^-,Nal^R, Ap^R,Km^R,Tc^R	PPW37(R68.45) x PPW4
PPW39	pUU31	"	"
PPW40	pUU32	"	"
PPW41	pUU33	"	"
PPW42	pUU34	"	"
PPW43	pUU35	"	"
PPW44	pUU36	"	"
PPW45	pUU37	"	"
PPW46	pUU38	"	"
PPW47	pUU39	"	"

CHAPTER 3

THE FORMATION AND CHARACTERIZATION OF THE R-PRIME pUU2

Chapter 3

The Formation and Characterization of the R-Prime pUU2

3.1 Introduction

Pseudomonas putida PP3 was isolated by Senior *et al.* (1976) by its ability to grow on 2-monochloropropionic acid (2MCPA) and 2,2-dichloropropionic acid (22DCPA) as its sole carbon and energy sources. PP3 has been shown to possess two dehalogenases (Weightman, *et al.*, 1979a; Weightman and Slater, 1980). Dehalogenases, also known as halidohydrolases, catalyse the hydrolytic removal of halides from halogenated alkanoic acids, particularly chlorinated acetic and propionic acids (Goldman *et al.*, 1968; Little and Williams, 1971; Slater *et al.*, 1979).

The plasmid R68.44, a derivative of the broad host-range plasmid R68, exhibits enhanced chromosome mobilizing ability (Haas and Holloway, 1976). In addition to chromosome mobilization R68.44 is able to form R-prime plasmids (Hedges and Jacob, 1977). R68.44 has been shown to be physically indistinguishable from its more stable derivative, R68.45 (Leemans *et al.*, 1980). R68.45 differs from R68 by a 2.12 kb duplicated region which is correlated with the possession of enhanced chromosome-mobilizing ability (Riess *et al.*, 1980a, b; Leemans *et al.*, 1980; Willetts *et al.*, 1981).

This chapter describes the introduction of R68.44 into PP3 and the formation of an R-prime plasmid bearing a dehalogenase gene. The derivative bacterial strains characterized and the plasmids involved were analysed with restriction endo-nucleases.

3.2.1 The introduction of R68.44 into *P. putida* PP3

Pseudomonas aeruginosa PAC174 containing R68.44 was kindly provided by

Professor P.H. Clarke (University College, London) (Table 3.1). PAC174 was a lysine auxotroph and was shown to be unable to grow on 2MCPA as sole carbon and energy source. Pseudomonas putida PP3 had never been shown to possess a plasmid, and was therefore assumed to be plasmid minus.

PAC174 (R68.44) was membrane mated with PP3 and transconjugants were selected by their ability to grow on minimal media containing 2MCPA in the presence of the antibiotics ampicillin (Ap), kanamycin (Km) and tetracycline (Tc) at concentrations of $50\mu gml^{-1}$, $50\mu gml^{-1}$ and $150\mu gml^{-1}$ respectively. The transconjugants produced the characteristic growth of P. putida on King's A and B media (King and Phillips, 1978). Screening for plasmids by the method of Wheatcroft and Williams (1981) indicated the presence of a plasmid of the same size as R68.45.[1] One of these plasmid containing derivatives of PP3 was designated PPW1 and was used in subsequent experiments. Frequencies for plasmid transfer were not obtained from this experiment. However in comparable matings frequencies $1x10^{-6}$ transconjugants per donor cell were obtained for the transfer of R68.45 from Pseudomonas aeruginosa PA08 into PP3.

The above mating was performed by Andrea Filipuik.

3.2.2 The Formation of Pseudomonas putida PPW2

Pseudomonas putida PaW340 was kindly provided by Dr. P.A. Williams (University of Bangor). PaW340 was a tryptophan auxotroph which was resistant to high concentrations of streptomycin (Sm). It was shown to be unable to grow on 2MCPA.

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1. All the comparative work was performed using R68.45 instead of R68.44. By the time this work was carried out the original strain PAC174 (R68.44) had been lost. As mentioned above (Section 3.1) R68.44 is physically identical to R68.45. R68.45 was obtained from Pseudomonas aeruginosa PA025 kindly provided by Dr. A.J. Weightman (University of Geneva).

TABLE 3.1

Bacterial strains and plasmids used or derived in Chapter 3

Strain	Species[1]	Plasmid	Genotype/Phenotype	Source
PAC174	P.a.	R68.44	Lys^-, $2Mcpa^-$, Ap^R, Km^R, Tc^R	P.H. Clarke
PP3	P.p.	-	$2Mcpa^+$	Senior *et al.* (1976)
PPW1	P.p.	pUU1	$2Mcpa^+$, Ap^R, Km^R, Tc^R	PAC174(R68.44) x PP3
PAO25	P.a.	R68.45	*leu*10 *arg*F10 Ap^R, Km^R, Tc^R	A.J. Weightman
PAW340	P.p.	-	Trp^-, $2Mcpa^-$, Sm^R	P.A. Williams
PPW2	P.p.	pUU2	Trp^-, $2Mcpa^+$, Sm^R, Ap^R, Km^R, Tc^R	PPW1 x PaW340
PAO1162	P.a.	-	*leu*$^{-38}$, *rmo*11	K.N. Timmis
PAU2	P.a.	-	*leu*$^{-38}$, *rmo*11, Rif^R	Mutant of PAO1162

1. Species:

   P.a. : *Pseudomonas aeruginosa*

   P.p. : *Pseudomonas putida*

P. putida PPW1 was mated with PaW340 with selection for the growth of transconjugants on 2MCPA supplemented with tryptophan ($25\mu gml^{-1}$), Ap ($50\mu gml^{-1}$), Km ($50\mu gml^{-1}$), Tc ($150\mu gml^{-1}$) and Sm ($750\mu gml^{-1}$). Transconjugants were obtained at a frequency of 1.3×10^{-10} per donor cell. This figure compared with a frequency of 1×10^{-6} transconjugants per donor cell when selection was made for transfer of the plasmid alone.

The transconjugants proved to be tryptophan auxotrophs, confirming that they were derivatives of PaW340 as opposed to Sm^R mutants of PP3. One of the transconjugants was designated PPW2 and was used for further study. PPW2 was shown to contain a plasmid of approximately the same size as R68.45 in a screening gel.

The above mating was performed by Dr. A.J. Weightman, see Weightman (1981).

3.3.1 Substrate Activity Profile of P. putida PPW2

Crude cell-free extracts of PPW2 grown on 2MCPA exhibited dehalogenase activity. However dehalogenase activity towards dichloroacetic acid (DCA) was substantially lower than the activity of crude cell-free extracts of PP3 and PPW1 (Table 3.2). In fact the substrate activity profile of PPW2 was comparable to that of P. putida PP411-006, a mutant of PP3 producing only fraction I dehalogenase (Weightman et al., 1979), and to the activity profile of a partially purified fraction I dehalogenase (Weightman, et al., 1982). These results suggest that PPW2 produced dehalogenase I alone.

3.3.2 Discontinuous Polyacrylamide Electrophoresis of the Dehalogenase from P. putida PPW2

Discontinuous polyacrylamide gel electrophoresis (disc-PAGE) on non-

TABLE 3.2

Dehalogenase specific activities and substrate activity ratios for various strains of *P. putida*

Strain	Dehalogenase specific activities {μmol substrate min^{-1} (mg protein)$^{-1}$}[1]			
	MCA	DCA	2MCPA	22DCPA
P. putida PP3	0.45	0.63	0.15	0.08
	(1.00)	(1.40)	(0.33)	(0.18)
P. putida PPW1	0.32	0.40	0.14	0.08
	(1.00)	(1.28)	(0.44)	(0.26)
P. putida PPW2	0.66	0.08	0.28	0.14
	(1.00)	0.12	(0.42)	(0.21)
P. putida PP4011-006[2]	ND	ND	ND	ND
	(1.00)	(0.17)	(0.49)	ND
Partially purified Fraction I dehalogenase[3]	0.62	0.007	0.40	0.19
	(1.00)	(0.11)	(0.65)	(0.31)

ND: Not determined

1: Numbers in parenthesis are dehalogenase substrate activity ratios with respect to MCA

2: Data from Weightman *et al.* (1979b)

3: Data from Weightman *et al.* (1982)

denaturing gels was performed on crude cell-free extracts of PP3, PPW1 and PPW2 grown on 2MCPA. The gels were incubated in 2MCPA and DCA and stained so as to reveal the presence of fraction I and II dehalogenases.

The lanes corresponding to extracts of PP3 and PPW1 showed two bands (Plate 3.1) which were identified as corresponding to fraction I and fraction II dehalogenases (Weightman *et al.*, 1979a). However the lane corresponding to the extract of PPW2 contained only one band which had migrated the same distance as the upper, dehalogenase fraction I, band of PP3.

These results confirmed the conclusion in Section 3.2.1 that PPW2 contained only the fraction I dehalogenase of PP3. These data suggest that the plasmid had transferred chromosomal DNA containing the gene or genes for dehalogenase fraction I from the plasmid containing derivative of PP3, PPW1, to PaW340 to form PPW2. The gene or genes for dehalogenase fraction I will be referred to as *dehI*.

Dehalogenase I in PPW2 was inducible rather than constitutive. Extracts from cells grown on succinate showed no dehalogenase activity. This suggested that the regulating genes controlling the synthesis for dehalogenase I had also been transferred to PPW2.

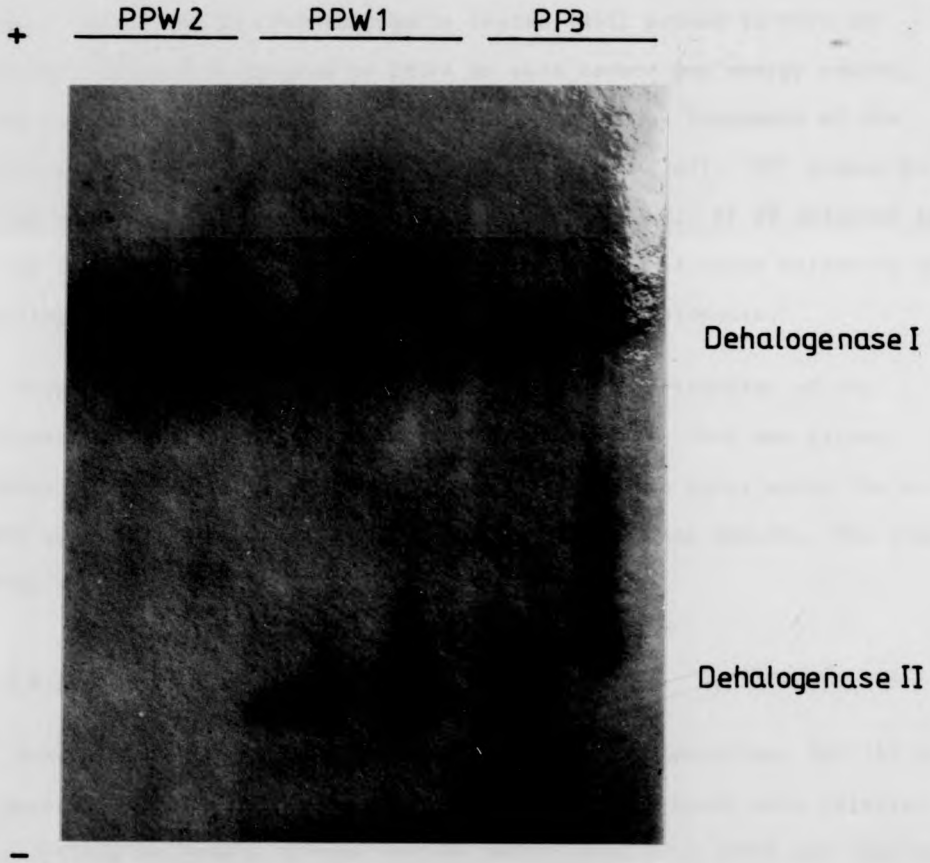
3.4.1 *P. putida* PPW2 Mated with *Pseudomonas aeruginosa* PAO

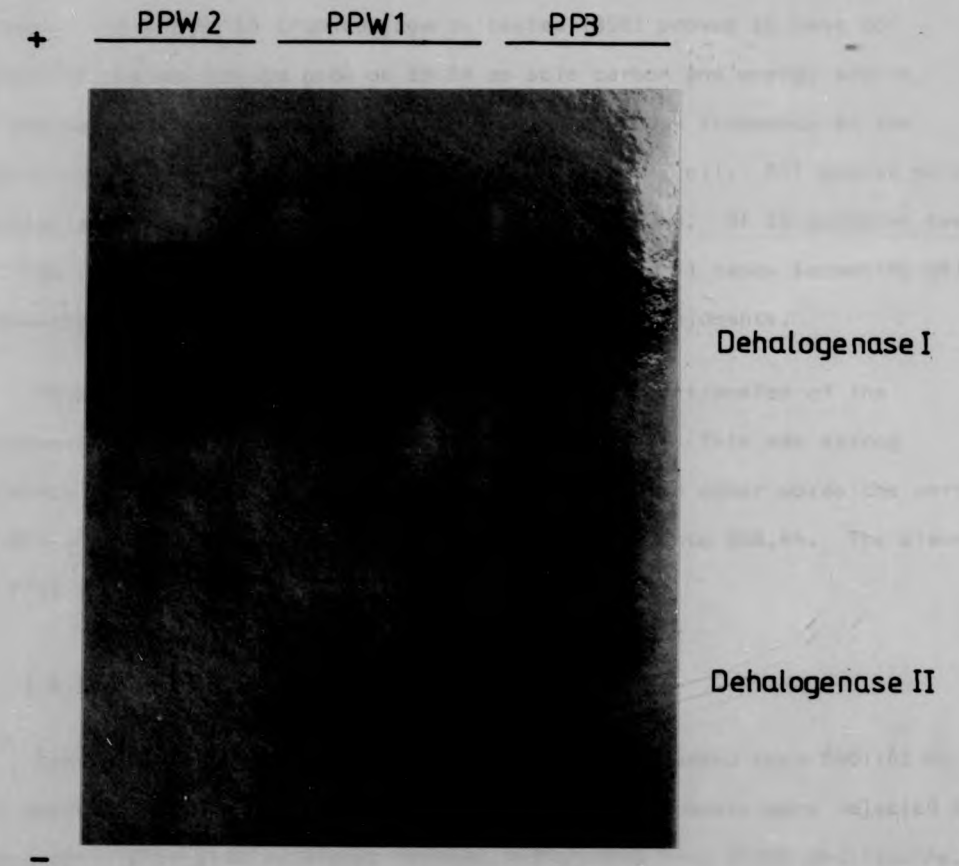
Pseudomonas aeruginosa PAO1162 was kindly provided by Professor K.N. Timmis (University of Geneva). PAO1162 had the genotype *leu*-38, *rmo* 11 (Dunn and Holloway, 1976). Its' important feature as a mating recipient was that it had a deficient restriction system. PAO1162 could not use 2MCPA as sole carbon and energy source.

PPW2 was membrane mated with a rifampicin (rif) resistant mutant of

PLATE 3.1

Polyacrylamide gel electrophoresis of *P. putida* PP3, PPW1 and PPW2 crude cell-free extracts


+
PPW 2
PPW1
PP3
Dehalogenase I
Dehalogenase II
−


+
PPW 2
PPW1
PP3
-
Dehalogenase I
Dehalogenase II
-

PA01162, PAU2. Selection was for transfer of the plasmid on nutrient agar supplemented with Ap ($50\mu gml^{-1}$), Km ($50\mu gml^{-1}$) Tc ($150\mu gml^{-1}$) and Rif ($280\mu gml^{-1}$) or for the ability to grow on 2MCPA on minimal medium containing 2MCPA and Leucine ($25\mu gml^{-1}$). In the former case, selection for transfer of the plasmid resistance markers alone, the frequency of plasmid transfer was 3.8×10^{-4} per donor cell. All the transconjugants proved to be leucine auxotrophs and gave the characteristic P. aeruginosa growth on King's A and B media. 19 of the 20 transconjugants tested (95%) proved to have co-inherited the ability to grow on 2MCPA as sole carbon and energy source. In the latter case, selection for growth on 2MCPA, the frequency of the acquisition of this phenotype was 5×10^{-5} per donor cell. All proved to be leucine auxotrophs and P. aeruginosa on King's A and B. Of 20 colonies tested all had inherited resistances to Ap, Km and Tc. In all cases screening gels demonstrated the presence of a plasmid in the transconjugants.

These results indicated a very high degree of co-transfer of the antibiotic markers and the ability to grown on 2MCPA. This was strong evidence for the plasmid in PPW2 being an R-prime. In other words the portion of PP3 chromosomal DNA containing dehI was inserted into R68.44. The plasmid in PPW2 was designated pUU2.

3.4.2 Transformation of plasmid pUU2

Purified pUU2, the plasmid from PPW2, was transformed into PA01162 by the method of Bagdasarian and Timmis (1982). Transformants were selected by their ability to grow on either minimal media containing 2MCPA and leucine, on nutrient agar containing Ap (50), Km (50) and Tc (150). Colonies grew on both sets of plates. No colonies grew on control plates which had received only competant cells. The transformants selected on 2MCPA proved to be resistant to the three antibiotics, while those selected for their antibiotic resistances proved to be capable of growth on 2MCPA. Screening gels confirmed

the presence of a plasmid in both sets of transformants.

These results demonstrated the coinheritance of the antibiotic resistance markers and the ability to utilize 2MCPA with the plasmid from PPW2, pUU2. These and the results presented in Section 3.4.1 were very strong evidence for pUU2 being an R-prime plasmid.

3.5.1 Restriction Endonuclease Analyses of the Plasmids R68.45, pUU1 and pUU2

The plasmid R68.45 from PA025 (R68.45), pUU1 the derivative plasmid of R68.44 in PPW1, and pUU2 the plasmid from PPW2 were purified by the crude lysis caesium chloride - ethidium bromide gradient method. The plasmids were digested with a range of restriction endonucleases, and the resultant fragments were separated and visualised by electrophoresis on agarose gels. The digest fragments were sized with reference to EcoRI/HindIII and HindIII digests of lambda (λ) DNA and to undigested lambda (Southern, 1979).

3.5.2 Comparison of Restriction Digests of R68.45 and pUU1

R68.45 was used instead of R68.44 because it is a more stable (Haas and Holloway, 1976) but physically identical (Leemans et al., 1980) derivative of R68.44. Also our version of R68.44 had been lost. The plasmid in PPW1, the strain of PP3 into which R68.44 had been mated, was designated pUU1 to distinguish it from the original R68.44.

The two plasmids were digested with the following restriction endonucleases: PstI, SmaI, KpnI, XhoI, EcoRI, HindIII and EcoRI/HindIII. The digests were compared with published maps of R68.45 (Nayudu and Holloway, 1981; Moore et al., 1983).

3.5.2.1 Digestion of plasmids R68.45 and pUU1 with PstI

RG68.45 and pUU1 were digested with the restriction endonuclease PstI

(Plate 3.2, and Figure 3.1). The band sizes were obtained from the mean sizes from several gels run under different conditions of time, voltage and percentage agarose so as to reveal different features of the digest patterns (Table 3.3).

pUU1 has lost band PE of R68.45 and band PF has become a single band, 1PF. These two bands were indicative of the second copy of IS21 which distinguished R68.45 and R68.44 from R68 (Riess et al., 1980a, b; Willetts et al., 1981). Band PB of R68.45 was missing in pUU1 and had been replaced by two novel bands, 1XP1 and 1XP2.

Apart from these differences, the remaining PstI generated bands of pUU1 were identical to those obtained with R68.45. The net size difference between pUU1 and R68.45, taking into consideration the loss of IS21, was a gain of 2.16kb.

These modifications in the PstI digest patterns of pUU1 were common to the plasmids of two independently isolated transconjugants from the mating of PAC174 (R68.44) with PP3.

3.5.2.2 Digestion of R68.45 and pUU1 with SmaI

R68.45 and pUU1 were digested with the restriction endonuclease SmaI (Plate 3.3, Figure 3.2). The restriction fragment sizes were obtained as in Section 3.5.2.1 (Table 3.4).

pUU1 has lost the SE band of R68.45 which was indicative of the second copy of IS21 (Riess et al., 1980a, b; Willetts et al., 1981). Band SB of R68.45 had been replaced by a larger band, 1X51. All the other SmaI generated bands of pUU1 were identical with those of R68.45. The net change in size of pUU1 with respect to R68.45, bearing in mind the loss of IS21, was an increase of 3.52kb.

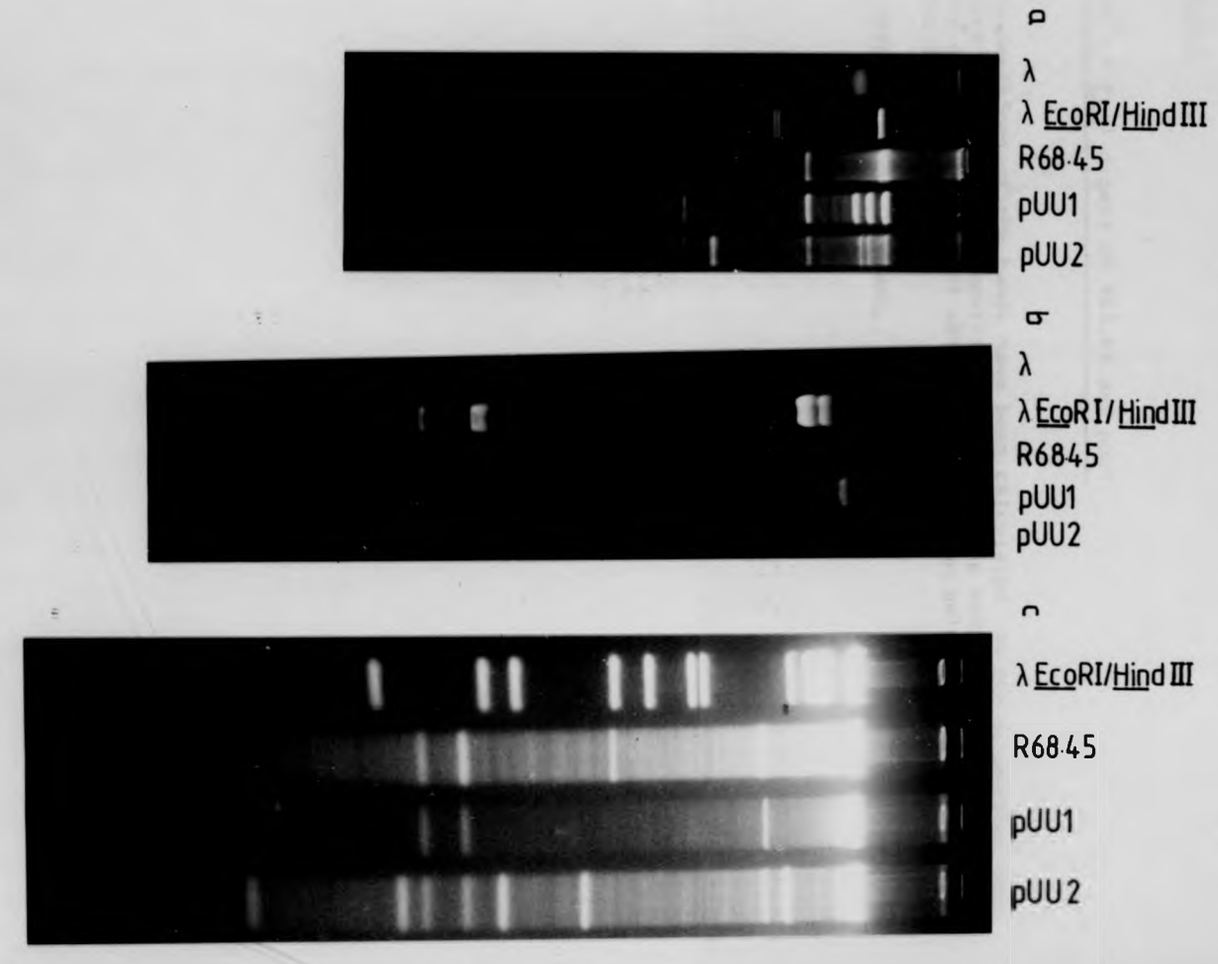
PLATE 3.2

Restriction digests of plasmids R68.45, pUU1 and pUU2 with PstI

a. 0.5% gel, $1Vcm^{-1}$, 16 h.

b. 0.5% gel, $1Vcm^{-1}$, 60 h.

c. 1.4% gel, $3Vcm^{-1}$, 5 h.


p
λ
λ EcoRI/HindIII
R68.45
pUU1
pUU2
q
λ
λ EcoRI/HindIII
R6845
pUU1
pUU2
r
λ EcoRI/HindIII
R68.45
pUU1
pUU2

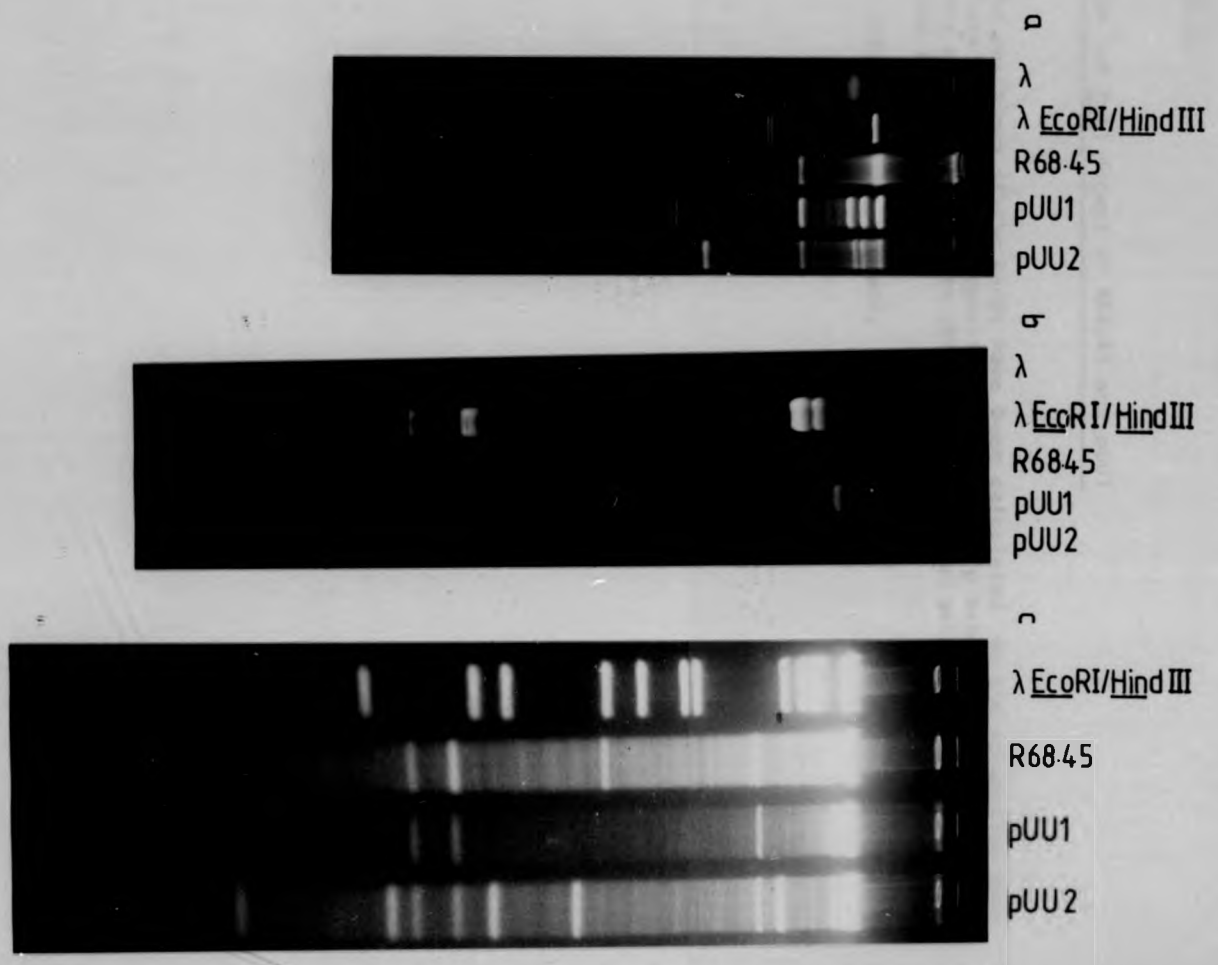

a
λ
λ EcoRI/HindIII
R68.45
pUU1
pUU2
b
λ
λ EcoRI/HindIII
R68.45
pUU1
pUU2
c
λ EcoRI/HindIII
R68.45
pUU1
pUU2

FIGURE 3.1

Diagram[1] of PstI digest of R68.45 and pUU1

1. The positions of the bands have been calculated on the assumption that there is an absolute logarithmic relationship between size and mobility. This enables the results obtained from several gels to be incorporated into the same diagram.

2. * indicates a double band.

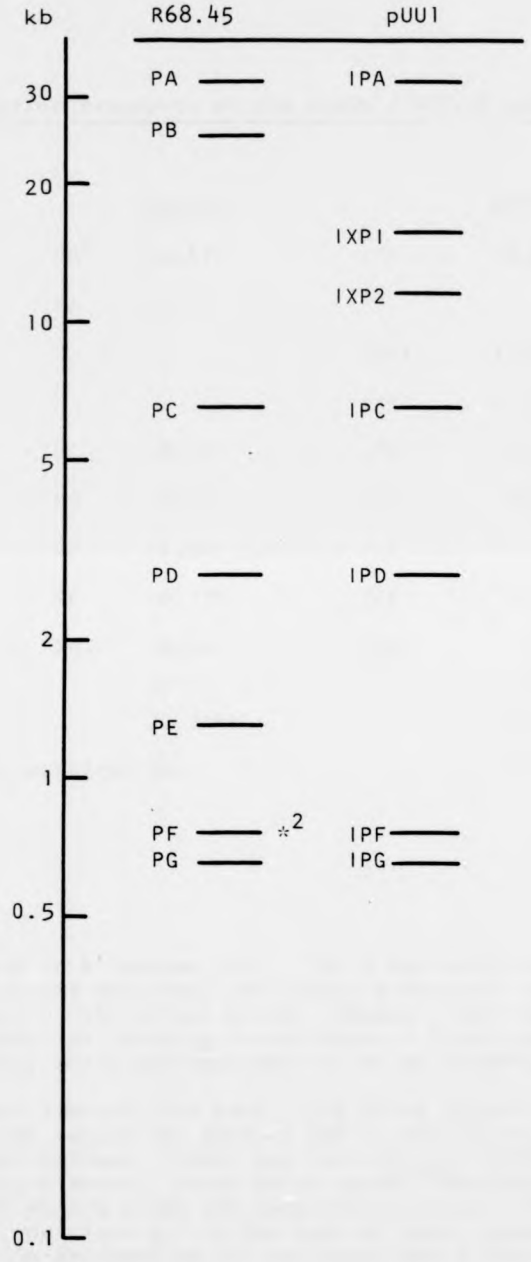

kb
R68.45
pUU1
30
20
10
5
2
1
0.5
0.1
PA
PB
PC
PD
PE
PF
PG
*2
IPA
IXP1
IXP2
IPC
IPD
IPF
IPG

TABLE 3.3

Sizes of restriction fragments of the plasmid R68.45 and pUU1 with PstI[1]

	R68.45		pUU1
PA[2]	32.67	IPA	32.67
PB	24.84		
		IXP1	15.68
		IXP2	11.32
PC	6.52	IPB	6.52
PD	2.77	IPC	2.77
PE	1.34		
PF	0.77*	IPF	0.77
PG	0.66	IPG	0.66
Size	70.34kb		70.39kb
Size of newly acquired DNA			2.16kb

1. The sizes are in kilobases (kb). The 2 decimal places do not imply that such accuracy was obtained, the larger bands were probably within a few kilobases of the values given. However, for comparative purposes they are useful as rounding up and down of sizes can blur differences between bands, which are apparent on the gel itself.

2. These letters identify the band. The first letter identifies the enzyme and the second the band of R68 or R68.45 as given in the maps of Nayudu and Holloway (1981) and Moore et al. (1983). In the case of derivative plasmids, bands which appear identical in size to bands in R68.45 are given the same letters plus a prefixing number to identify the plasmid. In the case of novel bands the band identification includes an 'X' for extra and a figure instead of a letter at the end.

3. * identifies a double band.

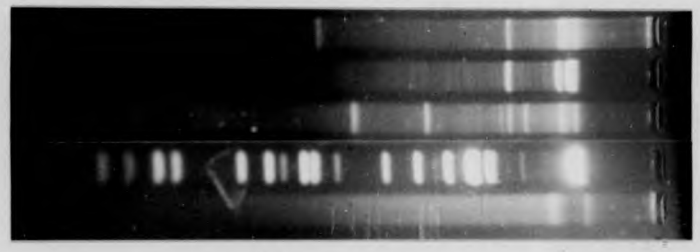
PLATE 3.3

Restriction digest of plasmids R68.45, pUU1 and pUU2 with *Sma*I

a. 0.5% gel, $1Vcm^{-1}$, 16 h.

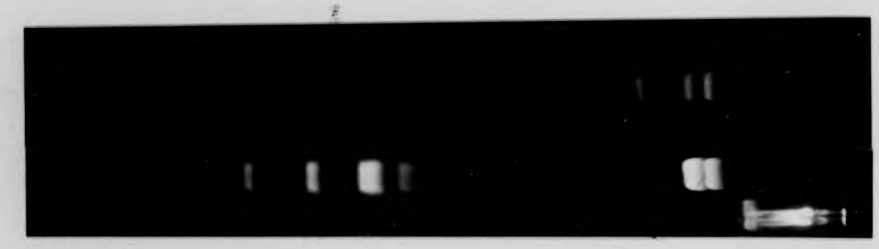
b. 0.5% gel, $1Vcm^{-1}$, 60 h.

a



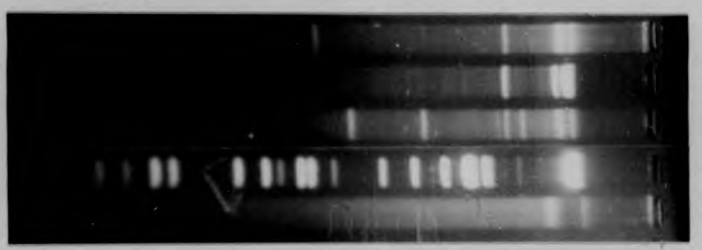
R68.45
pUU1
pUU2
λEcoRI/HindIII
λ

b



R68.45
pUU1
pUU2
λEcoRI/HindIII
λ

a

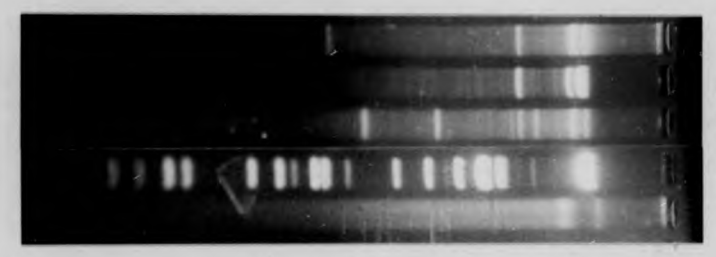


R68.45
pUU1
pUU2
λEcoRI/HindIII
λ

b

R68.45
pUU1
pUU2
λEcoRI/HindIII
λ

a

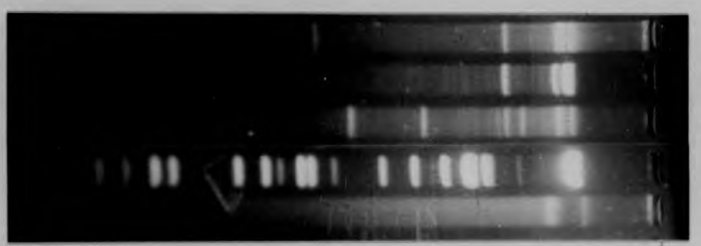


R68.45
pUU1
pUU2
λEcoRI/HindIII
λ

b

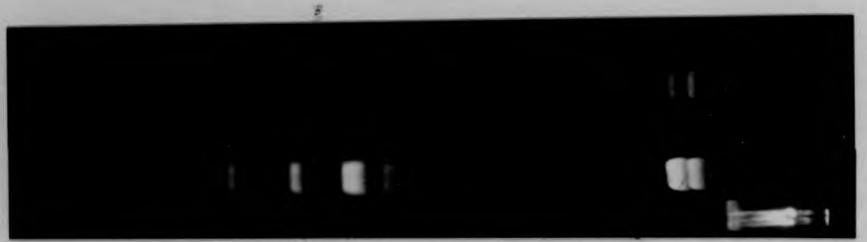
R68.45
pUU1
pUU2
λEcoRI/HindIII
λ

b



R68·45
pUU1
pUU2
λEcoRI/HindIII
λ

a



R68·45
pUU1
pUU2
λEcoRI/HindIII
λ

FIGURE 3.2

Diagram of *Sma*I digest of R68.45 and pUU1

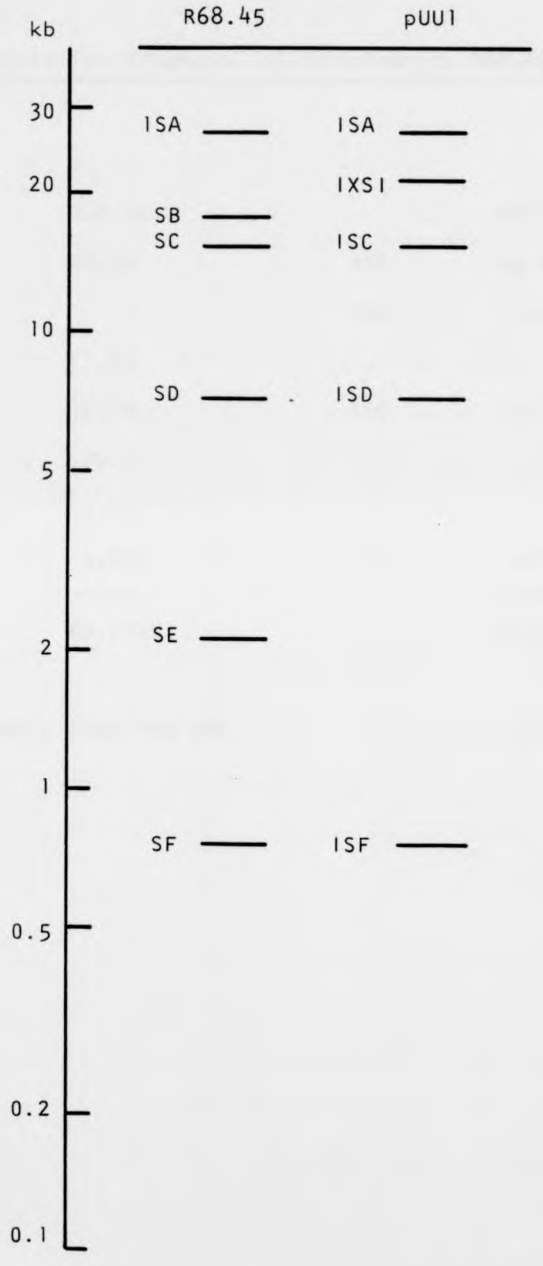

kb
R68.45
pUU1
30
20
10
5
2
1
0.5
0.2
0.1
ISA
SB
SC
SD
SE
SF
ISA
IXS1
ISC
ISD
ISF

TABLE 3.4

Sizes of restriction fragments of the plasmids R68.45 and pUU1 with *SmaI*

	R68.45		pUU1
SA	26.52	ISA	26.52
		IXSI	21.07
SB	17.55		
SC	15.10	ISC	15.10
SD	7.15	ISD	7.15
SE	2.12		
SF	0.76	ISF	0.76
Size	69.20kb		70.60kb
Size of newly acquired DNA			3.52kb

These modifications in the SmaI digest pattern of pUU1 were common to the plasmids of the two independently isolated transconjugants from the mating of PAC174 (R68.44) with PP3, mentioned in Section 3.5.2.1.

3.5.2.3 Digestion of R68.45 and pUU1 with KpnI

R68.45 and pUU1 were digested with the restriction endonuclease KpnI and the bands sized (Plate 3.4; Figure 3.3; Table 3.5).

The KD band of R68.45, indicative of the second copy of IS21 (Riess et al., 1980a, b; Willetts et al., 1981), was missing in pUU1. The KB band of R68.45 was missing in pUU1 and had been replaced by two smaller bands, IXK1 and IXK2. The net change in size of pUU1 with respect to R68.45, taking the loss of IS21 into consideration, was an increase of 2.75kb.

3.5.2.4 Digestion of R68.45 and pUU1 with XhoI, EcoRI, HindIII and EcoRI/HindIII

R68.45 and pUU1 were digested with the restriction endonucleases XhoI, EcoRI, HindIII and EcoRI/HindIII and the bands sized (Plate 3.5; Table 3.6).

The plasmid sizes obtained from these digests were of low accuracy as the fragments were extremely large, and in the cases of EcoRI and HindIII were based on extrapolation beyond the size of undigested lambda (49kb). However they were of comparative use, and all indicated a slight increase in size of pUU1 over R68.45. One of the pair of XhoI bands of R68.45 had increased in size in pUU1, 1XX1.

pUU1 retained the single EcoRI and HindIII sites of R68.45. A double digest with EcoRI and HindIII revealed that in pUU1 the E/HA band of R68.45 had increased in size to give IXE/HI.

PLATE 3.4

Restriction digest of R68.45, pUU1 and pUU2 with *Kpn*I

a. 0.5% gel, $1Vcm^{-1}$, 16 h.

b. 0.5% gel, $1Vcm^{-1}$, 60 h.


a
R 68.45
pUU1
pUU2
λ HindIII
λ


b
R 68.45
pUU1
pUU2
λ HindIII
λ

a

R 68.45
pUU1
pUU2
λ Hind III
λ

b

R 68.45
pUU1
pUU2
λ Hind III
λ

FIGURE 3.3

Diagram of KpnI digest of R68.45 and pUU1


R68.45
pUU1
kb
30
20
10
5
2
1
0.5
0.2
0.1
KA
KB
KC
KD
KE
IKA
IKC
IXK1
IXK2
IKE

TABLE 3.5

Sizes of restriction fragments of the plasmids R68.45 and pUU1 with KpnI

	R68.45		pUU1
KA	34.44	IKA	34.44
KB	15.66		
KC	13.96	IKC	13.96
		IXK1	11.18
		IXK2	7.23
KD	2.11		
KE	1.61	IKE	1.61
Size	67.78kb		68.42kb

Size of newly acquired DNA 2.75kb

PLATE 3.5

Restriction digests of R68.45, pUU1 and pUU2 with *Xho*I, *Eco*RI, *Hin*dIII and *Eco*RI/*Hin*dIII.

a. 0.5% gel, $1Vcm^{-1}$, 16h.
b. 0.5% gel, $1Vcm^{-1}$, 60h.

a
λ
λHind III
R68.45
pUU1
pUU2
R68.45
pUU1
pUU2
R68.45
pUU1
pUU2
R68.45
pUU1
pUU2
EcoRI
Hind III
EcoRI/Hind III
XhoI
b
λ
λHind III
R68.45
pUU1
pUU2
R68.45
pUU1
pUU2
R68.45
pUU1
pUU2
R68.45
pUU1
pUU2
EcoRI
Hind III
EcoRI/Hind III
XhoI

a

λ
λHind III
R68.45
pUU1
pUU2
EcoRI
HindIII
EcoRI/HindIII
XhoI

b

λ
λHind III
R68.45
pUU1
pUU2
EcoRI
HindIII
EcoRI/HindIII
XhoI

TABLE 3.6

Sizes of restriction fragments of the plasmids R68.45 and pUU1 with a. XhoI, b. EcoRI, c. HindIII and d. EcoRI/HindIII

a. XhoI		R.68.45		pUU1
			IXXI	38.17
	XA	33.98*	IXA	33.98
b. EcoRI		R68.45		pUU1
	EA	55.61	IXEI	56.96
c. HindIII		R68.45		pUU1
	HA	58.30	IXHI	58.95
d. EcoRI/HindIII		R.68.45		pUU1
			IXE/HI	43.51
E/HA		42.02		
E/HB		23.93	IE/HB	23.93

3.5.2.5 Interpretation of the restriction digests of R68.45 and pUU1

From the data presented above it is apparent that R68.44 had undergone some modifications in PP3 in addition to the loss of the second copy of IS21.

R68.44 was unstable with respect to its enhanced chromosome-mobilization (E.C.M.) ability compared to R68.45 (Haas and Holloway, 1976). E.C.M. ability was correlated with the possession of a duplicated 2.12kb portion of R68 known as IS21 (Riess et al., 1980a, b; Willetts et al., 1981). R68.45 had been shown to be particularly unstable with respect to its E.C.M. ability after transfer to a new host, several subculturings with selection for E.C.M. were required before R68.45 became stably established (Haas and Riess, 1983). It was therefore not surprising that R68.44 lost the second copy of IS21, especially as no selection for E.C.M. was carried out after the transfer of R68.44 to PP3. It was not possible to tell at what stage R68.44 had lost the second copy of IS21 as the original strain PAC174 (R68.44) was no longer available for examination and the restriction digests presented here were performed six months to a year after the matings described in this section were carried out. During this intervening period PPW1 (the derivative of PP3 into which R68.44 had been transferred) was routinely subcultured, without selection for E.C.M., every 14 days. However during the subsequent 2 to 3 years when this work was carried out no further changes in pUU1 were detected.

In addition to this reversion of R68.44 to the R68 form, pUU1 had undergone some other modification which included an increase in size of about 2.16kb to 3.52kb. This modification had included the acquisition of at least two new restriction sites.

It should be pointed out that the sizes of the restriction fragments of R68.45 obtained here do not correspond exactly with those in the literature (Riess et al., 1980a,b; Currier and Morgan, 1981; Nayudu and Holloway, 1981;

Moore et al., 1983). This was particularly the case with the larger fragments. However these apparent differences were probably due to differences in protocol in running the agarose gels and to different approaches in measuring the migration distances. Due to the approximately logarithmic relationship between molecular weight and migration distance, slight variations in measuring technique can have a considerable effect on the estimated size of the larger bands. In fact the sizes given for the larger bands by Currier and Morgan (1981) were up to 5kb different in size from those given by Riess et al. (1980a, b); Nayudu and Holloway (1981); and Moore et al. (1983). The important features of the band sizes was that they were consistent and comparable internally within one worker's set of results and that they were comparable, at least in terms of the relative position of the fragments with the results of other workers.

For the interpretation of the results presented here the published maps of R68 and R68.45 have been used as regards the relative order of the bands and restriction sites (Nayudu and Holloway, 1981; Moore et al., 1983). The discrepancy between the relative positions of the KpnI, KB and KC bands of R68 in the maps of Riess et al. (1980a) and those of Nayudu and Holloway (1981) and Moore et al. (1983) should be pointed out. This is discussed in Nay du and Holloway (1981) and their positioning is followed here.

The PstI generated PB (24.84kb) band of R68.45 had been replaced by two smaller bands in pUU1, 1XP1 (15.68kb) and 1XP2 (11.32) (Plate 3.2, Figure 3.1, Table 3.3). This suggested that a 2.16kb piece of DNA containing one PstI site had inserted itself into the PB band of R68.44 so that the new PstI site was 15.68kb from one end and 11.32kb from the other. The orientation of these two derivative fragments could not be determined from these data alone (Figure 3.4). This 2.16kb inserted piece of DNA was designated I1, for 'insert one'.

SB (17.55kb) an SmaI generated fragment of R68.45 had increased in size

FIGURE 3.4

Interpretation of the restriction data to determine the location of I1 in pUU1

a. Positions of various restriction sites within the PstI generated PB band of R68.45.[1]

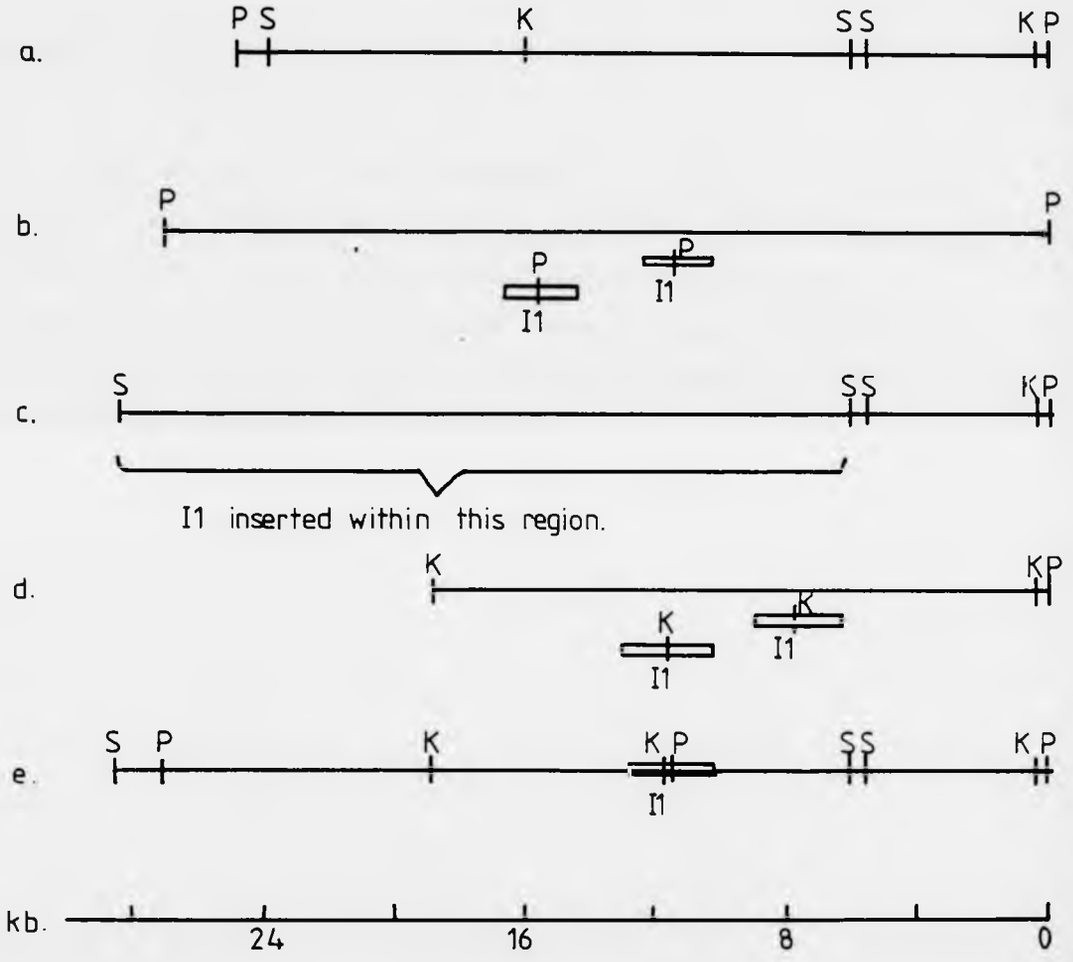
b. Alternative positions of the new PstI site and I1 in pUU1 as determined from the PstI digest data.

c. Region within which I1 could be inserted as determined by the SmaI digest data.

d. Alternative positions of the new KpnI site and I1 in pUU1 as determined from the KpnI digest data.

e. Positioning of I1 and its internal restriction sites from the combined restriction data.

1. For the purposes of this figure the 2 o'clock PstI site (Figure 3.5) was taken as 0kb and the 6 o'clock PstI site was at 24.8kb. The relative positions of the restriction sites in the PB fragment of R68.45 were determined from the sizes of fragments presented here, and where necessary from a regression analysis of these sizes and the sizes given by Moore et al. (1983). This led to the misplacement of the 23.8kb SmaI site to the right of the 24.8kb PstI site. This discrepancy did not affect the interpretation of the results presented here.



K = KpnI

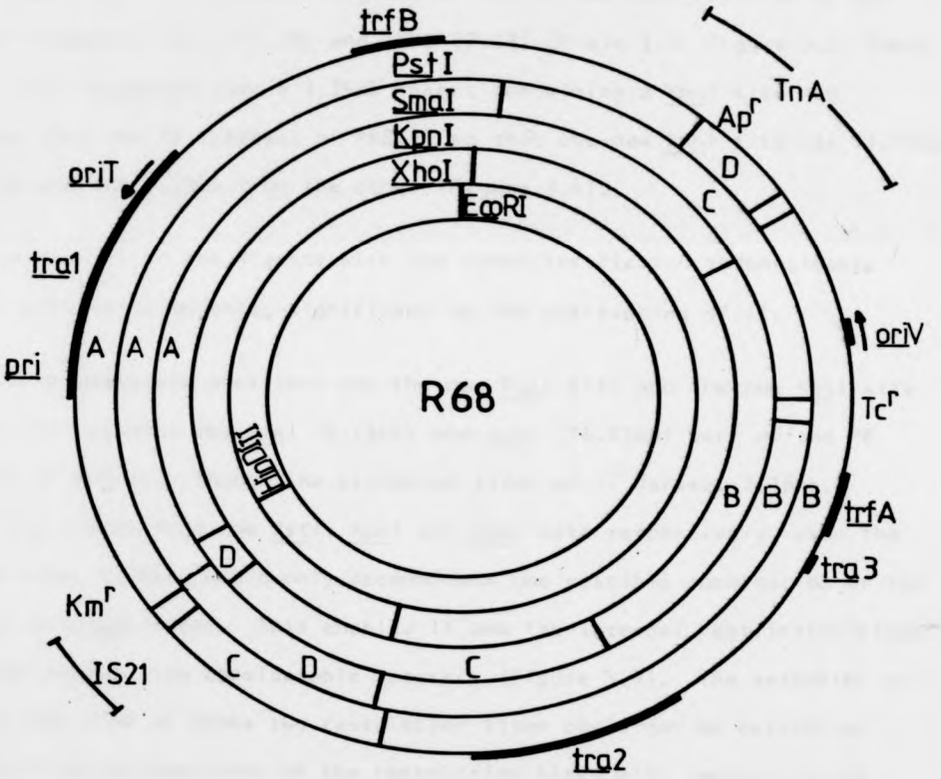
P = PstI

S = SmaI

FIGURE 3.5

Map of plasmid R68

Based on the maps of R68, RP4 and RK2 given by Nayudu and Holloway (1981), Thomas (1981a), Lanka and Barth (1981) and Guiney and Yakobson (1983). OriV: origin of unidirectional vegetative plasmid DNA replication. OriT: origin of conjugal transfer. tra: regions required for conjugal transfer. trf: regions specifying trans-acting replication or maintenance functions.


trfB
PstI
SmaI
KpnI
XhoI
EcoRI
TnA
Ap^r
D
C
oriT
tra1
pri
A
A
A
oriV
R68
Tc^r
HindIII
B
B
B
trfA
tra3
D
Km^r
IS21
C
D
C
tra2

in pUU1 by 3.52kb producing IXS1 (21.07kb), no new SmaI sites had been generated (Plate 3.3, Figure 3.2, Table 3.4). The PB and SB fragments of R68.45 overlapped (Figure 3.5). It was therefore possible to combine this SmaI data with the PstI to narrow down the possible site of insertion of I1 (Figure 3.4).

In pUU1 the KB (15.66kb) fragment of R68.45 had been replaced by two smaller fragments IXK1 (11.18) and IXK2 (7.23) (Plate 3.4, Figure 3.3, Table 3.5). This suggested that a 2.75kb insert containing a KpnI site had inserted into the KB fragment of R68.44 so that the new KpnI site was 11.18kb from one end and 7.23kb from the other (Figure 3.4).

The results of the digests with the other restriction endonucleases did not contribute anything significant to the positioning of I1.

The two possible positions for the new PstI site and the new KpnI site of PUU1 fell between the SmaI (6.13kb) and KpnI (16.03kb) part of the PB fragment of R68.45. Though the estimated sizes of I1 varied, 2.16kb, 2.75kb and 3.52kb from the PstI, KpnI and SmaI data respectively, even the maximum size, 3.52kb, would only accommodate one possible combination of the new PstI and KpnI sites. This enabled I1 and its internal restriction sites to be positioned with considerable accuracy (Figure 3.4). The extension of I1 on either side of these two restriction sites could not be determined accurately as the positions of the restriction sites with respect to the extremities of I1 were unknown.

Thomas (1981a) positions various genetic regions on his map of RK2. Two of these trfA and tra3 map within the fragment in which I1 had inserted. It was possible to include these two genetic regions in the diagram of this part of R68.44 (Figure 3.6). These two genetic regions fell precisely into that part of the SmaI-KpnI fragment into which I1 had inserted (Figure 3.6). TrfA was required for initiation of replication while tra3 was required for

FIGURE 3.6

Possible site for the insertion of I1 with respect to *trfA* and *tra3*

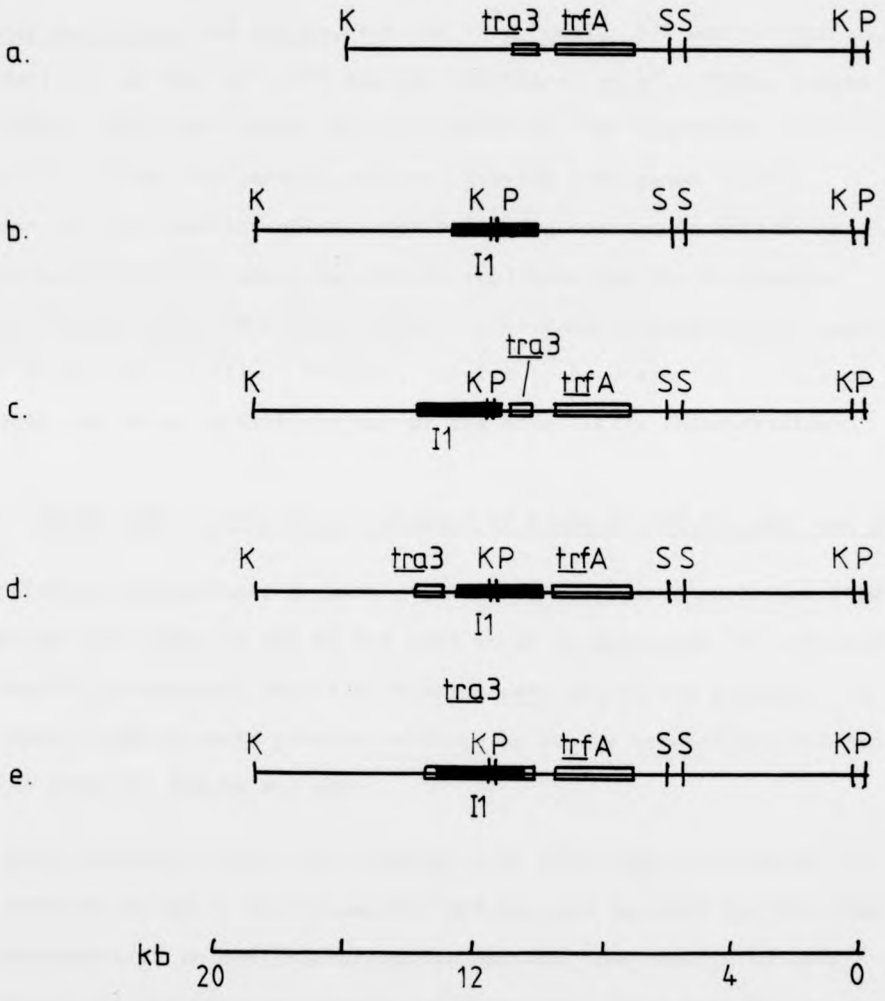
a. Location of *trfA* and *tra3* (Thomas, 1981a) between the 3 o'clock *Pst*I site and the 5 o'clock *Kpn*I site of R68.45.

b. Location of *Kpn*I and *Pst*I sites of I1 in pUU1.

c. Location of I1 to the left of *trfA* and *tra3*.

d. Location of I1 between *trfA* and *tra3*.

e. Location of I1 within *tra3*.


a.
K
tra3
trfA
S S
K P
b.
K
K P
S S
K P
I1
c.
tra3
K
K P
trfA
S S
K P
I1
d.
K
tra3
K P
trfA
S S
K P
I1
e.
tra3
K
K P
trfA
S S
K P
I1
kb
20
12
4
0

conjugal transfer (Thomas, 1981a, b; Thomas *et al.*, 1982). Therefore it was unlikely that I1 had inserted into *trfA*, as such a plasmid would be unable to replicate. Cowan and Krishnapillai (1982) however isolated a Tn7 insertion mutant into *trfA* of R18 which was capable of replication in *Pseudomonas aeruginosa* and *putida*, but not in *E. coli*. R18 was similar to, if not identical to R68, RP1, RP4 and RK2 (Burkhardt *et al.*, 1979a; Stokes *et al.*, 1981). This left three possible positions for insertion of I1; i) to the left of the two genetic regions, leaving both genes intact; ii) between the two genetic regions, again leaving both genes intact; or iii) into *tra3*, such a plasmid would be able to replicate but not to transfer conjugally (Figure 3.6). The restriction digest data presented here supports all three hypotheses equally. However, results to be presented in Chapter 4 suggest that the third possibility may be the more likely interpretation.

3.5.3 Comparison of Restriction Digests of Plasmids R68.45, pUU1 and pUU2

Restriction endonuclease digests of the R-prime plasmid pUU2 from PPW2 were compared with those of R68.45 and pUU1 so as to determine the site and size of the PP3 chromosomal insertion bearing *dehI* within the plasmid. In addition these digests would provide evidence as to the connections between the R-prime pUU2 and R68.44 and pUU1.

The most coherent picture will emerge if at this stage the results for pUU2 are compared directly with those for R68.45 ignoring pUU1 for the time being. Subsequently, in the interpretative section, the results for all three plasmids will be brought together and compared. This approach is necessary due to the linear presentation of a subject which has horizontal ramifications. Though it has disadvantages, it does avoid the confusion which could occur in presenting results and interpretation concurrently.

3.5.3.1 Digestion of plasmids R68.45 and pUU2 with *PstI*

R68.45 and the R-prime plasmid pUU2 from PPW2 were digested with the

restriction endonuclease PstI and the restriction fragment sized (Plate 3.2; Figure 3.7; Table 3.7).

pUU2 had lost the band PE and one of the pair PF which were indicative of the second copy of IS21 in R68.45. In addition pUU2 had lost the PB band of R68.45; this had been replaced by two large single bands, 2XP1 and 2XP2, and five sets of smaller double bands. All the other PstI bands of R68.45 were present in pUU2. The net change in size of pUU2 with respect to R68.45, taking into account the loss of IS21, was an increase of 14.72kb.

3.5.3.2 Digestion of R68.45 and pUU2 with SmaI

The two plasmids were digested with SmaI and the bands sized (Plate 3.3; Figure 3.8; Table 3.8).

pUU2 had lost band SE of R68.45 which was indicative of the presence of the second copy of IS21. Band SB of R68.45 was also missing; it had been replaced by several smaller bands. Due to the small number of bands and the distances between them on the gel it was difficult to determine with any degree of certainty whether there were any doubles present amongst the new bands. However bands 2XS3 and SXS6 of pUU2 were probably double bands. In which case the net change in size of pUU2 with respect to R68.45 as determined from the SmaI digest data and taking the loss of IS21 into consideration was 20.12kb.

3.5.3.3 Digestion of R68.45 and pUU2 with KpnI

The two plasmids were digested with KpnI and the bands sized (Plate 3.4; Figure 3.9; Table 3.9).

The KD band, indicative of the second copy of IS21, of R68.45 was missing in pUU2. The KB band of R68.45 had been replaced in pUU2 by one

FIGURE 3.7

Diagram of PstI digests of R68.45 and pUU2

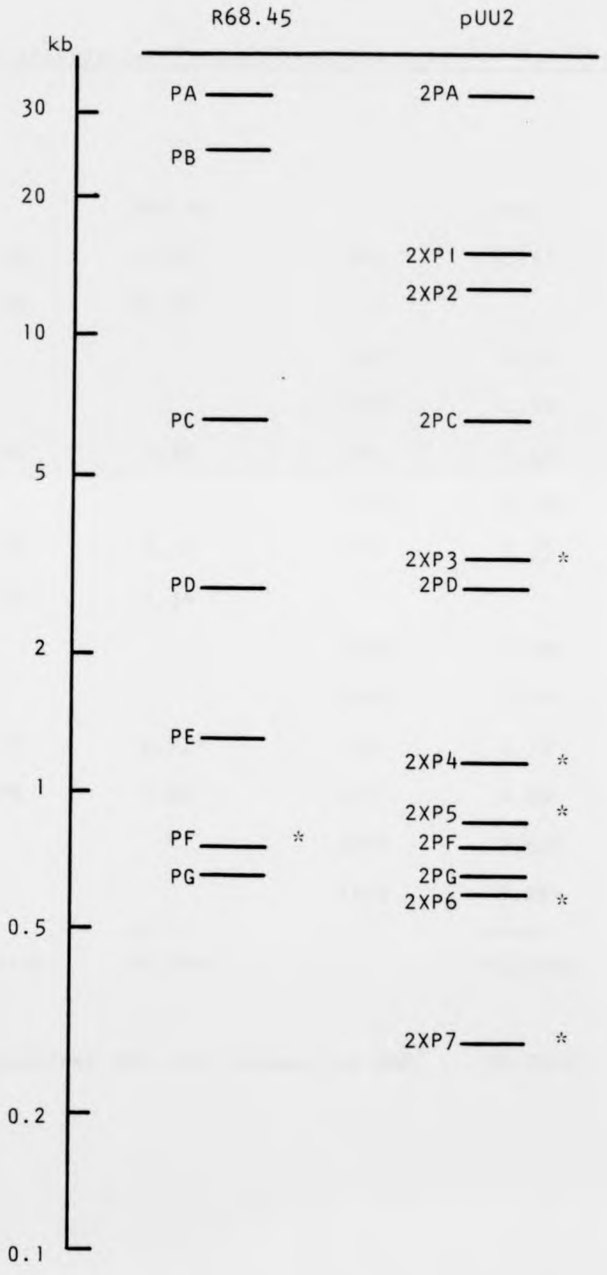

R68.45
pUU2
kb
30
20
10
5
2
1
0.5
0.2
0.1
PA
PB
PC
PD
PE
PF
PG
2PA
2XP1
2XP2
2PC
2XP3
2PD
2XP4
2XP5
2PF
2PG
2XP6
2XP7
*

TABLE 3.7

Sizes of the restriction fragments of the plasmids R68.45 and pUU2 with PstI

	R68.45		pUU2
PA	32.67	2PA	32.67
PB	24.84		
		2XP1	14.82
		2XP2	12.38
PC	6.52	2PC	6.52
		2XP3	3.23*
PD	2.77	2PD	2.77
PE	1.34		
		2XP4	1.19*
		2XP5	0.87*
PF	0.77*	2PF	0.77
PG	0.66	2PG	0.66
		2XP6	0.60*
		2XP7	0.29*
Size	70.34kb		82.95kb
Acquired DNA with respect to R68			14.72kb

FIGURE 3.8

Diagram of *Sma*I digests of R68.45 and pUU2

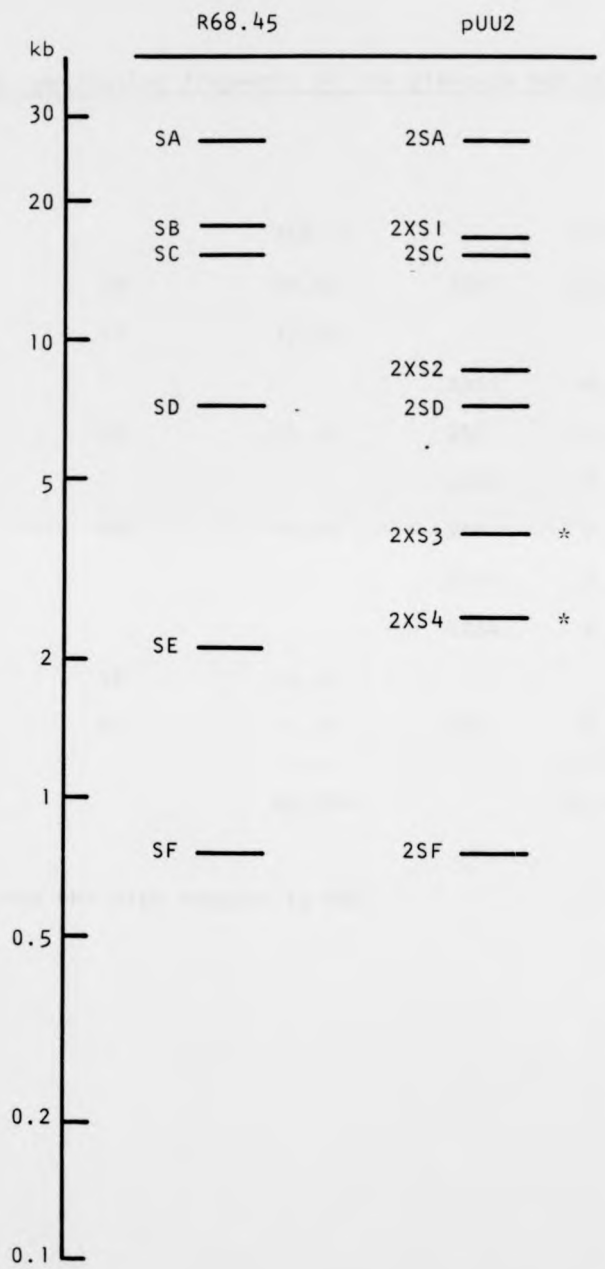

R68.45
pUU2
kb
30
20
10
5
2
1
0.5
0.2
0.1
SA
SB
SC
SD
SE
SF
2SA
2XS1
2SC
2XS2
2SD
2XS3
*
2XS4
*
2SF

TABLE 3.8

Sizes of the restriction fragments of the plasmids R68.45 and pUU2 with SmaI

	R68.45		pUU2
SA	26.52	2SA	26.52
SB	17.55		
		2XS1	16.60
SC	15.10	2SC	15.10
		2XS2	8.45
SD	7.15	2SD	7.15
		2XS3	3.80*
		2XS4	2.51*
SE	2.12		
SF	0.76	2SF	0.76
	69.20kb		87.20kb

Acquired DNA with respect to R68 20.12kb

TABLE 3.9

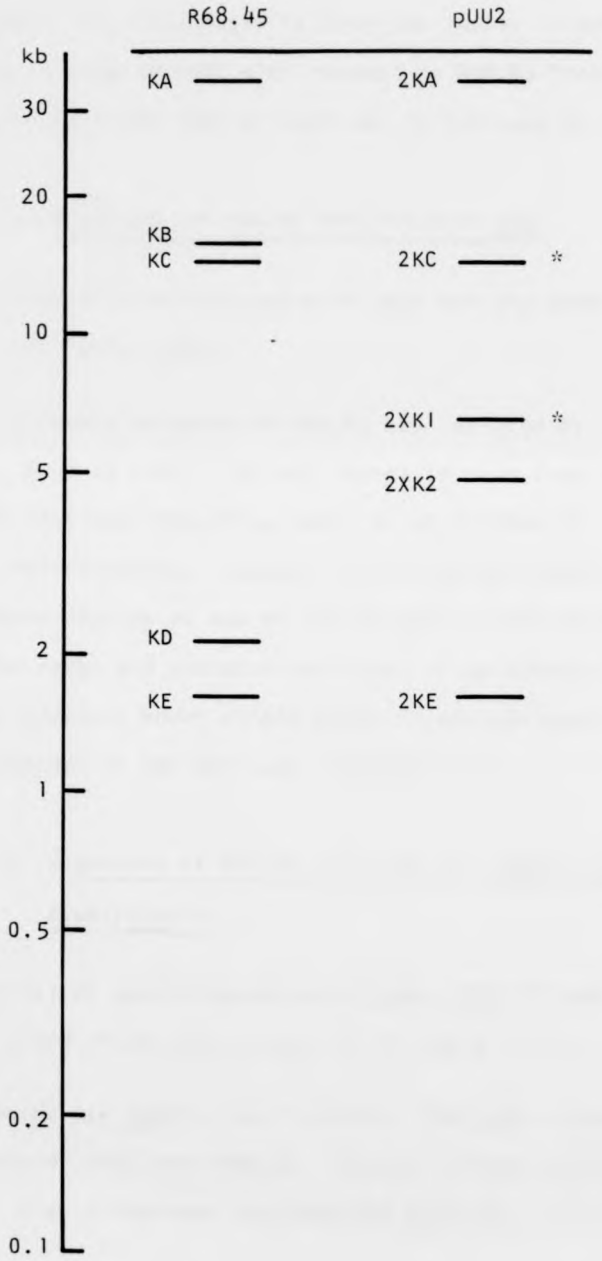
Sizes of the restriction fragments of the plasmids R68.45 and pUU2 with KpnI

	R68.45		pUU2
KA	34.44	2KA	34.44
KB	15.66		
KC	13.96	2KC	13.96*
		2XK1	6.49*
		2XK2	4.79
KD	2.11		
KE	1.61	2KE	1.61
Size	67.78kb		81.74kb

Acquired DNA with respect to R68 16.07kb

FIGURE 3.9

Diagram of KpnI digests of R68.45 and pUU2


R68.45
pUU2
kb
30
20
10
5
2
1
0.5
0.2
0.1
KA
KB
KC
KD
KE
2KA
2KC
2XK1
2XK2
2KE
*
*

double and two single bands in pUU2. The larger new single band of 13.96kb appeared as virtually identical in size to the KC band of R68.45 and the 2KC band of pUU1. To distinguish it from the latter it was designated 2KC[1]. The net change in size of pUU2 with respect to R68.45 from the KpnI digest data, bearing in mind the loss of IS21 was in increase in size of 16.07kb.

3.5.3.4. Digestion of R68.45 and pUU2 with XhoI

The two plasmids were digested with XhoI and the bands sized (Plate 3.4; Figure 3.10; Table 3.10).

One of the double XA bands of R68.45 was replaced by two smaller bands and one double band in pUU2. The net change in size from these data was an increase of 25.78kb over R68.45 by pUU2, or of 27.90kb if the loss of IS21 is taken into consideration. However little emphasis must be given to the accuracy of these figures as two of the fragments from which they were determined were large and therefore unlikely to be accurately sized. Because of the lack of adjacent known single bands it was not possible to determine conclusively whether or not 2XX3 was a double band.

3.5.3.5 Digestion of R68.45 and pUU2 with EcoRI, HindIII and EcoRI/HindIII

The two plasmids were digested with EcoRI, HindIII and EcoRI/HindIII and the bands sized (Plate 3.5; Figure 3.11; Table 3.11).

There were no new EcoRI sites in pUU2. The EcoRI digest showed an increase in size of pUU2 over R68.45. However little reliability can be placed in the actual size difference (see Section 3.5.2.4).

pUU2 possessed five new HindIII sites which generated two large single band and two smaller double bands. The identification of 2XE/H2 and 2XB/H3

FIGURE 3.10

Diagram of XhoI digests of R68.45 and pUU2

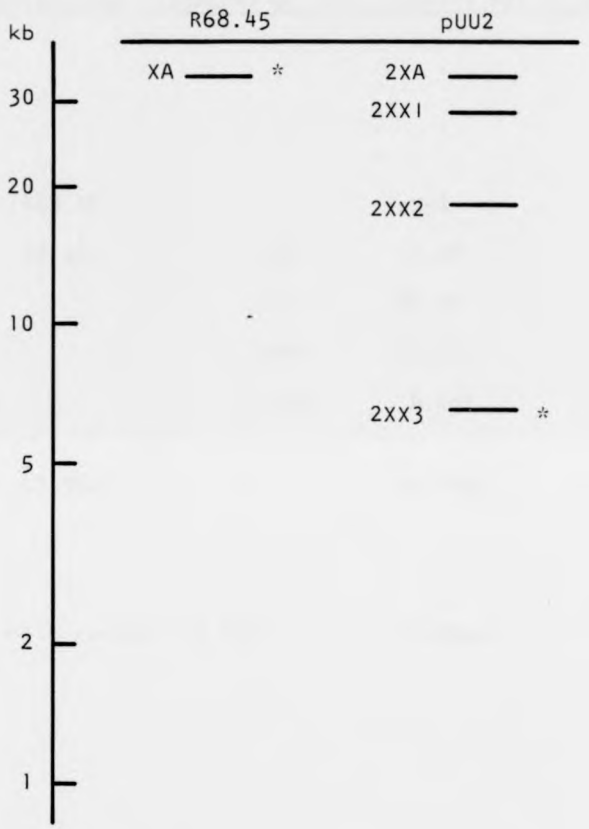

kb
R68.45
pUU2
XA
*
2XA
2XX1
2XX2
2XX3
*
30
20
10
5
2
1

TABLE 3.10

Sizes of the restriction fragments of the plasmids R68.45 and pUU2 with XhoI

	R68.45		pUU2
XA	33.98*	2XA	33.98
		2XX1	28.46
		2XX2	18.20
		2XX3	6.55*
Size	67.96kb		93.74kb
Acquired DNA with respect to R68			27.90kb

FIGURE 3.11

Diagram of EcoRI, HindIII and EcoRI/HindIII digests of R68.45 and pUU2

a. EcoRI

b. HindIII

c. EcoRI/HindIII

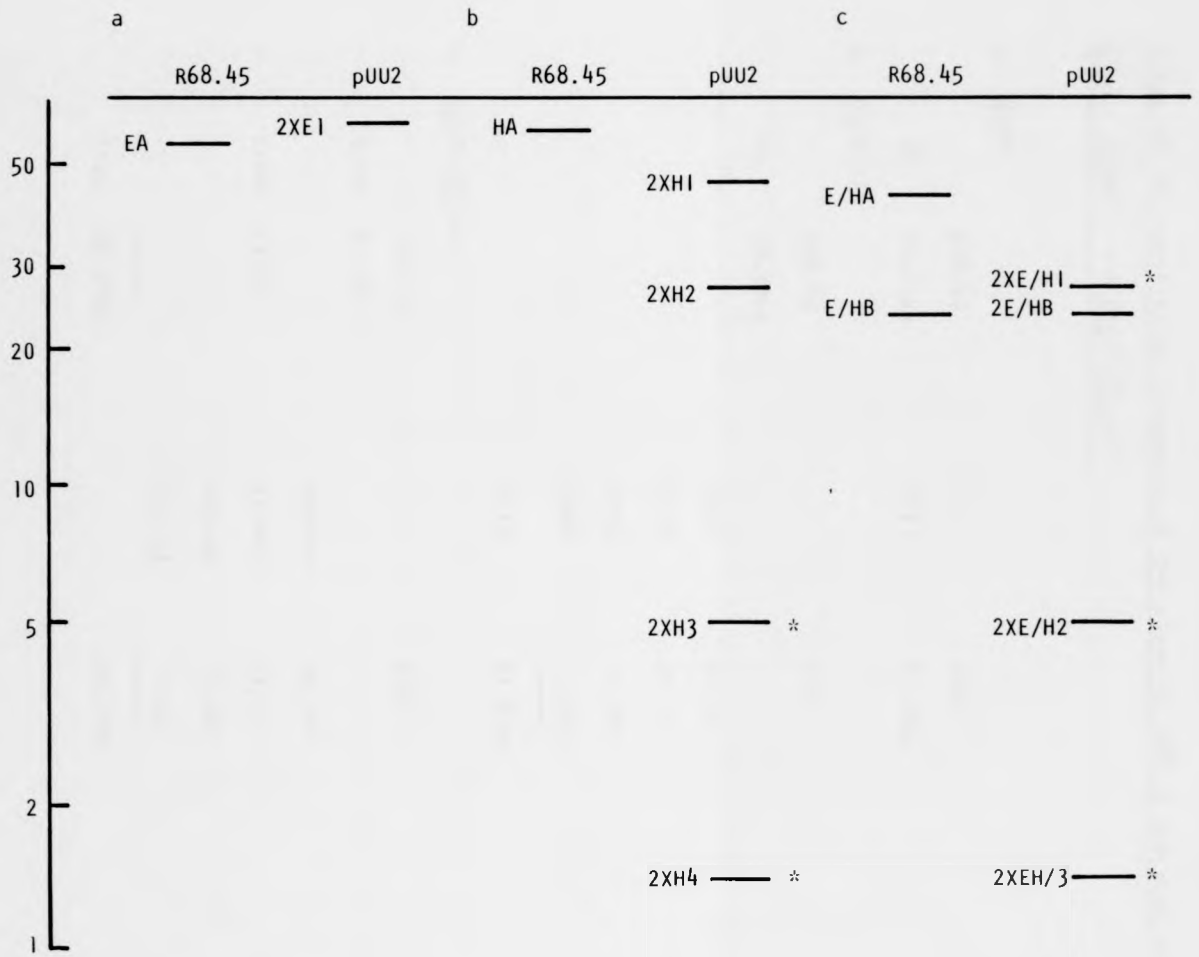

a
b
c
R68.45
pUU2
R68.45
pUU2
R68.45
pUU2
EA
2XE1
HA
2XH1
E/HA
2XH2
E/HB
2XE/H1
2E/HB
2XH3
2XE/H2
2XH4
2XEH/3
50
30
20
10
5
2
1

TABLE 3.11

Sizes of the restriction fragments of the plasmids R68.45 and pUU2 with EcoRI, HindIII and EcoRI/HindIII

a. EcoRI

	R68.45		pUU2
EA	55.61kb	2XEI	61.78kb

b. HindIII

	R68.45		pUU2
HA	58.30kb		
		2XH1	44.79
		2XH2	26.70
		2XH3	5.04*
		2XH4	1.43*
		Size	83.83kb

c. EcoRI/HindIII

	R68.45		pUU2
E/HA	42.02		
		2XE/H1	26.70*
E/HB	23.93	2E/HB	23.93
		2XE/H2	5.04*
		2XE/H3	1.43*
Size	65.95kb		90.27kb

as doubles was subject to the reservations mentioned in preceding sections. However, a derivative plasmid of pUU2, pUU3, definitely possessed a double band corresponding to 2XE/H2 (see Chapter 6). The HindIII estimated size of pUU2 was greater than that for R68.45, but again little reliability can be placed on the measured size difference.

The EcoRI/HindIII digest data confirmed the presence of an EcoRI site in the 2XHIB band produced by the digestion with HindIII alone. This gave two bands, one, 2XE/HI, appeared as virtually identical in size to the 2XH2 (26.70kb) band produced by the HindIII digest alone. The second band, 2EH/B, was similar in size to the smaller of the two bands of R68.45, E/HB, produced by EcoRI/HindIII digestion. Again, an increase in size of pUU2 over R68.45 was shown by those data.

3.5.3.6 Interpretation of the Digests of Plasmids R68.45, pUU1 and pUU2

The relationships between pUU1 and R68.44 have been established (Section 3.5.2.5). However the relationships between pUU2 and the other two plasmids remain undetermined. In the preceding sections the differences between pUU2 and R68.45 have been catalogued but references to pUU1 were omitted. This section will deal firstly with the relationships between the three plasmids and secondly with the location of the insert of PP3 chromosomal DNA bearing dehI, I2.

3.5.3.6.1 The Relationships Between Plasmids R68.45, pUU1 and pUU2

If pUU2 was derived from pUU1 common features would be expected between the restriction digest patterns after discounting the bands which both plasmids share with R68.45.

A comparison of the PstI digest patterns of pUU1 and pUU2 with that of R68.45 (Figure 3.12) revealed that in both cases the PB band of R68.45 had been replaced by other smaller bands. Therefore I1, the insert in pUU1, and I2, the dehI bearing insert of pUU2, were in the same region of the plasmid. In pUU1 the PB band of R68.45 had been replaced by two bands 1XP1 and 1XP2. Therefore if in pUU2 only one new insert, I2, had been introduced into pUU1 and no other modification had occurred, it would be expected that one of these new bands of pUU1, 1XP1 or 1XP2, would have disappeared and been replaced by another band or other bands, and that the other new band of pUU1 would be retained in pUU2. This was initially believed to have been the case; that 1XP1 of pUU1 had been retained by pUU2, while 1XP2 was absent and had been replaced by other bands (Beeching et al., 1983), which suggested that I2 had inserted into 1XP2. However running low percentage gels (0.5% agarose) at low voltages ($1Vcm^{-1}$) for a long time (60 h.) proved that this was not the case. Neither 1XP1 nor 1XP2 were present in pUU2. Therefore at least two modifications had occurred in pUU1 in the formation of pUU2, or pUU2 had been formed, in some way, directly from R68.44.

The SmaI digests of R68.45, pUU1 and pUU2 indicated that in the two latter plasmids the SB band of R68.45 had been replaced by one or more new bands (Figure 3.13). Therefore both I1 in pUU1 and I2 in pUU2 had inserted into the same region of R68.44. However, as in pUU1 the SB band of R68 had increased in size by the addition of I1 but no new SmaI sites had been generated, these digests provided no evidence as to whether or not pUU2 was formed via pUU1.

The KpnI digests indicated that both pUU1 and pUU2 had been formed by modifications of the KB fragment of R68.45 (Figure 3.14). In pUU1 KB had been replaced by two bands 1XK1 and 1XK2. Therefore, if pUU2 was derived from pUU1 by a simple single insertion into pUU1, either 1XK1 or 1XK2 from pUU1 would be expected to be present in pUU2. In fact neither 1XK1 nor

FIGURE 3.12

Diagram of PstI digests of R68.45, pUU1 and pUU2

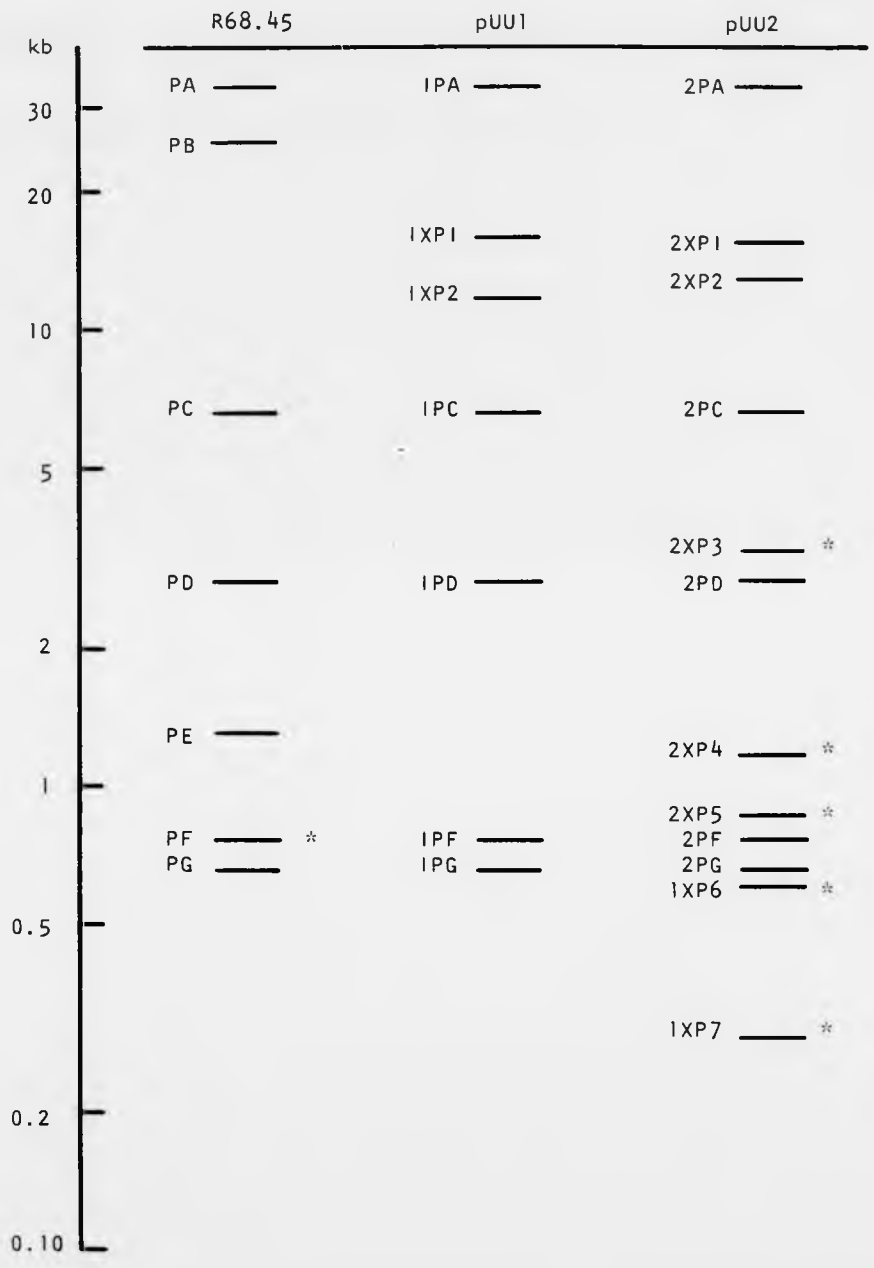

R68.45
pUU1
pUU2
kb
30
20
10
5
2
1
0.5
0.2
0.10
PA
PB
PC
PD
PE
PF
PG
1PA
1XP1
1XP2
1PC
1PD
1PF
1PG
2PA
2XP1
2XP2
2PC
2XP3
2PD
2XP4
2XP5
2PF
2PG
1XP6
1XP7
*

FIGURE 3.13

Diagram of SmaI digests of R68.45, pUU1 and pUU2

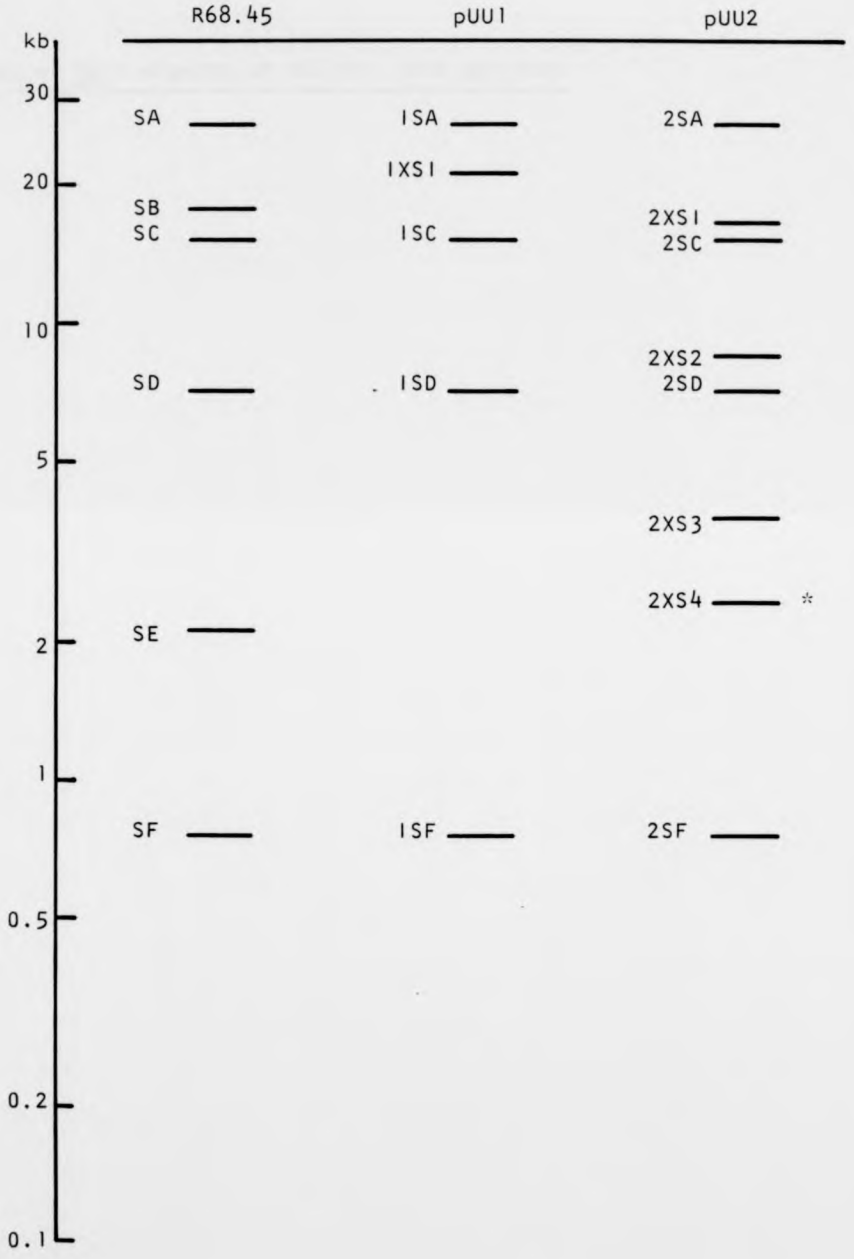
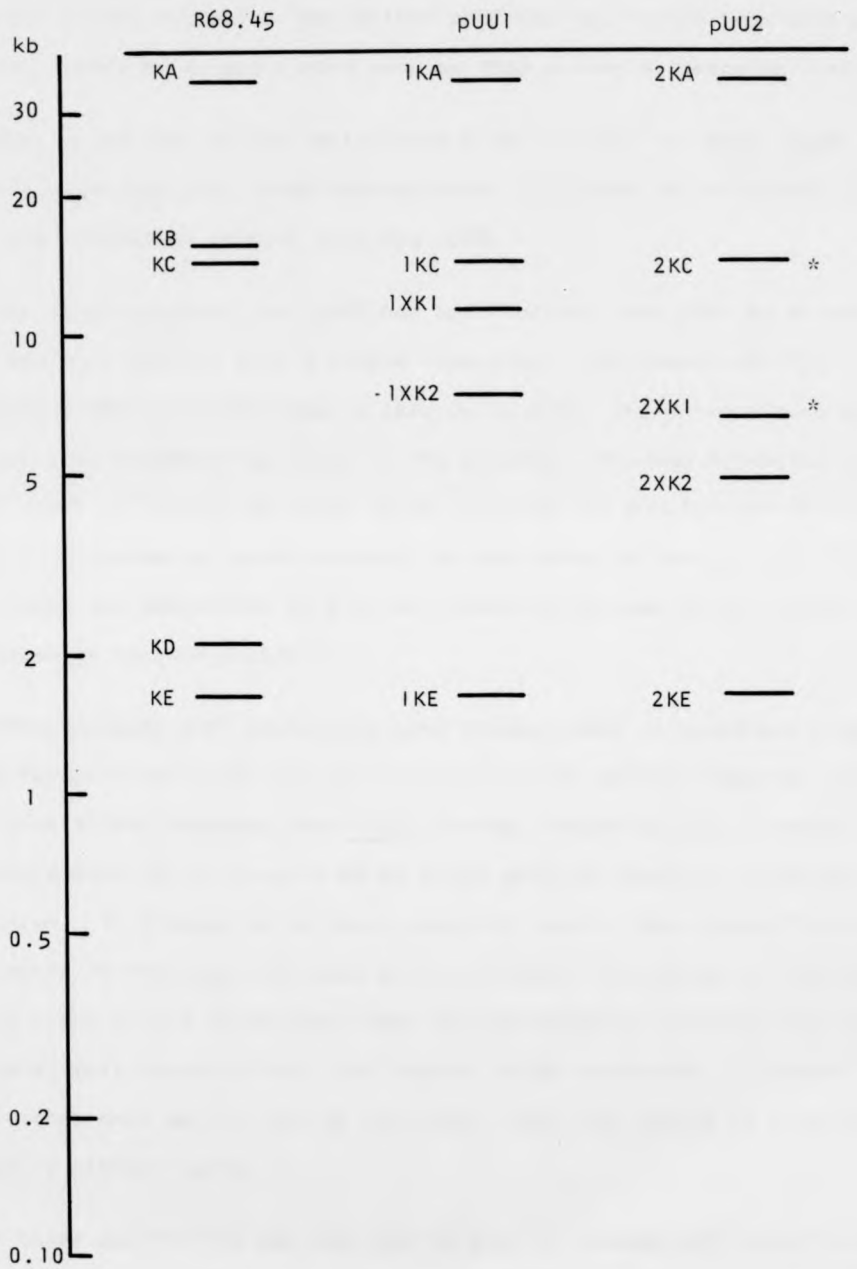

R68.45
pUU1
pUU2
kb
30
20
10
5
2
1
0.5
0.2
0.1
SA
SB
SC
SD
SE
SF
ISA
IXS1
ISC
ISD
ISF
2SA
2XS1
2SC
2XS2
2SD
2XS3
2XS4
*
2SF

FIGURE 3.14

Diagram of KpnI digests of R68.45, pUU1 and pUU2


R68.45
pUU1
pUU2
kb
30
20
10
5
2
1
0.5
0.2
0.10
KA
KB
KC
KD
KE
1KA
1KC
1XK1
1XK2
1KE
2KA
2KC
2XK1
2XK2
2KE
*
*

IXK2 were present in pUU2. These KpnI digests, like the PstI digest data discussed above, suggested that either pUU2 was not formed from pUU1 or that pUU2 was formed by an event more complex than a single insertion into pUU1.

Due to the lack of new restriction sites in pUU1 for XhoI, EcoRI and HindIII, digestion with these endonucleases furnished no additional information as to the connection between pUU1 and pUU2.

The above suggests that pUU2 had been derived from pUU1 by an event which was more complex than a single insertion. Two pieces of PP3 chromosomal DNA could have been picked up by pUU1, these two pieces could have inserted into neighbouring sites in the plasmid. R-prime formation is a rare enough event in itself; an event which required the acquisition of two pieces of DNA simultaneously, would probably be even more unlikely. Insufficient is known about the mechanisms of R-prime formation to come to any definite conclusion as to this possibility.

Alternatively pUU1 could have gone through some intermediate stage prior to the formation of pUU2. If possibility iii) is correct (Section 3.5.2.5) i.e., that I1 had inserted into tra 3, thereby rendering pUU1 transfer deficient, pUU1 would have had to lose I1 so as to be able to transfer itself and form an R-prime. This seems an unlikely chain of events; the probability against pUU1 losing I1 from tra 3 followed by that plasmid picking up I2 (the dehI bearing piece of PP3 chromosomal DNA) and subsequently transferring to the recipient, must be very high. Yet results to be presented in Chapter 4 suggest that this may in fact be the case. pUU1 was unable to transfer conjugally without losing I1.

A third possibility was that R68.44 did not become pUU1 immediately after its transfer to PP3. In this case the R-prime pUU2 could have been formed from R68.44 instead of pUU1. There is no way of checking this hypothetical series of events directly. However plasmids in PP3 have been

examined shortly after other mating experiments in which R68.45 was transferred into PP3. These all proved to have undergone modifications similar to those which had occurred in pUU1.

The data do not conclusively support any one of the possible paths by which pUU2 may have been formed. Certainly pUU2 was not formed by one single simple insertion into pUU1. Therefore I propose to treat pUU2 as if it was derived from R68.44 regardless of whether or not it passed directly or indirectly through a pUU1 form.

3.5.3.6.2 The Location of I2 on pUU2

The PstI, SmaI and KpnI digest data indicated that pUU2 had lost the second copy of IS21 of R68.44. The same arguments apply to this event in pUU2 as to the same event in pUU1 (Section 3.5.2.5).

The PstI, SmaI and KpnI data showed that pUU2 had increased in size over R68.45, taking the loss of IS21 into consideration, by 14.72kb, 20.18kb and 16.07kb respectively. These sizes were dependent on the interpretation of some gel bands as singles and others as doubles; this interpretation was by its very nature subjective, especially if there were few bands and they were far apart. The discrepancies in the sizes of the insert I2 as determined from PstI, SmaI and KpnI may be due to the incorrect determination of single bands as doubles or to the difficulty of accurately determining the sizes of large restriction fragments with any certainty.

A PstI digest of R68.45 and pUU2 revealed that pUU2 had lost the PB (24.84kb) band of R68.45 and had gained two large single bands, 2XP1 (14.82kb) and 2XP2 (12.38kb) and five sets of smaller double bands (Plate 3.2; Figure 3.7; Table 3.7). I2 as estimated from the PstI data was 14.72kb in size. Fragment 2XP1 (14.82kb) was too large to be an internal fragment of I2, and therefore probably contained one of the two regions flanking I2. The same

argument applied to 2XP2 (12.38kb). Though smaller than I2, 2XP2 plus all but one of the double bands would be too large to be internal to I2. It was not possible to tell from the PstI data alone which ends these two fragments were at (Figure 3.15). However as the region between the two PstI sites at the extremes of I2 was 12.36kb and the total size of I2, from PstI data, was 14.72kb, the SmaI sites and the extreme righthand KpnI site could be located with respect to I2. However it was not possible, from these data alone to determine which side of I2 the KpnI site at 16kb in the R68.44 PB band was located in pUU2. The remaining nine PstI sites of I2 must lie between these outer two sites, their exact location could not be determined.

The presence of double bands, especially of so many small ones, was possibly indicative of some duplicated features within I2 rather than being due to coincidence. Double bands featured in the digests of the I2 region with other endonucleases, which lends support to this possibility.

The SmaI digest data showed the presence of five new bands in pUU2 replacing the SB band of R68.45 (Plate 3.3; Figure 3.8; Table 3.8). The largest of these new bands 2XS1 (16.60kb) probably included a region flanking I2. If the second largest new SmaI fragment, 2XS2 (8.45kb) was the SmaI fragment containing the other flanking region it would extend into both the possible positions for I2 as determined from the PstI data if it were to occupy the right-hand position, but would fall short by about 3kb if it were to occupy the left-hand position. The remaining new SmaI fragments, the doubles 2XS3 (3.8kb) and 2XS4 (2.51kb), would fall short at either end. Therefore one can conclude that 2XS1 and 2XS2 included the regions flanking I2, and that 2XS1 probably occupied the lefthand flanking region and 2XS2 probably occupied the right hand. Though the opposite alternative must not be dismissed entirely (Figure 3.15). The remaining three SmaI sites of I2 must lie between the outer sites. The 7.5kb of I2 not included between the two outer SmaI sites of I2 must extend to the right and left of them.

FIGURE 3.15

Location of I2 in pUU2

a. PB fragment of R68.44.

b. Possible location of the outer PstI sites of I2.

c. Possible location of the outer PstI sites of I2.

d. Probable location of the outer SmaI sites of I2.

e. Possible, though unlikely, location of the outer SmaI sites of I2.

f. Probable location of the KpnI sites of I2.

g. Discounted locations of the KpnI sites of I2.

FIGURE 3.15

Location of I2 in pUU2

a. PB fragment of R68.44.

b. Possible location of the outer PstI sites of I2.

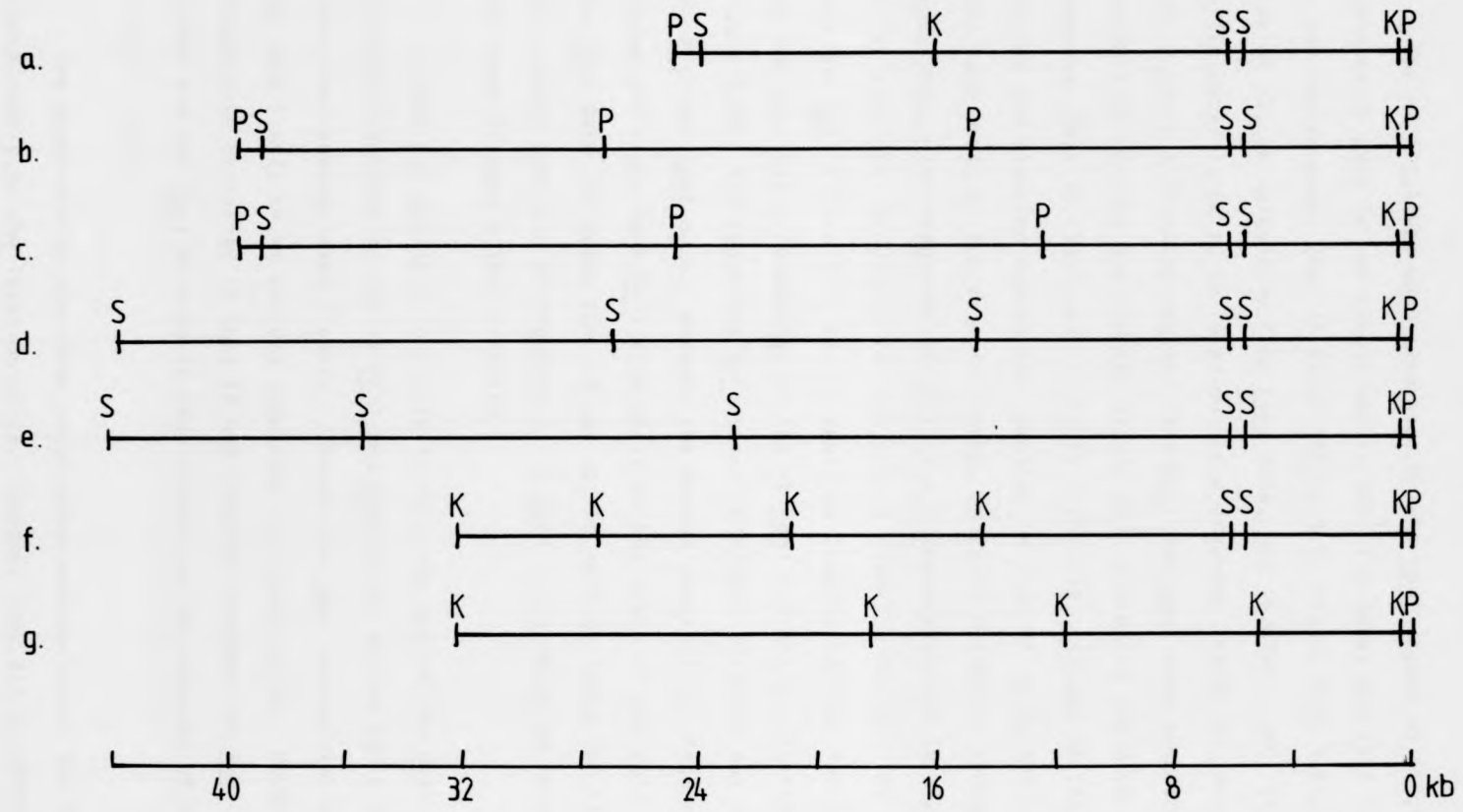
c. Possible location of the outer PstI sites of I2.

d. Probable location of the outer SmaI sites of I2.

e. Possible, though unlikely, location of the outer SmaI sites of I2.

f. Probable location of the KpnI sites of I2.

g. Discounted locations of the KpnI sites of I2.


a.
P S
K
S S
K P
b.
P S
P
P
S S
K P
c.
P S
P
P
S S
K P
d.
S
S
S
S S
K P
e.
S
S
S
S S
K P
f.
K
K
K
K
S S
K P
g.
K
K
K
K
K P
40
32
24
16
8
0 kb

The KB (15.66kb) band generated by the KpnI digest of R68.45 had been replaced in pUU2 by two single bands, $2KC^1$ (13.96kb) and 2XK2 (4.79kb), and by one double band, 2XKI (6.49kb) (Plate 3.4; Figure 3.9; Table 3.9). The size of I2, as calculated from KpnI data, was 16.07kb. $2KC^1$ (13.96kb) was sufficiently large to be ruled out as a fragment inside I2, and therefore must contain a flanking region. The PstI and SmaI data suggested the possibility of some duplication within I2. Therefore the double band fragments 2XKI (6.49kb) were probably within I2. If one of 2XKI was to include the flanking region the identity in size of it to the other internal 2XKI band would be due to coincidence, which is unlikely, thought not impossible, with fragments of this size. Therefore 2XK2 (4.79kb) was left as the strongest candidate for the second flanking region. As there remained only one KpnI site within I2, it could be located between the outer sites. The two possible arrangements of the I2 KpnI sites are illustrated in Figure 3.15. The suggested KpnI sites in Figure 3.15f did not conflict with the PstI and SmaI data. However the second possibility, Figure 3.15g, placed the right hand KpnI site of I2 to the right of the pair of SmaI sites. The SmaI data indicated that I2 was to the left of these two sites. Therefore this second possible arrangement of I2 KpnI sites could be ruled out, and the other (Figure 3.15f) accepted.

It was not possible to similarly rule out one of the PstI or SmaI possible locations of the I2 PstI and SmaI sites on the basis of the evidence from other endonuclease digests. Though the SmaI interpretation in Figure 3.15d was more likely to be correct than that in Figure 3.15e. However what can be stated with certainty is that I2 had inserted between the SmaI site at 6.46kb and the KpnI site at 16.01kb within the PB fragment of R68.44 (Figure 3.15a).

The digests with the other restriction endonucleases did not contribute significantly to the location of I2. However they did provide some additional

information. As mentioned above, Riess et al. (1980), Nayudu and Holloway (1981) and Moore et al. (1983) give different positionings of the KpnI KB and KC fragments in R68. The data presented here indicate that I2 was inserted into the second largest KpnI band of R68.45, namely KB. The XhoI digest data of pUU2 (Table 3.10) indicated that I2 was about 18kb from 6 o'clock XhoI site (Figure 3.5). The HindIII digest data of pUU2 indicated that I2 was about 26kb from the single HindIII site of R68. The PstI and SmaI data showed that these distances must be measured in an anti-clockwise direction. These distances would place I2, and by implication KB, beyond its location by Riess et al. (1980) to that given by Nayudu and Holloway (1981) and Moore et al. (1983). The map used here is based on those of Nayudu and Holloway (1981) and Moore et al. (1983) (Figure 3.5).

These digest data also indicated that I2 contained at least two XhoI sites, 5 HindIII sites and no EcoRI sites.

It did not prove possible to locate I2 with the same precision as I1. Therefore it was not possible to position I2 relative to the genetic regions mapped by Thomas (1981a). However, presumably the same three possibilities which applied to I1 could equally be applied to I2 (Figure 3.6).

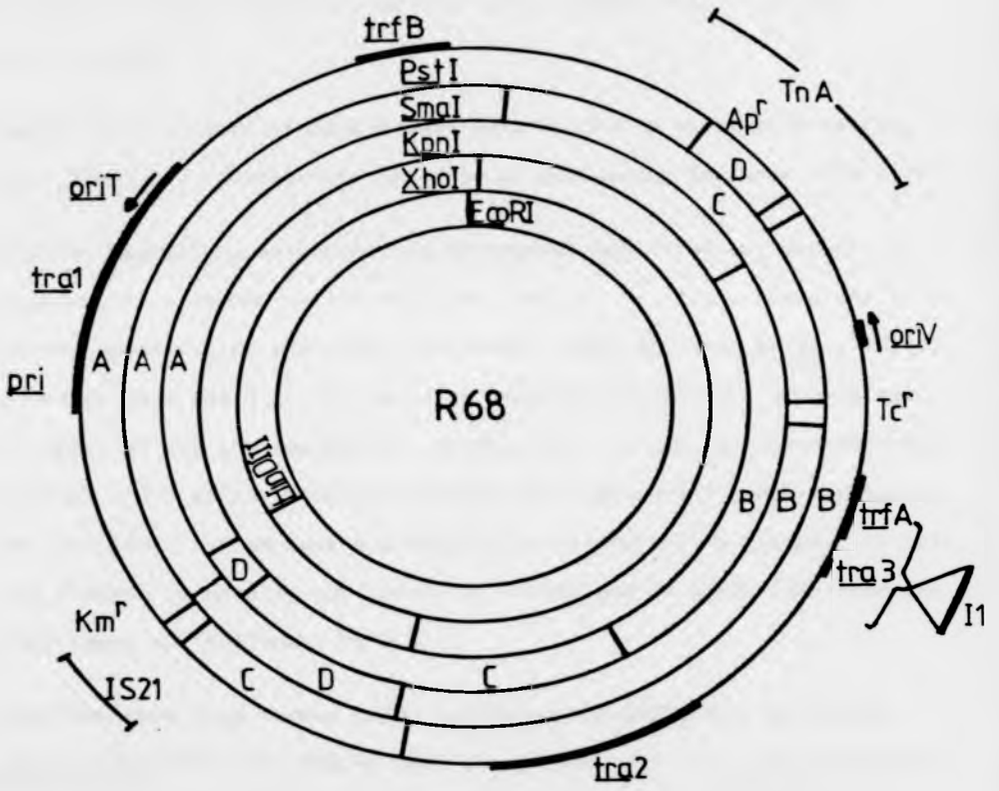
3.6 Discussion

On its transfer into P. putida PP3, R68.44 lost the second copy of IS21 and acquired a small insert, 2.16 - 3.52kb in size, known as I1. It was inserted into the plasmid, between 3 o'clock and 4 o'clock (Figure 3.16). This derivative plasmid was known as pUU1 and its host as PPW1.

PPW2 was formed by mating P. putida PPW1 with P. putida PaW340 with selection for growth on 2MCPA. PPW2 contained an R-prime derivative of R68.44. This plasmid, pUU2, contained an insert of presumably PP3 DNA of 14.72 - 20.12kb in size, I2. This insert carried the dehalogenase I gene of PP3

FIGURE 3.16

Map of R68 showing position of I1 in R68


trfB
PstI
SmaI
KpnI
XhoI
EcoRI
TnA
Ap^r
D
C
oriT
tra1
pri
A A A
oriV
Tc^r
R68
HindIII
B B B
trfA
tra3
I1
D
Km^r
IS21
C D C
tra2

and presumably its control sequences; these were known as dehI. I2 had inserted into approximately the same position in the plasmid as I1 in pUU1 (Figure 3.17).

The precise relationships between pUU1 and pUU2 could not be determined. The main possibilities were: that pUU2 was formed from pUU1 by an event more complex than a single insertion, or that pUU1 somehow lost I1 in the formation of pUU2.

Restriction digests of pUU2 showed several double bands originating from I2. These may indicate the presence of duplicated features within I2.

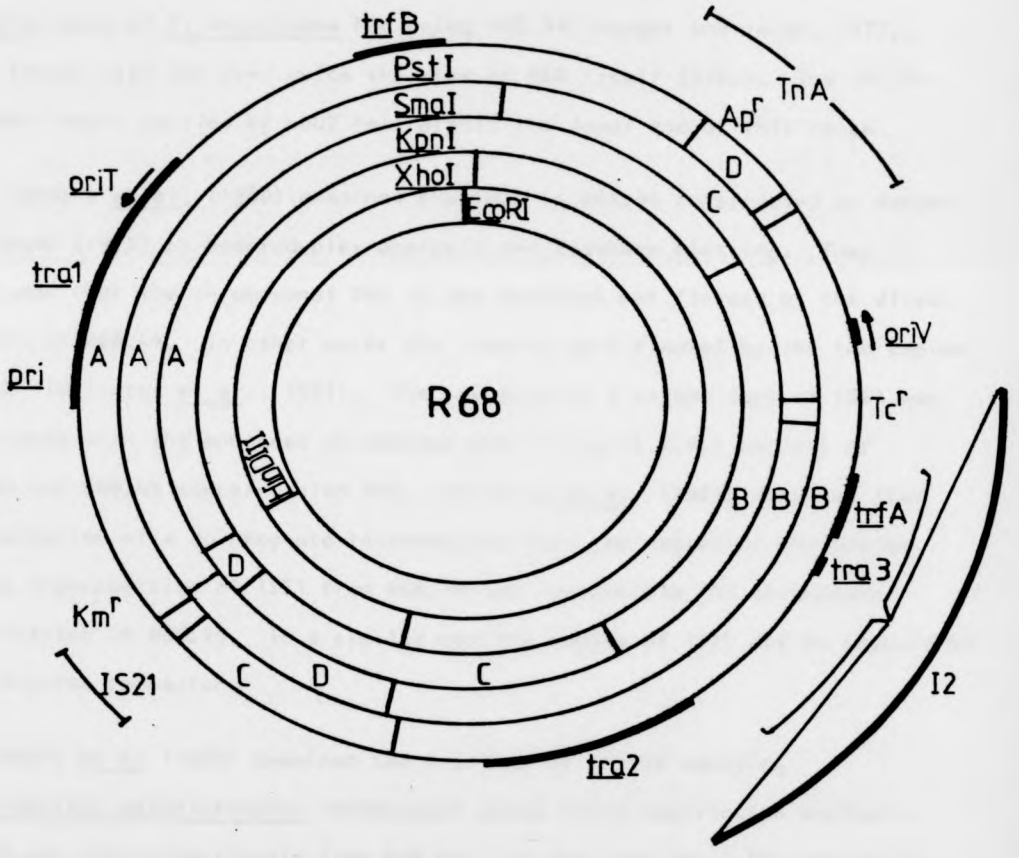
R-prime formation, as opposed to chromosome mobilization, occurs if the recipient is a recombination deficient mutant, or if the recipient is of a different species from the donor (Holloway, 1978; Holloway et al., 1979). In the latter case there is little or no homology between the transferred genetic material and the recipients' chromosome. In the case of chromosome mobilization there exists homology between the transferred genetic material and the recipients' chromosome and homologous recombination occurs. In this case the plasmid is usually not inherited intact and is often lost from the recipient (Haas and Holloway, 1976).

R-primes have been formed using recombination deficient recipients in P. aeruginosa PAO using R68.45 (Holloway, 1978) and in E. coli with RPI (Olsen and Gonzalez, 1974). The sizes of the inserts carried by these plasmids were estimated as small. The inserts were unstable especially in the absence of selection for those markers carried.

R-primes using R68.44, R68.45 or closely related plasmids have been formed in interspecific matings carrying genes from Rhizobium leguminosarium and R. meliloti (Johnston et al., 1978a and b; Kowalczuk et al., 1981; Kiss et al., 1980), Klebsiella pneumoniae (Dixon et al., 1976), P. putida PPN

FIGURE 3.17

Map of R68 showing position of I2 in pUU2


trfB
PstI
SmaI
KpnI
XhoI
EcoRI
TnA
Apr
D
C
oriT
tra1
oriV
pri
A A A
R68
Tcr
HindIII
B B B
trfA
tra3
D
Kmr
C D C
IS21
I2
tra2

(Morgan, 1982), P. aeruginosa PAC (Hedges and Jacob, 1977; Hedges et al., 1977) and Methylophilus methyotrophus (Moore et al., 1983). In some of these cases the size of the inserted DNA has been estimated, and in a few cases the position of insertion has been determined. The size of the extra DNA carried by an R-prime is very variable from 2.3kb carrying the cys-46 gene of Rhizobium meliloti using R68.45 (Kiss et al., 1980) to about 140kb carrying the ArgA gene of P. aeruginosa PAC using R68.44 (Hedges and Jacob, 1977). This larger size was over twice the size of R68 itself (57kb). The 14.72 - 20.12kb insert carried by pUU2 fell within the lower end of this range.

Leemans et al. (1980) examined R-primes in R68.44 constructed by Hedges and Jacob (1977) by heteroduplex analysis and Southern blotting. They concluded that the chromosomal DNA in the R-primes was flanked by the direct repeats of R68.44. In other words the inserts were flanked by the two copies of IS21 (Willetts et al., 1981). The presence of a second copy of IS21 was correlated with the enhanced chromosome mobilizing (E.C.M.) ability of R68.44 and R68.45 compared with R68. Willetts et al. (1981) proposed that the formation of a cointegrate intermediate with the bacterial chromosome during transposition of IS21 from R68.45 was responsible for chromosome mobilization in R68.45. In a similar way the copies of IS21 may be associated with R-prime formation.

Moore et al (1983) examined two R-primes of pM0170 carrying Methylophilus methylotrophus chromosomal genes using restriction analysis. pM0170 was indistinguishable from R68.45. In one case the 4.8kb insertion had inserted nearby but not precisely between the duplicated regions, and one copy of IS21 had been lost. They could not determine whether this deletion had occurred in the process of, or subsequent to, the insertion.

Riess et al. (1983) examined the mobilization of pACYC184 and pBR325 by R68.45 in E. coli. In recA recipient strains they did not detect R68.45

and pACYC184 but isolated cointegrates between the two plasmids. In the co-integrate pACYC184 was located between the two copies of IS21. When the cointegrate resolved into two plasmids they recovered R68, not R68.45, and pACYC184 containing one copy of IS21. This copy was found in several sites within the plasmid but always in the same orientation. In R-primes derived from R68.45 containing E.coli chromosomal DNA, they observed the insert flanked by two copies of IS21 in the same orientation. They proposed that co-integrate formation was a model for R-prime formation.

In other R-primes the chromosomal insertion was not associated with the duplicated portion of R68.44 or R68.45. Johnston et al. (1978a) performed SmaI digests on three R-primes of R68.45 carrying Rhizobium leguminosarum genes. In two cases the insert was into the largest SmaI fragment (SA). In the third case the insert was into the fourth largest SmaI fragment (SD). It must be pointed out that these two SmaI fragments were on either side of the duplicated region. In all cases their R-primes had lost the second copy of IS21. However, so had the plasmid which they label as R68.45. Kiss et al. (1980) also performed SmaI digests on their R-prime derivatives of R68.45 carrying genes from Rhizobium meliloti. They found in one case that the 27.5kb insert was into the SD fragment. Again, their R-primes had lost the second copy of IS21. Kowalczuk et al. (1981) created an R-prime using R68.45 carrying the modulation genes of Rhizobium meliloti. They did not map the insert but assume that it was probably near the ampicillin resistance gene.

The above survey shows that though none of the mapped R-primes possessed inserts in the same position as that in pUU2, they were not all confined to the duplicated region of R68.44/R68.45. Therefore though IS21 may play a role in the formation of R-primes, it did not appear necessary for the inserted DNA to be directly associated with the duplicated region.

R68.45 was derived from R68 in matings in *P. aeruginosa* in which selection was made for an arginine marker (Haas and Holloway, 1976). The two plasmids differed physically solely by the presence of the second copy of IS21 in R68.45. R68.45 did not possess any *P. aeruginosa* PAO DNA. Therefore Willetts *et al*. (1981) concluded that the formation of R68.45 from R68 was entirely an R68 dependent result. Though the puzzle as to why R68.45 should be formed at higher frequencies in certain matings than in others remained.

Nayudu and Holloway (1981) isolated enhanced chromosome mobilizing (E.C.M.) variants of R68 in *E. coli* by the same methods as those used to produce R68.45 in *P.aeruginosa* PAO. These exhibited chromosome mobilizing ability (Cma) in *E. coli* but not in PAO. They examined two of the E.C.M. plasmids and found that they both differed from R68 by the possession of 1.2kb additional DNA. In neither case was this extra DNA associated with IS21. In one case it was inserted 6kb anticlockwise from the single *Eco*RI site, in the other near the TnA region. The inserts showed no homology with IS21 but did show homology with the *E. coli* chromosome. The inserts did not show restriction patterns similar to a range of insertion sequences known to be harboured in the *E. coli* chromosome. However Nayudu and Holloway (1981) did suggest that the inserts were probably transposable elements and that as such they could be involved with Cma.

I1, the 2.16 - 3.52kb insert in pUU1, may be a transposable element. It was capable of insertion into several sites in R68, though its location in pUU1 may be its most probable position (see Chapter 4). As such I1 may act as a centre for R-prime formation, as the inserts in the *E. coli* derived E.C.M. plasmids (described above) did for Cma, and IS21 itself did for Cma and R-prime formation in R68.45. In most cases of R-prime formation using R68.45, the second copy of IS21 was lost (Johnston *et al*., 1978a; Kiss *et al*., 1980; Moore *et al*., 1983). This may be due to the mechanism of R-prime formation. The absence of I1 in pUU2 may be similarly explained.

Cma and R-prime formation was enhanced in many plasmids by the insertion into the plasmid of DNA from another source by either *in vivo* or *in vitro* techniques. These constructed R-primes in R68 or its relatives contained either bacterophage Mu or lambda DNA (Denarie *et al.*, 1977; Grinter, 1981; Van-Gijsegen and Toussaint, 1982), a transposon (Pemberton and Bowen, 1981) or chromosomal DNA (Nagahari *et al.*, 1977; Barth, 1979b; Julliot and Boistard, 1979; Beck *et al.*, 1982). This phenomenon had also been observed with F-primes and primes with other plasmids (Pittard and Adelberg, 1963; Low, 1972; Hopwood and Wright, 1976a, b). Cma and R-prime formation in these plasmids would appear to be due to homology between the insert in the plasmid and sites in the donor's chromosome. Certainly in these cases Cma was polarized, there being a gradient of declining Cma for markers at greater distances for the region of homology.

The insert I1 of pUU1 was presumably of PP3 chromosomal origin. Therefore the possibility arises that it too, as in the case of the constructed primes discussed above, may enhance C.m.a. and R-prime formation from regions of the PP3 chromosome adjoining homologous regions to I1 in the chromosome.

It is unfortunate that the results presented in this chapter do not support one of the above speculations more than another. I1 may even play no role in the formation of pUU2.

CHAPTER 4

THE FORMATION OF DERIVATIVES OF PLASMID pUU1

CHAPTER 4

THE FORMATION OF DERIVATIVES OF PLASMID pUU1

4.1 Introduction

In order to investigate the influence of I1 on pUU1 and its role in R-prime formation, this plasmid was transferred to other bacterial species. These matings provided information on the relationship between I1 and *tra*3 and on the nature of I1 itself.

4.2.1 Mating of *P. putida* PPW1 with *P. aeruginosa* PAU1 and *P. putida* PaW340

P. putida PPW1, the PP3 derivative containing pUU1, was mated separately with PAU1, a streptomycin resistant mutant of *P. aeruginosa* PAO1162, and *P. putida* PaW340. In both cases recombinants were selected for on nutrient agar containing Ap ($50\mu g ml^{-1}$), Km ($50\mu g ml^{-1}$), Tc ($150\mu g ml^{-1}$) and Sm($1mg ml^{-1}$). Recombinants were purified by streaking out on selective medium and subsequently checked for the auxotrophic markers of the recipients and, in the case of PAU1, for characteristic growth on King's A and B media. The frequencies of recombinants per donor cell were as follows:

PPW1 X PAU1: 1.4×10^{-5}

PPW1 X PaW340: 1.2×10^{-6}

The plasmids from 6 recombinants from each mating were purified by the rapid method and digested with *Pst*I and *Sma*I (Plate 4.1). None of the recombinants contained plasmids which resembled pUU1. All the recombinants contained plasmids which give identical digest patterns. These digest patterns were indistinguishable from that of R68 (R68.45 which has lost the

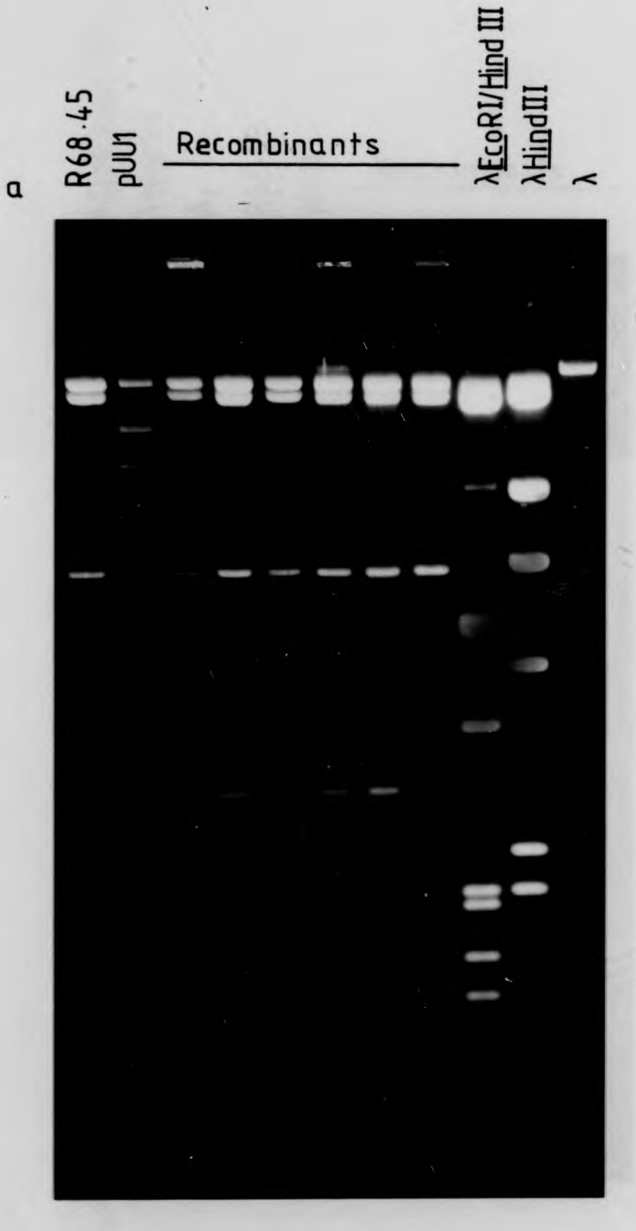
PLATE 4.1

Digests of plasmids from recombinants of the matings PPW1 x PAU1 and PPW1 x PaW340

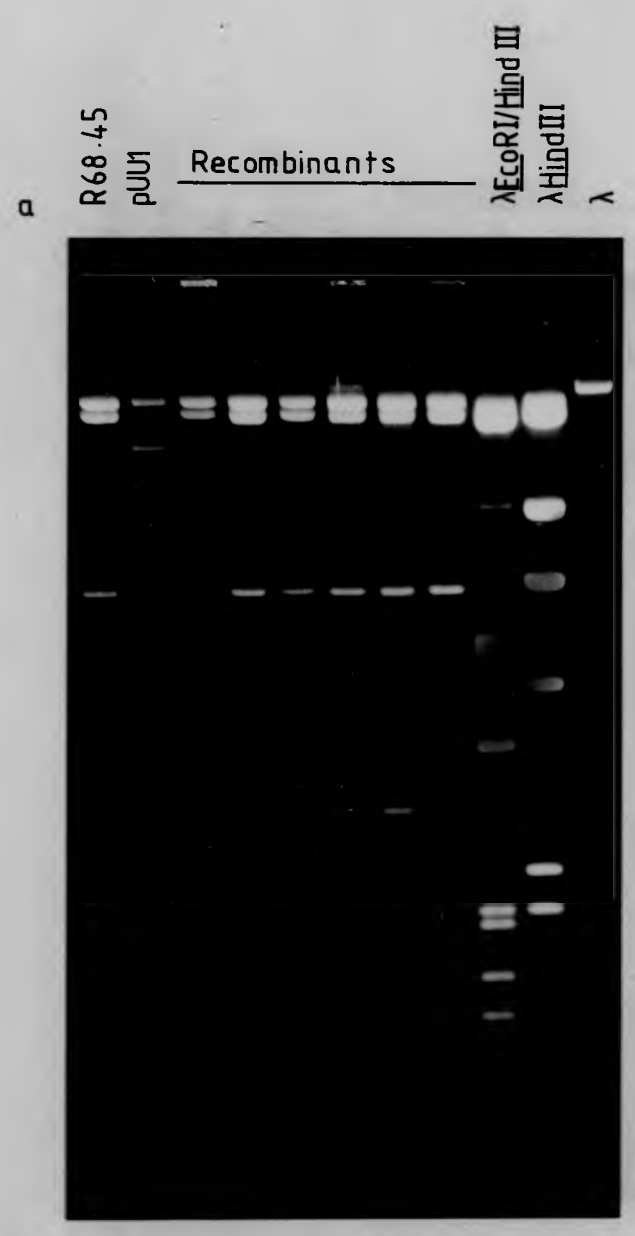
a. PstI

b. SmaI

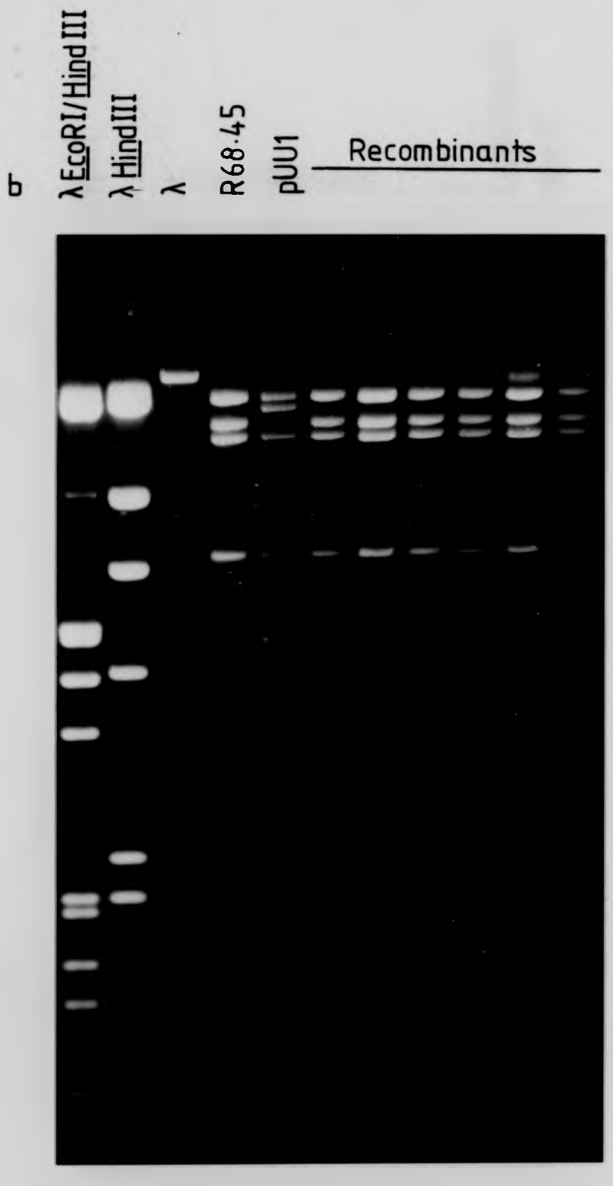
PPW1 × PAU1



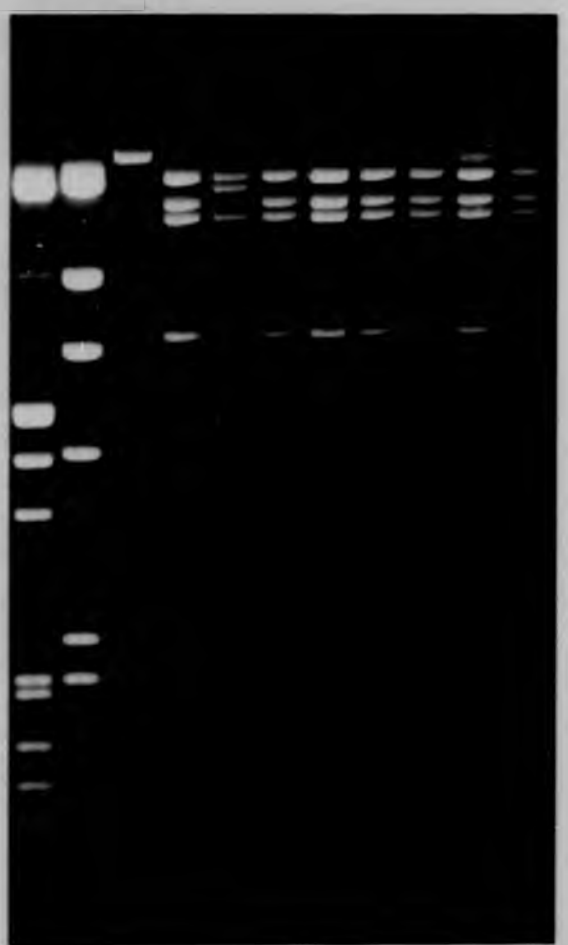
PPW1 × PAU1



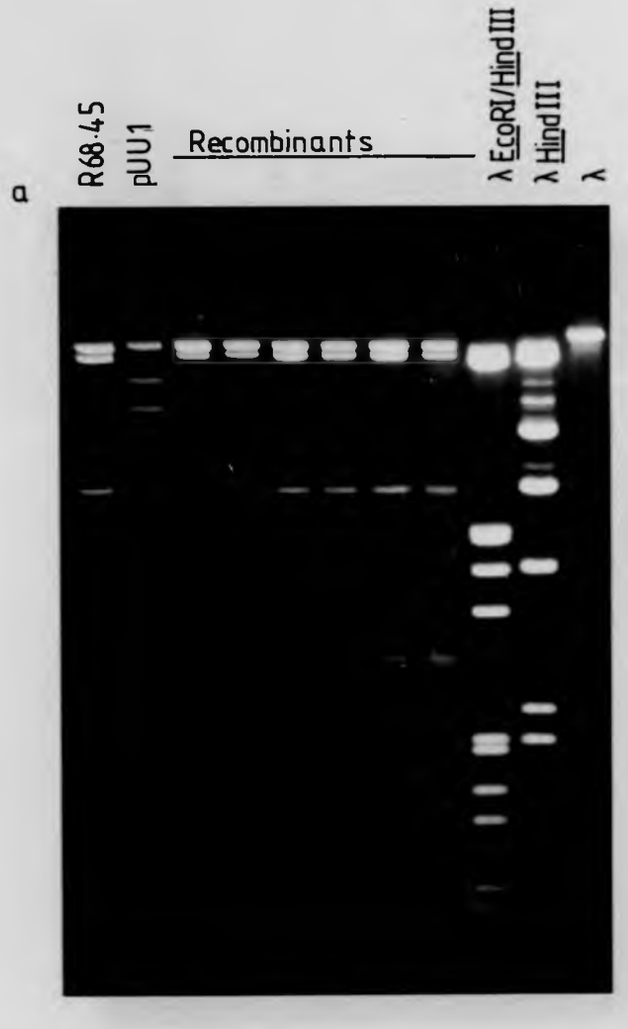
PPW1 × PAU1



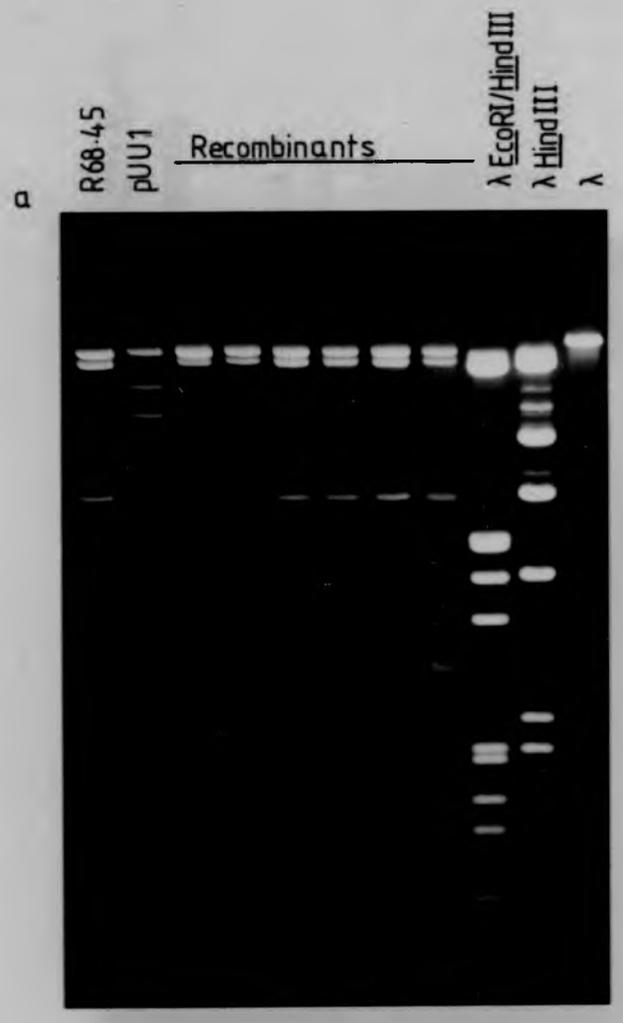
PPW1 × PAU1



PP W1 × PaW 340

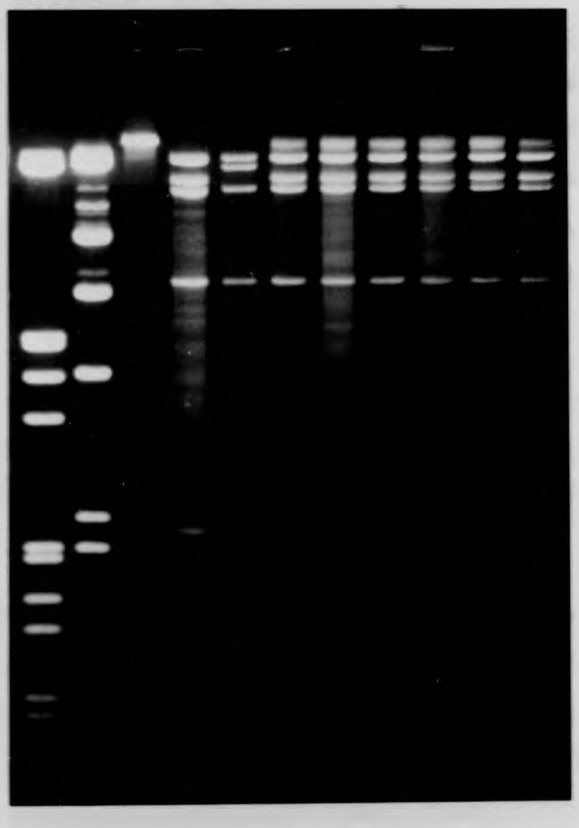


PPW1× PaW340

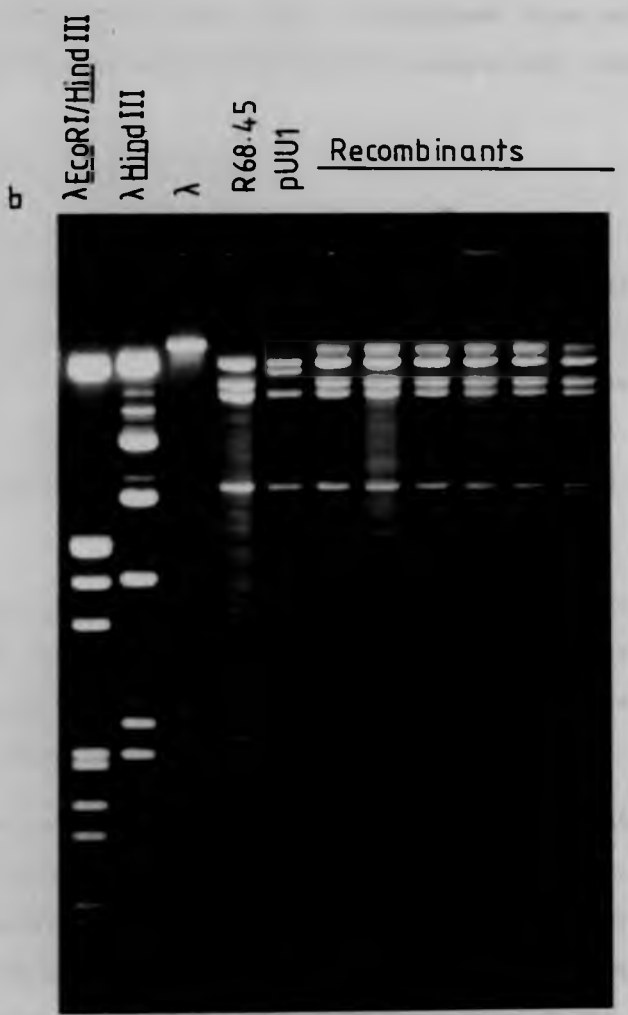


PPW1 × PaW340

b



PPW1 × PaW 340



second copy of IS21). It would appear that in the 12 cases examined that pUU1 had lost I1 and had reverted to a form indistinguishable from R68.

One recombinant from the mating of PPW1 with PAU1 was designated PAU8 and the plasmid it contained pUU6. A recombinant from the mating of PPW1 with PaW340 was designated PPW16 and its plasmid pUU7 (Table 4.1)

4.2.2 Mating of PAU8 with PPW5

P. aeruginosa PAU8 was mated with a histidine auxotrophic mutant of P. putida PP3, PPW5. Selection for recombinants was on minimal medium supplemented with 2MCPA, histidine (25µgml), Ap (50µgml^{-1}), Km (50µgml^{-1}) and Tc (150µgml^{-1}). After purification on selective medium the recombinants were checked for auxotrophic markers and characteristic growth in King's A and B media. Recombinants were obtained at a frequency of 6.8×10^{-7} per donor cell.

The plasmids from 10 recombinants (Table 4.1) were purified by the rapid method and digested with PstI and SmaI. The digest patterns of these plasmids were compared with those of R68.45, pUU1 and pUU6, the R68-like plasmid in PAU8 (Plate 4.2; Table 4.2; Figures 4.1, 4.2).

All the derivative plasmids, pUU8 - 17, differed from pUU6, the R68-like plasmid in PAU8, from which they were derived. pUU6 was modified in all cases on its transfer to the PP3 derivative PPW5. In all cases the PstI generated band 6PB in pUU6, which appeared identical to PB in R68.45, was missing, suggesting that some modification had occurred in this region of the plasmid (Figure 4.1). Similarly 6SB in pUU6, equivalent to SB in R68.45, was missing in the derivative plasmids (Figure 4.2). These two fragments overlap in R68 (Figure 4.3). This region of the plasmid includes that into which I1 inserted in pUU1 (Section 3.5.2.5)

TABLE 4.1

Bacterial strains and plasmids used or derived in Chapter 4

Strain	Species[1]	Plasmid	Genotype/Phenotype	Derivation
PPW1	P.p.	pUU1	2Mcpa$^+$,Ap^R,Km^R,Tc^R	PAC174 (R68.44)xPP3
PA01162	P.a.	-	leu 38,r$^-$,m$^+$	Dunn and Holloway (1976)
PAU1	P.a.	-	leu 38,r$^-$,m$^+$,Sm^R	Mutant of PA01162
PaW340	P.p.	-	Trp$^-$,Sm^R	P.A. Williams
PAU8	P.a.	pUU6	leu 38,r$^-$,m$^+$,Sm^R,Ap^R, Kp^R, Tc^R	PPW1 X PAU1
PPW16	P.p.	pUU9	Trp$^-$,Sm^R,Ap^R,Km^R,Tc^R	PPW1 X PaW340
PPW5	P.p.	-	2Mcpa$^+$,His$^-$	Mutant of PP3
PPW17	P.p.	pUU8	2Mcpa$^+$,His$^-$,Ap^R,Km^R,Tc^R	PAU8 X PPW5
PPW18	P.p.	pUU9	"	"
PPW19	P.p.	pUU10	"	"
PPW20	P.p.	pUU11	"	"
PPW21	P.p.	pUU12	"	"
PPW22	P.p.	pUU13	"	"
PPW23	P.p.	pUU14	"	"
PPW24	P.p.	pUU15	"	"
PPW25	P.p.	pUU16	"	"
PPW26	P.p.	pUU17	"	"
PPW27	P.p.	pUU18	2Mcpa$^+$,His$^-$,Ap^R,Km^R,Tc^R	PPW16 X PPW5
PPW28	P.p.	pUU19	"	"
PPW29	P.p.	pUU20	"	"
PPW30	P.p.	pUU21	"	"
PPW31	P.p.	pUU22	"	"
PPW32	P.p.	pUU23	"	"
PPW33	P.p.	pUU24	"	"
PA08	P.a.	R68	Met$^-$,Ilv$^-$,Sm^R	B.W. Holloway
PPW34	P.p.	pUU25	2Mcpa$^+$,Ap^R,Km^R,Tc^R	PA08 (R68) X PP3
PPW35	P.p.	pUU26	"	"
PPW36	P.p.	pUU27	"	"

1. Species: P.p.: Pseudomonas putida; P.a.: P. aeruginosa

TABLE 4.1

Bacterial strains and plasmids used or derived in Chapter 4

Strain	Species[1]	Plasmid	Genotype/Phenotype	Derivation
PPW1	P.p.	pUU1	2Mcpa$^+$,Ap^R,Km^R,Tc^R	PAC174 (R68.44)xPP3
PA01162	P.a.	-	leu 38,r$^-$,m$^+$	Dunn and Holloway (1976)
PAU1	P.a.	-	leu 38,r$^-$,m$^+$,Sm^R	Mutant of PA01162
PaW340	P.p.	-	Trp$^-$,Sm^R	P.A. Williams
PAU8	P.a.	pUU6	leu 38,r$^-$,m$^+$,Sm^R,Ap^R, Kp^R, Tc^R	PPW1 X PAU1
PPW16	P.p.	pUU9	Trp$^-$,Sm^R,Ap^R,Km^R,Tc^R	PPW1 X PaW340
PPW5	P.p.	-	2Mcpa$^+$,His$^-$	Mutant of PP3
PPW17	P.p.	pUU8	2Mcpa$^+$,His$^-$,Ap^R,Km^R,Tc^R	PAU8 X PPW5
PPW18	P.p.	pUU9	"	"
PPW19	P.p.	pUU10	"	"
PPW20	P.p.	pUU11	"	"
PPW21	P.p.	pUU12	"	"
PPW22	P.p.	pUU13	"	"
PPW23	P.p.	pUU14	"	"
PPW24	P.p.	pUU15	"	"
PPW25	P.p.	pUU16	"	"
PPW26	P.p.	pUU17	"	"
PPW27	P.p.	pUU18	2Mcpa$^+$,His$^-$,Ap^R,Km^R,Tc^R	PPW16 X PPW5
PPW28	P.p.	pUU19	"	"
PPW29	P.p.	pUU20	"	"
PPW30	P.p.	pUU21	"	"
PPW31	P.p.	pUU22	"	"
PPW32	P.p.	pUU23	"	"
PPW33	P.p.	pUU24	"	"
PA08	P.a.	R68	Met$^-$,Ilv$^-$,Sm^R	B.W. Holloway
PPW34	P.p.	pUU25	2Mcpa$^+$,Ap^R,Km^R,Tc^R	PA08 (R68) X PP3
PPW35	P.p.	pUU26	"	"
PPW36	P.p.	pUU27	"	"

1. Species: P.p.: Pseudomonas putida; P.a.: P. aeruginosa

PLATE 4.2

Digests of R68.45, pUU1, pUU6 and derivative plasmids

a. PstI

b. SmaI

a. PstI

16h.

60h.

a. PstI

16h.

60h.

16h

λ
λ HindIII
λ EcoRI/HindIII
R68·45
pUU1
pUU6
pUU13
pUU14
pUU15
pUU16
pUU17

60h

λ
λ HindIII
λ EcoRI/HindIII
R68·45
pUU1
pUU6
pUU13
pUU14
pUU15
pUU16
pUU17

16h

λ
λ HindIII
λ EcoRI/HindIII
R68.45
pUU1
pUU6
pUU13
pUU14
pUU15
pUU16
pUU17

60h

λ
λ HindIII
λ EcoRI/HindIII
R68.45
pUU1
pUU6
pUU13
pUU14
pUU15
pUU16
pUU17

b. SmaI

16h

60h

b. SmaI

16h

60h

16h
λEcoRI/HindIII
λHindIII
λ
pUU13
60h
λEcoRI/HindIII
λHindIII
λ
pUU13

16h
λEcoRI/HindIII
λHindIII
λ
pUU13
60h
λEcoRI/HindIII
λHindIII
λ
pUU13

16h
R68.45
pUU1
pUU6
pUU14
pUU15
pUU16
pUU17
λEcoRI/HindIII
λHindIII
λ
60h
R68.45
pUU1
pUU6
pUU14
pUU15
pUU16
pUU17
λEcoRI/HindIII
λHindIII
λ

16h
R68.45
pUU1
pUU6
pUU14
pUU15
pUU16
pUU17
λEcoRI/HindIII
λHindIII
λ
60h
R68.45
pUU1
pUU6
pUU14
pUU15
pUU16
pUU17
λEcoRI/HindIII
λHindIII
λ

TABLE 4.2

Sizes[1] of restriction fragments of R68.45, pUU1, pUU6 and derivative plasmids digested with PstI and SmaI

a. PstI

Strain		PPW1	PAU8	PPW17	PPW18
Plasmid	R68.45	pUU1	pUU6	pUU8	pUU9
	32.67	32.67	32.67	32.67	32.67
	24.84	15.68	24.84	15.68	14.15
	6.52	11.32	6.52	11.32	11.32
	2.77	6.52	2.77	6.52	6.52
	1.34	2.77	0.77	2.77	2.77
	0.77*	0.77	0.66	0.77	0.77
	0.66	0.66		0.66	0.66
	70.34kb	70.39kb	68.23kb	70.39kb	70.39kb

Strain	PPW19	PPW20	PPW21	PPW22	PPW23
Plasmid	pUU10	pUU11	pUU12	pUU13	pUU14
	32.67	32.67	32.67	32.67	32.67
	14.15	12.97	10.89	15.68	15.68
	11.88	12.18	7.39	7.39	11.32
	6.52	6.52	6.52	6.94	6.52
	3.32	4.95	3.32	6.52	2.77
	2.77	2.77	2.77	2.77	0.77
	1.22	0.77	1.22	0.77	0.66
	0.91	0.66	0.91	0.66	
	0.77		0.77		
	0.66		0.66		
	74.87kb	73.49kb	67.12kb	73.4kb	70.39kb

Strain	PPW24	PPW25	PPW26
Plasmid	pUU15	pUU16	pUU17
	32.67	32.67	32.67
	14.15	14.15	15.68
	11.88	11.88	11.32
	6.52	6.52	6.52
	2.77	4.71	4.37
	0.77	2.77	1.82
	0.66	1.96	1.13
		1.22	0.77
		0.91	0.66
		0.77	
		0.66	
	69.42kb	78.22kb	74.94kb

b. SmaI

Strain		PPW1	PAU8	PPW17	PPW1
Plasmid	R68.45	pUU1	pUU6	pUU8	pUU9
	26.52	26.52	26.52	26.52	26.52
	17.55	21.07	17.55	21.07	21.07
	15.10	15.10	15.10	15.10	15.10
	7.15	7.15	7.15	7.15	7.15
	2.12	0.76	0.76	0.76	0.76
	0.76				
	69.20kb	70.6kb	67.08kb	70.60kb	70.60kb

Strain	PPW19	PPW20	PPW21	PPW22	PPW23
Plasmid	pUU10	pUU11	pUU12	pUU13	pUU14
	26.52	26.52*	26.52	26.52*	26.52
	16.65	15.10	15.10	15.10	21.07
	15.10	7.15	8.37	7.15	15.10
	8.17	0.76	8.17	0.76	7.15
	7.15		7.15		0.76
	2.49		2.49		
	0.76		0.76		
	76.62kb	76.05kb	68.56kb	76.05kb	70.60kb

Strain	PPW24	PPW25	PPW26
Plasmid	pUU15	pUU16	pUU17
	26.52	26.52	26.52
	21.07	20.09	21.07
	15.10	15.10	19.11
	7.15	8.17	7.15
	2.49	7.15	0.76
	0.76	2.49	
		0.76	
	73.09kb	79.26kb	74.61kb

1. Sizes are in kilobases (kb). These were estimated from photographic enlargements and by comparison with the sizes of restriction fragments of plasmids discussed in Chapter 3.

* Double band.

FIGURE 4.1

Diagram of PstI generated restriction fragments of R68.45, pUU1, pUU6 and derivative plasmids

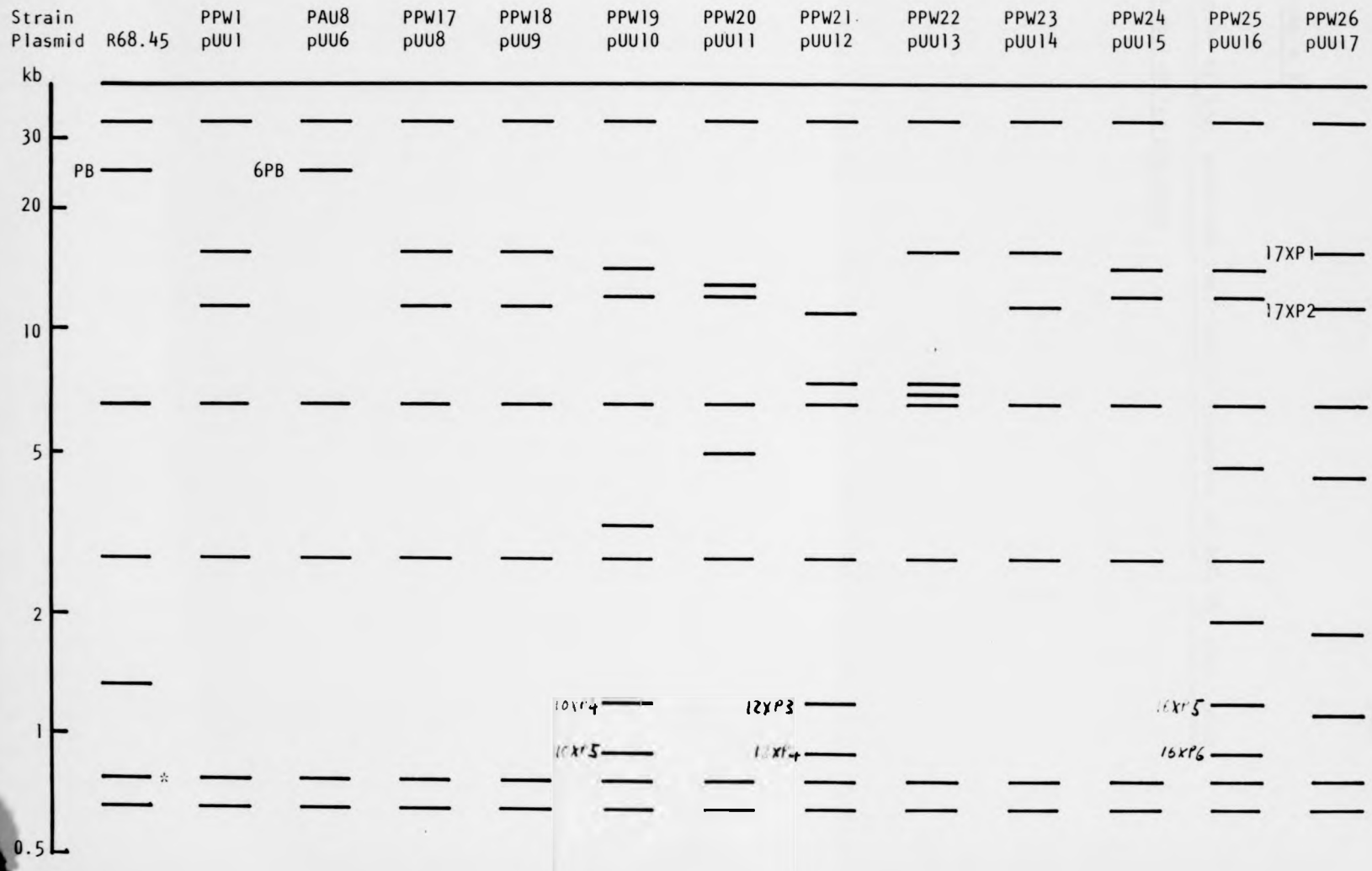

Strain
Plasmid
R68.45
PPW1
pUU1
PAU8
pUU6
PPW17
pUU8
PPW18
pUU9
PPW19
pUU10
PPW20
pUU11
PPW21
pUU12
PPW22
pUU13
PPW23
pUU14
PPW24
pUU15
PPW25
pUU16
PPW26
pUU17
kb
30
20
10
5
2
1
0.5
PB
6PB
17XP1
17XP2
12XP3
16XP6
*

FIGURE 4.2

Diagram of SmaI generated restriction fragments of R68.45, pUU1, pUU6 and derivative plasmids

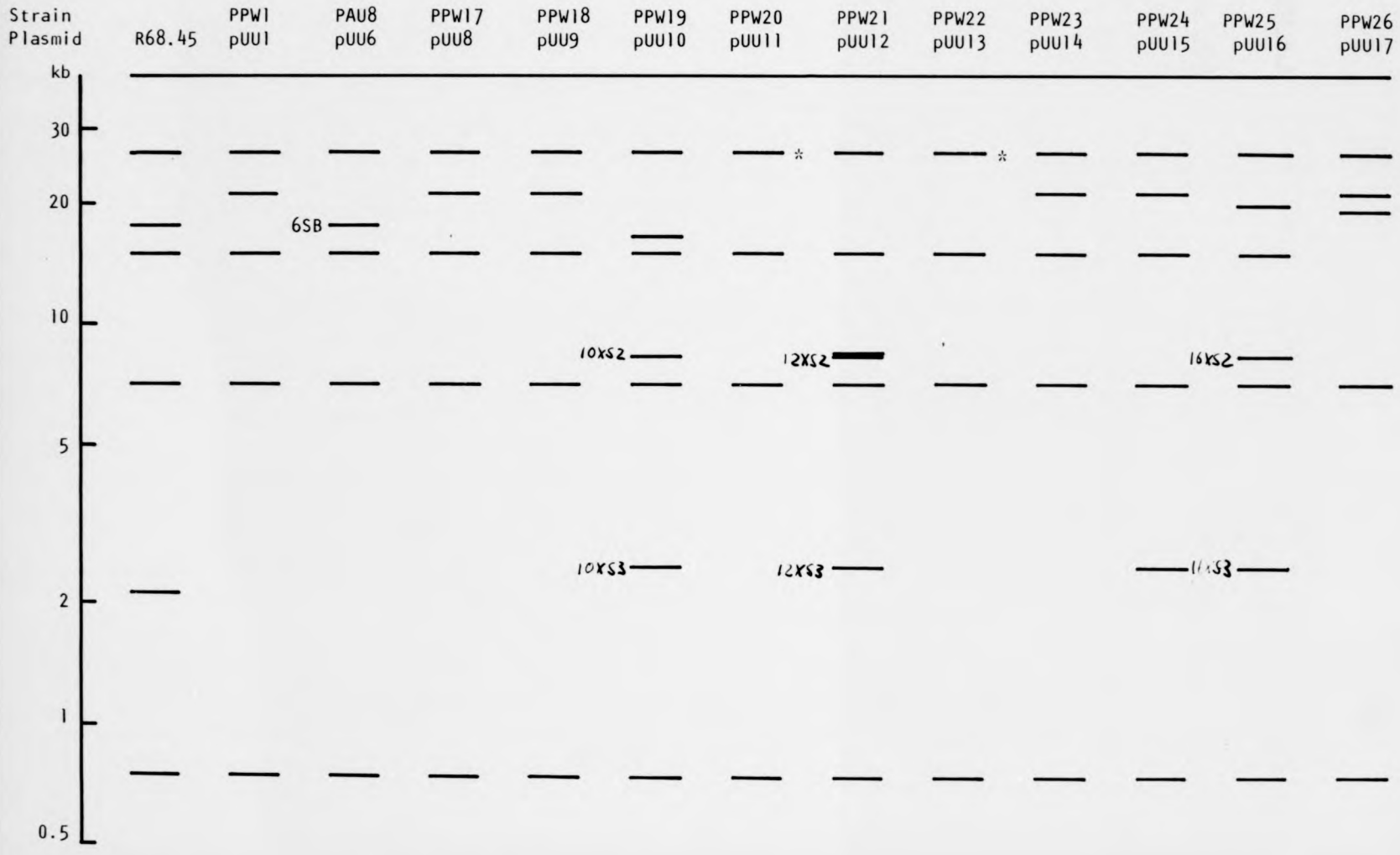
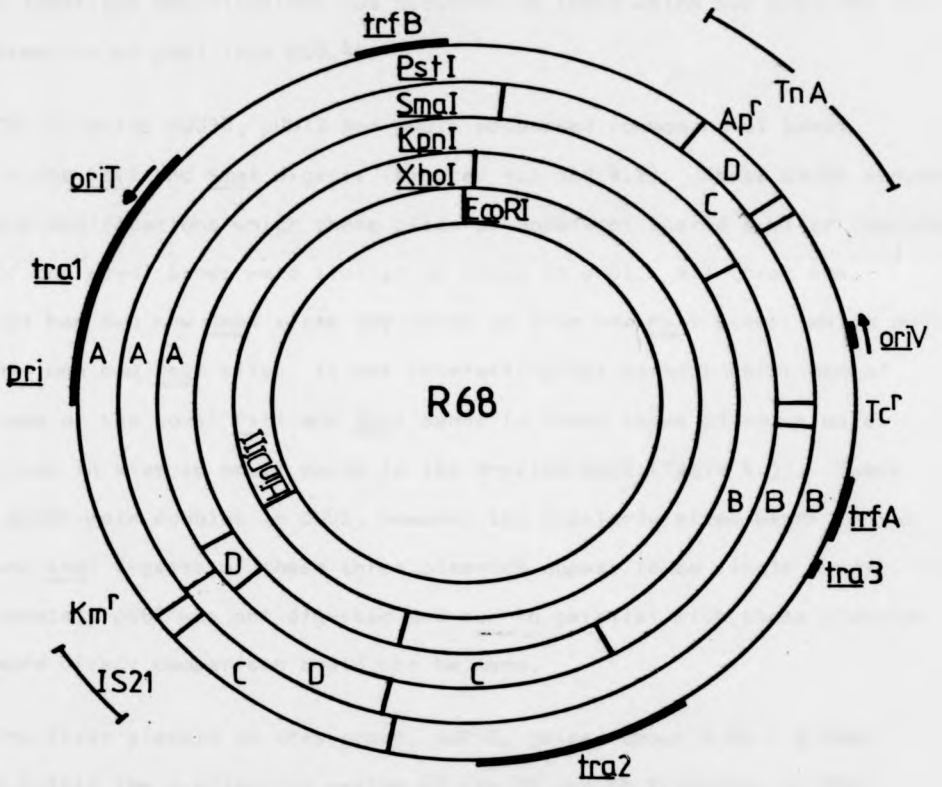

Strain
Plasmid
R68.45
PPW1 pUU1
PAU8 pUU6
PPW17 pUU8
PPW18 pUU9
PPW19 pUU10
PPW20 pUU11
PPW21 pUU12
PPW22 pUU13
PPW23 pUU14
PPW24 pUU15
PPW25 pUU16
PPW26 pUU17
kb
30
20
10
5
2
1
0.5
6SB
10XS2
12XS2
16XS2
10XS3
12XS3
16XS3
*
*

FIGURE 4.3

Map of R68


trfB
PstI
SmaI
KpnI
XhoI
EcoRI
TnA
Ap^r
D
C
oriV
Tc^r
B B B
trfA
tra3
tra2
C D C
IS21
Km^r
D
HincII
A A A
pri
tra1
oriT
R68

Rather than dwelling on the minutiae of the size differences between the plasmids, which may not be very accurate being based on few gels, it is more profitable to compare the broader digest patterns of the different plasmids. Three of the derivative plasmids, pUU8, pUU9 and pUU14, gave restriction patterns with PstI and SmaI which were indistinguishable from those of pUU1. These results suggested that in these three plasmids similar if not identical modifications had occurred to those which had occurred in the formation of pUU1 from R68.44.

The plasmids pUU10, pUU12 and pUU16 possessed common novel bands both in the PstI and SmaI digests (Figures 4.1 and 4.2). These bands suggest that the modifications which these plasmids underwent shared similar features. None of the novel bands were similar to those in pUU1. All three new plasmids had two new SmaI sites and three to five new PstI sites, while pUU1 had only one new PstI site. It was interesting but perhaps coincidental that some of the novel PstI and SmaI bands in these three plasmids were very close in size to novel bands in the R-prime pUU2 (Table 4.3). These novel bands were doubles in pUU2, however the similarly sized bands in the PstI and SmaI digests of these three plasmids appear to be single bands. Unfortunately pUU2 was not digested and run in parallel with these plasmids so a more direct comparison could not be made.

The first plasmid in this group, pUU10, gained about 6.64 - 9.54kb of DNA within the overlapping region of the PB and SB fragments of R68. This new DNA contained four PstI sites and two SmaI sites.

pUU12 gained a very small piece of DNA if any at all; in fact the PstI digest data would appear to indicate a small loss. This plasmid however did gain three new PstI sites and two new SmaI sites. These new restriction sites could not be all confined within any gained DNA as the sizes of the new fragments generated were too large to be accommodated within such a small gain in DNA. Two of the new fragments would have to fit within any inserted

TABLE 4.3

Comparison of the sizes of some digest fragments of pUU2, pUU10, pUU12 and pUU16

	pUU2		pUU10, pUU12, pUU6	
	Band	Size, kb	Band	Size, kb
PstI				
			10XP4	1.22
	2XP4	1.19	12XP3	1.22
			16XP5	1.22
			10XP5	0.91
	2XP5	0.87	12XP4	0.91
			16XP6	0.91
SmaI				
			10XS2	8.17
	2XS2	8.45	12XS2	8.17
			16XS2	8.17
			10XS3	2.49
	2XS4	2.51	12XS3	2.49
			16XS3	2.49

DNA. Therefore either the modification to this plasmid somehow produced a scattering of new restriction sites, or there had been a concomitant loss of native plasmid DNA with a gain of some large novel DNA.

The third plasmid in this group, pUU16, had gained about 9.99 - 12.18kb of DNA. This modification was accompanied by the acquisition of five new PstI sites and two new SmaI sites. These new restriction sites could all be accommodated within the gained DNA.

Plasmids pUU11, pUU13 and pUU15 showed different modifications to the same region of the plasmid. In all cases the changed digest patterns indicated the insertion of DNA containing varying numbers of restriction sites for PstI and SmaI. pUU11 had gained about 5.26 - 8.97kb containing two PstI sites and no SmaI sites. pUU13 had gained about 5.17 - 8.97kb containing two PstI sites and no SmaI sites. pUU15 had gained about 1.19 - 6.01kb containing one PstI site and one SmaI site.

The plasmid pUU17 stood out from the other new plasmids as it showed a modification to the PD and SC bands of R68, in addition to a modification PB and SB bands. The PD and SC bands overlap within the TnA region (Figure 4.3). This region included the ampicillin resistance gene. However this gene was unaffected by the modification in this region of the plasmid as PPW26, the strain harbouring pUU17, was selected for on medium containing ampicillin. Thus in pUU17 two modifications had occurred in distinct parts of the plasmid. It was not possible to identify which novel bands came from which insertion, though the two largest PstI generated novel bands, 17XP1 and 17XP2, were probably derived from the modification of the PB band of R68. Overall there had been a gain of about 6.71 - 7.53kb of DNA, accompanied by three new PstI sites and no new SmaI sites.

4.2.3 Mating of *P. putida* PPW16 with *P. putida* PPW5

P. putida PPW16, the derivative of PaW340 containing the R68-like

plasmid pUU7 (Section 4.2.1), was mated with the histidine auxotrophic mutant of P. putida PP3, PPW5. Recombinants were selected for on minimal medium supplemented with 2MCPA, histidine, Ap, Km and Tc at the same concentrations as used previously (Section 4.2.2). After purification the recombinants were checked for the appropriate auxotrophic markers. Recombinants were obtained at a frequency of 2×10^{-4} per donor cell.

The plasmids from seven of these recombinants were purified by the rapid method and digested with PstI and SmaI. The digest patterns of these plasmids were compared with those of R68.45, pUU1 and pUU7, the R68-like plasmid from which they were derived (Plate 4.3; Table 4.4; Figures 4.4 and 4.5).

In all cases the PstI and SmaI digests of these new plasmids differed from those of pUU7, the R68-like plasmid from which they were derived. The bands 7PB and 7SB of pUU7, equivalent to PB and SB of R68 had each been replaced by one or more novel bands in all seven new plasmids. Therefore, as with the new plasmids discussed in the previous section (Section 4.2.2), all these new plasmids had undergone a modification within the same region of the plasmid that had been modified by I1 in the plasmid pUU1 from PPW1.

Two of the new plasmids, pUU20 and pUU21, had undergone modifications, which from the PstI and SmaI digest data, appeared to be indistinguishable from those of pUU1.

Plasmids pUU18 and pUU19 yielded indistinguishable digest patterns indicating the acquisition of 2.92 - 4.80kb of DNA containing one PstI site and no SmaI sites.

Plasmids pUU22 and pUU24 gave virtually identical digest patterns. The PstI patterns gave slightly different sizes for the third largest band, while the other PstI bands were indistinguishable. All the SmaI bands were indistinguishable. These two plasmids had gained about 2.94 - 4.10kb of

PLATE 4.3

Digests of R68.45, pUU1, pUU7 and derivative plasmids

PstI
16h
λ
λ HindIII
R68.45
pUU1
pUU7
pUU18
pUU19
60h
λ
λHindIII
R68.45
pUU1
pUU7
pUU18
pUU19

PstI
16h
λ
λ HindIII
R68.45
pUU1
pUU7
pUU18
pUU19
60h
λ
λ HindIII
R68.45
pUU1
pUU7
pUU18
pUU19

PstI

16h
λEcoRI/HindIII
λHindIII
λ
R68·45
pUU1
pUU7
pUU20
pUU21
pUU22
pUU23
pUU24
60h
λEcoRI/HindIII
λHindIII
λ
R68·45
pUU1
pUU7
pUU20
pUU21
pUU22
pUU23
pUU24

PstI

16h
λEcoRI/HindIII
λHindIII
λ
R68·45
pUU1
pUU7
pUU20
pUU21
pUU22
pUU23
pUU24
60h
λEcoRI/HindIII
λHindIII
λ
R68·45
pUU1
pUU7
pUU20
pUU21
pUU22
pUU23
pUU24

SmaI

16h

R68.45
pUU1
pUU7
pUU18
pUU19
λHindIII
λ

60h

R68.45
pUU1
pUU7
pUU18
pUU19
λHindIII
λ

SmaI

16h

R68.45
pUU1
pUU7
pUU18
pUU19
λHindIII
λ

60h

R68.45
pUU1
pUU7
pUU18
pUU19
λHindIII
λ

SmaI
16h
λ
λHindIII
λEcoRI/HindIII
R68·45
pUU1
pUU7
pUU20
pUU21
pUU22
pUU24
pUU23
60h
λ
λHindIII
λEcoRI/HindIII
R68·45
pUU1
pUU7
pUU20
pUU21
pUU22
pUU24
pUU23

SmaI

16h

λ
λHindIII
λEcoRI/HindIII
R68·45
pUU1
pUU7
pUU20
pUU21
pUU22
pUU24
pUU23

60h

λ
λHindIII
λEcoRI/HindIII
R68·45
pUU1
pUU7
pUU20
pUU21
pUU22
pUU24
pUU23

TABLE 4.4

Sizes of restriction fragments of R68.45, pUU1, pUU7 and derivative plasmids digested with PstI and SmaI

a. PstI

Strain		PPW1	PPW16	PPW27	PPW28
Plasmid	R68.45	pUU1	pUU7	pUU18	pUU19
	32.67	32.67	32.67	32.67	32.67
	24.84	15.68	24.84	15.26	15.26
	6.52	11.32	6.52	12.50	12.50
	2.77	6.52	2.77	6.52	6.52
	1.34	2.77	0.77	2.77	2.77
	0.77*	0.77	0.66	0.77	0.77
	0.66	0.66		0.66	0.66
	70.34kb	70.39kb	68.23kb	71.15kb	71.15kb

Strain	PPW29	PPW30	PPW31	PPW32	PPW33
Plasmid	pUU20	pUU21	pUU22	pUU23	pUU24
	32.67	32.67	32.67	32.67	32.67
	15.68	15.68	16.04	16.04	16.04
	11.32	11.32	11.89	11.59	11.74
	6.52	6.52	6.52	6.52	6.52
	2.77	2.77	2.77	3.63	2.77
	0.77	0.66	0.66	2.56	0.77
	0.66			0.77	0.66
				0.66	
	70.39kb	70.39kb	71.32kb	74.44kb	71.17kb

b. SmaI

Strain		PPW1	PPW16	PPW27	PPW28
Plasmid	R68.45	pUU1	pUU7	pUU18	pUU19
	26.52	26.52	26.52	26.52	26.52
	17.55	21.07	17.55	22.35	22.35
	15.10	15.10	15.10	15.10	15.10
	7.15	7.15	7.15	7.15	7.15
	2.12	0.76	0.76	0.76	0.76
	0.76				
	69.20kb	70.60kb	67.08kb	71.88kb	71.88kb

Strain	PPW29	PPW30	PPW31	PPW32	PPW33
Plasmid	pUU20	pUU21	pUU22	pUU23	pUU24
	26.52	26.52	26.52	26.52	26.52
	21.07	21.07	21.65	21.65	21.65
	15.10	15.10	15.10	18.64	15.10
	7.15	7.15	7.15	7.15	7.15
	0.76	0.76	0.76	0.76	0.76
	70.60kb	70.60kb	71.18kb	74.72kb	71.18kb

FIGURE 4.4

Diagram of PstI generated restriction fragments of R68.45, pUU1, pUU7 and derivative plasmids

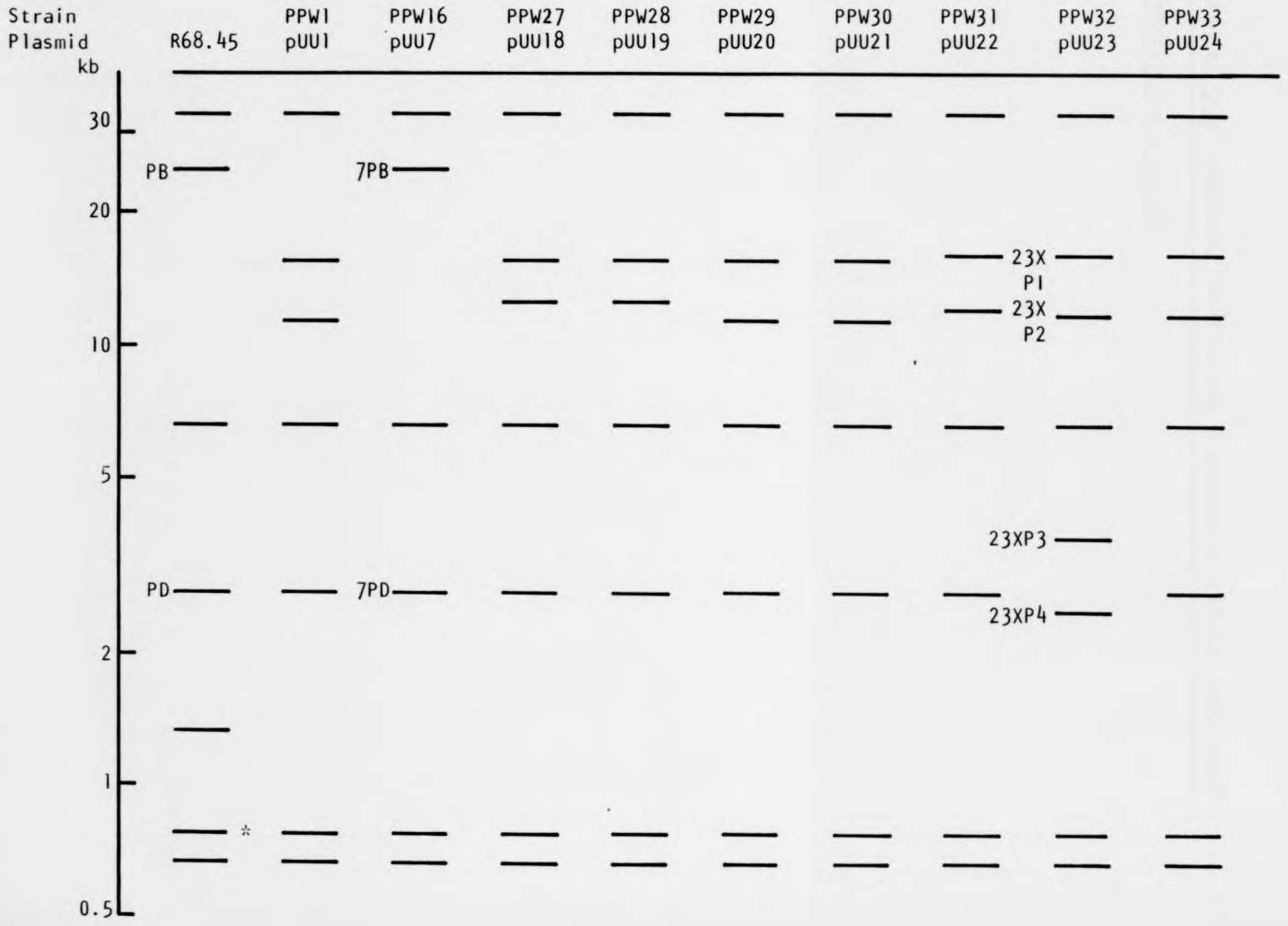
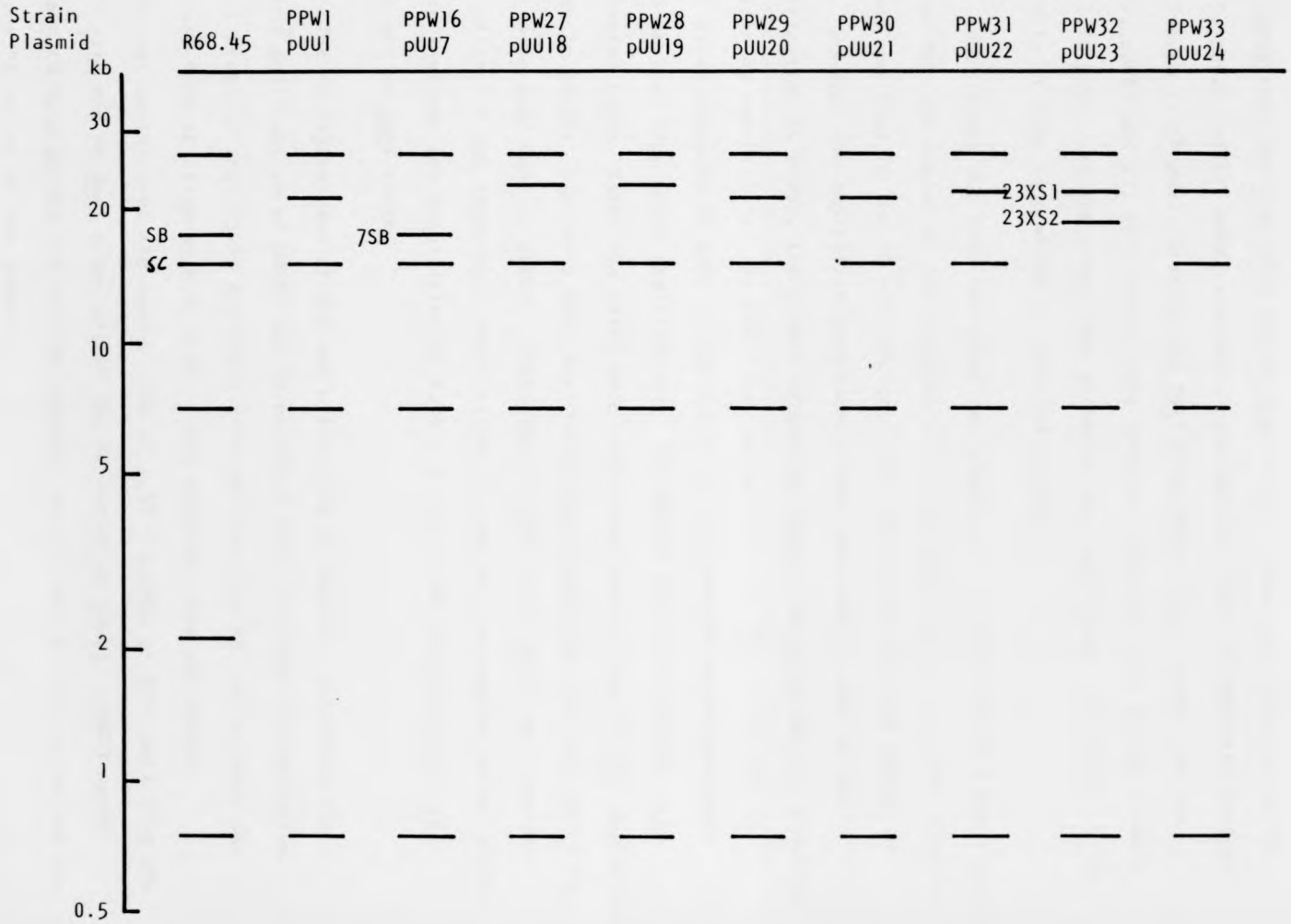

Strain
Plasmid
kb
R68.45
PPW1
pUU1
PPW16
pUU7
PPW27
pUU18
PPW28
pUU19
PPW29
pUU20
PPW30
pUU21
PPW31
pUU22
PPW32
pUU23
PPW33
pUU24
30
20
10
5
2
1
0.5
PB
7PB
PD
7PD
23X
P1
23X
P2
23XP3
23XP4
*

FIGURE 4.5

Diagram of SmaI generated restriction fragments of R68.45, pUU1, pUU7 and derivative plasmids


Strain
Plasmid
R68.45
PPW1
pUU1
PPW16
pUU7
PPW27
pUU18
PPW28
pUU19
PPW29
pUU20
PPW30
pUU21
PPW31
pUU22
PPW32
pUU23
PPW33
pUU24
kb
30
20
10
5
2
1
0.5
SB
SC
7SB
23XS1
23XS2

DNA containing one PstI site and no SmaI sites. These six plasmids, pUU18, pUU19, pUU20, pUU21, pUU22 and pUU4, received an insert of DNA very similar in size to I1 of pUU1, bearing one PstI site and no SmaI sites. In two cases, pUU20 and 21, this insert went into an identical site in the plasmid as I1 in pUU1. In the other four plasmids the insert went into sites in the vicinity of that into which I1 inserted in pUU1.

pUU23 stood out from the other new plasmids. In addition to a modification in the PB - SB region of the plasmid, which was modified in the other plasmids, it was modified in the PD - SC region. This was within the TnA region of R68. However the ampicillin gene cannot have been inactivated by the modification as PPW32, the strain harbouring pUU23, was selected for a medium containing ampicillin. As pUU23 had gained only two PstI sites and no SmaI sites compared to pUU7 it was possible to determine which new bands were derived from which modification. The second and third largest PstI generated bands, 23XP1 and 23XP2 were indistinguishable from the corresponding bands in pUU24. Similarly 2XS1 was indistinguishable from the corresponding SmaI generated band in pUU24. These data suggest that pUU23 had undergone a similar, if not identical, modification in the PB - SB region as had pUU24, which involved the acquisition of 2.94 - 4.1kb of DNA containing one PstI site and no SmaI sites.

The PD region band of R68 was within the SC region. Therefore the second modification of pUU23 can be narrowed down to within the PD region. It is probable that 23XP3 and 23XP4 were derived from PD, while 23XS2 was derived from SC (Figures 4.4, 4.5). This suggests that the second modification involved the acquisition of 3.42 - 3.54kb of DNA containing one PstI site and no SmaI sites within the PD region of pUU23. pUU23 would appear to have gained two I1-like inserts, one in the PB - SB region and one in the PD region of the plasmid.

4.2.4 Mating of *P. aeruginosa* PA08 (R68) with *P. putida* PP3

pUU6 and pUU7 were indistinguishable from R68 in terms of their PstI and SmaI digest patterns. On the mating of these plasmids back into the PP3 derivative PPW5 they produced modified plasmids which, while some appeared indistinguishable from pUU1 from which pUU6 and pUU7 were derived, others differed from pUU1 to varying extents (Sections 4.2.2 and 4.2.3). Yet two independently isolated transconjugants of the original mating of PAC174 (R68.44) with PP3 gave plasmids whose PstI and SmaI digest patterns were indistinguishable from those of pUU1 (Chapter 3). The possibility therefore arises that the different modifications in the pUU6 and pUU7 derived plasmids was due at least in part to some undetected change in pUU6 and pUU7 rather than solely to whatever phenomenon produced the original modification in pUU1. To examine this possibility R68 was mated into PP3.

Pseudomonas aeruginosa PA08 *met*28, *ilv*202, *str*-1 containing R68 was kindly provided by Professor B.W. Holloway (Monash University). PA08 (R68) was mated with *Pseudomonas putida* PP3 and transconjugants selected for on minimal media containing 2MCPA, Ap ($50\mu g ml^{-1}$), Km ($50\mu g ml^{-1}$) and Tc ($150\mu g ml^{-1}$). Transconjugants were obtained at a frequency of 1×10^{-6} per donor cell. The transconjugants were purified by streaking out on the selective medium. Purified colonies gave the characteristic growth of *P. putida* on King's A and B media. Three of the transconjugants were designated PPW34, PPW35 and PPW36; their plasmids were designated pUU25, pUU26 and pUU27 respectively (Table 4.1).

pUU25, pUU26 and pUU27 were purified by the rapid method and their restrictions digests with PstI and SmaI were compared with those of R68.45 and pUU1 (Plate 4.4; Table 4.5; Figures 4.6, 4.7). In all three new plasmids a modification occurred involving the addition of DNA to the region of R68.45 where the PB and SB fragments overlap. This was the same region into which

PLATE 4.4

Digests of R68.45, pUU1 and derivative plasmids

16h

PstI | SmaI

R68·45 | pUU1 | pUU25 | pUU26 | pUU27 | R68·45 | pUU1 | pUU25 | pUU26 | pUU27 | λEcoRI/HindIII | λHindIII | λ

60h

PstI | SmaI

R68·45 | pUU1 | pUU25 | pUU26 | pUU27 | R68·45 | pUU1 | pUU25 | pUU26 | pUU27 | λEcoRI/HindIII | λHindIII | λ

16h

PstI | SmaI

R68·45 pUU1 pUU25 pUU26 pUU27 R68·45 pUU1 pUU25 pUU26 pUU27 λEcoRI/HindIII λHindIII λ

60h

PstI | SmaI

R68·45 pUU1 pUU25 pUU26 pUU27 R68·45 pUU1 pUU25 pUU26 pUU27 λEcoRI/HindIII λHindIII λ

TABLE 4.5

Sizes of restriction fragments of R68.45, pUU1 and derivative plasmids digested with PstI and SmaI

a. PstI

Strain		PPW1	PPW34
Plasmid	R68.45	pUU1	pUU25
	32.67	32.68	32.67
	24.84	15.68	16.27
	6.52	11.32	10.62
	2.77	6.52	6.52
	1.34	2.77	2.77
	0.77*	0.77	0.77
	0.66	0.76	0.66
	70.34kb	70.39kb	70.28kb

	PPW35	PPW36
	pUU26	pUU27
	32.67	32.67
	15.48	19.94
	11.21	9.51
	6.52	6.52
	3.76	2.77
	2.77	0.77
	0.77	0.66
	0.66	
	73.84kb	72.90kb

b. SmaI

Strain		PPW1	PPW34
Plasmid	R68.45	pUU1	pUU25
	26.52	26.52	26.52
	17.55	21.07	22.08
	15.10	15.10	15.10
	7.15	7.15	7.15
	2.12	0.76	0.76
	0.76		
	69.20kb	70.60kb	71.61kb

	PPW35	PPW36
	pUU26	pUU27
	26.52	26.52
	25.79	22.08
	15.10	15.10
	7.15	7.15
	0.76	0.76
	75.32kb	71.61kb

FIGURE 4.6

Diagram of PstI generated restriction fragments of R68.45, pUU1 and derivative plasmids

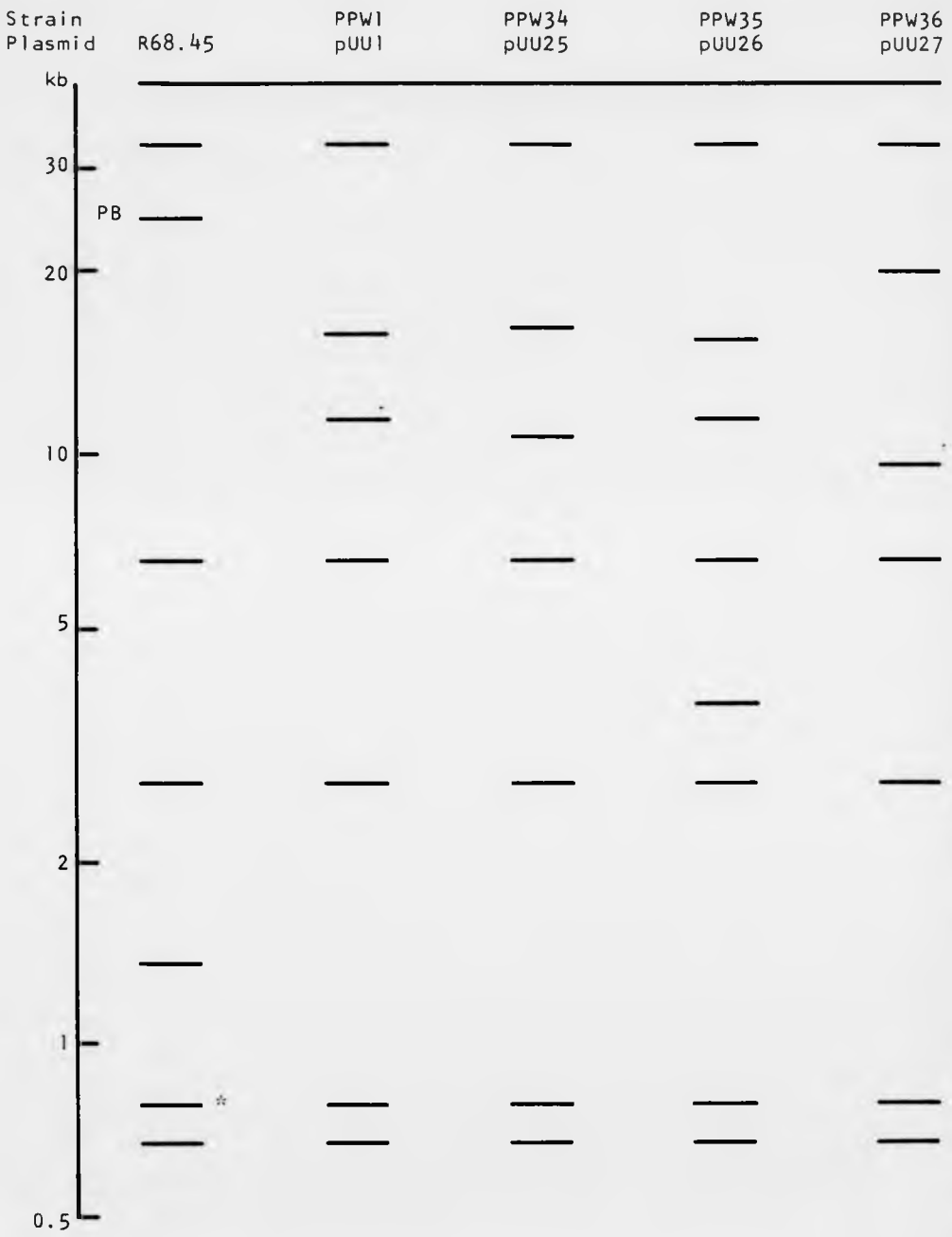
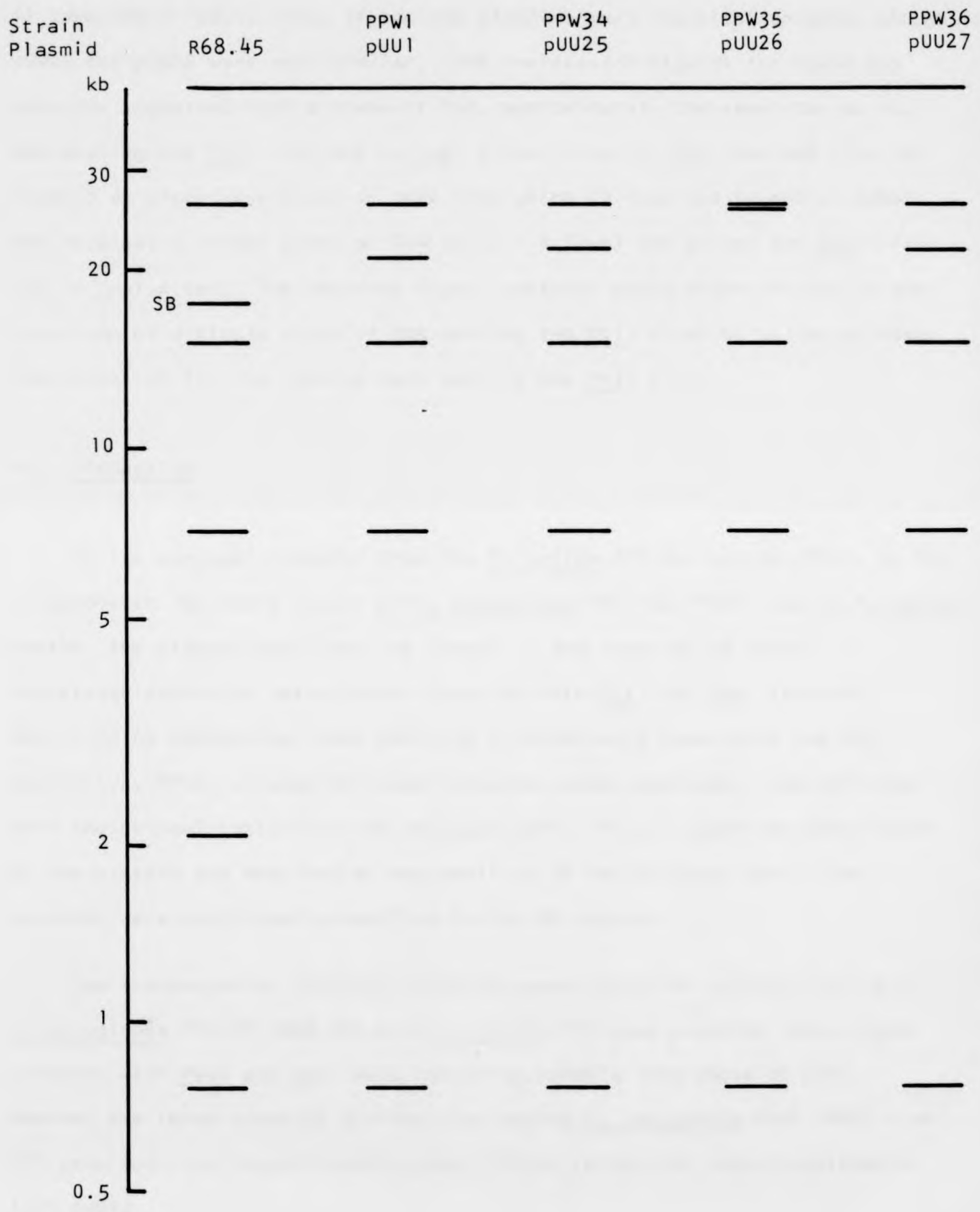

Strain
Plasmid
R68.45
PPW1
pUU1
PPW34
pUU25
PPW35
pUU26
PPW36
pUU27
kb
30
PB
20
10
5
2
1
0.5
*

FIGURE 4.7

Diagram of SmaI generated restriction fragments of R68.45, pUU1 and derivative plasmids


Strain
Plasmid
R68.45
PPW1
pUU1
PPW34
pUU25
PPW35
pUU26
PPW36
pUU27
kb
30
20
SB
10
5
2
1
0.5

I1 inserted in pUU1. None of the new plasmids were identical to pUU1, though pUU25 and pUU27 were very similar. The restriction digests for these two plasmids suggested that a piece of DNA, approximately the same size as I1, and bearing one PstI site and no SmaI sites, like I1, had inserted into the plasmid at sites very close to that into which I1 inserted in pUU1. pUU26 had received a larger piece of DNA (5.61 - 8.24kb) and gained two PstI sites but no SmaI sites. The observed digest patterns could either be due to the insertion of a single piece of DNA bearing two PstI sites or to two adjacent insertions of I1-like inserts each bearing one PstI site.

4.3 Discussion

On its conjugal transfer from the P. putida PP3 derivative, PPW1, to the streptomycin resistant mutant of P. aeruginosa PAO1162, PAU1, and to P. putida PaW340, the plasmid pUU1 lost the insert I1 and reverted to forms indistinguishable by restriction digestion with PstI and SmaI from R68. When strains containing these R68-like plasmids were mated with the PP3 derivative, PPW5, a range of mutant plasmids were generated. Some of these were indistinguishable from the original pUU1. In all cases the same region of the plasmid was modified as was modified in the original pUU1. Two plasmids were additionally modified in the PD region.

Two independently isolated transconjugants from the original mating of P. aeruginosa PAC174 (R68.44) with P. putida PP3 gave plasmids whose digest patterns with PstI and SmaI were indistinguishable from those of pUU1. However the three plasmids derived from mating P. aeruginosa PAO8 (R68) with PP3 gave modified plasmids which were similar to but not indistinguishable from pUU1.

There were two important features in these results: firstly, the loss of I1 from pUU1 and the revertion of that plasmid to a R68-like form on its

transfer to other species; and secondly, the nature of and the relationships between the modifications to the derivative plasmids.

pUU1 may not transfer because I1 interfered with the transfer mechanisms. I1 was shown to be closely associated with the tra3, if not actually within tra3 (Section 3.5.2.5). Alternatively, pUU1 may have transferred but be unable to replicate in the new host due to the presence of I1. An R68-like plasmid was detected in the recipients which must have been derived in some form or another from pUU1. The frequency of transfer of this plasmid was 1.4×10^{-5} per donor cell in the case of PPWI x PAU1, and 1.2×10^{-6} in the case of PPWI x PaW340. These were several orders of magnitude lower than values obtained for the intra specific transfer of R68 or R68.45; 0.3 - 1.5×10^{-1} per donor cell (Riess et al., 1980), 10^{-1} - 10^{-2} (Beringer et al., 1978). The differences in frequency obtained here may be due to the transfer being interspecific. However frequencies of interspecific transfer of R68 and R68.45 of 10^{-2} - 10^{-3} per donor cell have been observed (Johnston et al., 1978b).

The reduction in conjugal transfer frequencies observed here could be due to the action of restriction endonucleases. Nagahari and Sakaguchi (1978) obtained a 10^5 increase in transformation efficiency in restriction deficient E. coli over restriction-plus cells. A similar difference might be expected in conjugal transfer. P. aeruginosa PAU1 was restriction deficient; P. putida PaW340 was not, however the difference in transfer frequency, (1.4×10^{-5} and 1.2×10^{-6} per donor cell, respectively) was not so marked. The restriction systems of the recipients could not account for the 10^3 reduction in observed over expected transfer efficiency.

Cowan and Krishnapillai (1982) isolated Tn7 insertion mutants of R18 (= RK2 = R68) which affected the host range of the plasmid. These mutant plasmids showed reduced transfer ability from P. aeruginosa PAO to E. coli.

The mutants fell into two classes; one class had deficient transfer functions and the second defective replication and maintenance functions in the new host. The former insertions mapped near OriV and within trfA, while the latter were all within traI (Figure 4.3). The insert I1 of pUU1 mapped at the opposite side of the plasmid from these latter mutations in R18. This, together with the finding that I1 was close to if not within tra3 (Section 3.5.2.5) suggest that 'replication deficient in new host' hypothesis can be dismissed and the 'transfer deficient' hypothesis accepted. In which case only about $1 - 10^{3}$ bacteria in a population of PPWI contained plasmids which were transfer proficient, presumably because they were R68-like having lost I1. Presumably it was only these transfer proficient plasmids which could engage in R-prime formation. Therefore the frequency for the formation of the R-prime pUU2 could be given as 1.3×10^{-7} per proficient donor cell (Section 3.2.2). However the question remains unanswered as to why in the formation of the R-prime pUU2 from pUU1, I2, the dehI bearing insert, should become inserted in close proximity to, if not within, the site from which pUU1 ha lost I1 (Section 3.5.3.6.2), rather than in association with IS21 as other workers have found (see Section 3.6). Did I1 in some way become a centre for R-prime formation in an analogous fashion to IS21 in other cases? Or did the insertion of I1 into and its subsequent excision from pUU1 somehow 'modify' or 'prepare' the plasmid for R-prime formation at or near that site?

When the R68-like plasmids pUU6 and pUU7 were mated back into the PP3 derivative PPW5 mutant derivative plasmids were obtained. Five of these plasmids were indistinguishable from pUU1; these were pUU8, pUU9, pUU14, pUU20 and pUU21. Four of the plasmids, pUU18, pUU19, pUU22 and pUU24, gave digest patterns which indicated the insertion of a piece of DNA approximately the same size as I1 near the site of insertion of I1 in pUU1. One plasmid, pUU23 had undergone two I1-like insertions, one in the PB - SB region and the other in the PD region. These results indicated that I1, or a piece of

DNA very similar to I1, was capable of insertion into these two R68-like plasmids in closely located sites within the PB - SB region of the plasmid, and, more occasionally, also into the PD region. Similar results were obtained using a genuine R68 or R68.44, confirming that this phenomenon was not due to some intrinsic difference in the R68-like plasmids. These results lead to the conclusion that I1 was probably an insertion sequence capable of insertion into at least two areas of the plasmid.

Not all the modifications were due to I1-like insertions. The remaining seven plasmids had undergone modifications which involved the acquisition of SmaI sites which I1 did not possess. Usually these modifications involved larger pieces of DNA than I1. In one case pUU17, these modifications were both in the PB - SB and PD regions of the plasmid.

In three cases, pUU10, pUU12 and pUU16, the sizes of some of the new restriction bands were very close to those from the dehI bearing insert, I2, of pUU2. Preliminary results suggest that dehI is located on a transposable element (J.H. Slater, A.J. Weightman and B.G. Hall, unpublished observations). The modifications in these plasmids could indicate the insertion of this transposable element. If that is the case it indicates that this event was not rare; it occurred in three out of the twenty two plasmids examined.

CHAPTER 5

THE TRANSFER OF PLASMID pUU2 TO OTHER SPECIES

CHAPTER 5

THE TRANSFER OF PLASMID pUU2 TO OTHER SPECIES

5.1 Introduction

In order to investigate the effect of the dehI insert on the host-range and transfer ability of pUU2, the plasmid was transferred to a strain of Pseudomonas aeruginosa and two strains of Escherichia coli by conjugation and by transformation. Changes in plasmid structure were observed and interpreted in the light of the results obtained for pUU1 in Chapter 4.

5.2 Mating of PPW2 with Pseudomonas aeruginosa PAO

Pseudomonas putida PPW2 containing the R-prime pUU2 was mated with a rifampicin resistant mutant of Pseudomonas aeruginosa PAO 1162, PAU2 (see Section 3.4.1 for details). Transconjugants were selected at 5×10^{-5} per donor cell on minimal medium supplemented with 2MCPA and rifampicin, and at 3.8×10^{-4} on nutrient agar supplemented with rifampicin, ampicillin, kanamycin and tetracycline. The transconjugants showed a high (95%) co-inheritance of the plasmid markers for the ability to utilize 2MCPA as sole carbon and energy source and antibiotic resistance.

Two of the transconjugants, PAU9 and PAU10 (Table 5.1) isolated on nutrient agar and antibiotics were kept and subsequently maintained on 2MCPA minimal agar. Their plasmids, pUU28 and pUU29, respectively, were purified by the rapid method and digested with PstI and SmaI and their digest patterns compared with those obtained from R68.45 and pUU2 (Plate 5.1; Table 5.2; Figure 5.1).

The PstI digests of pUU28 and pUU29 showed that the novel double bands

TABLE 5.1

Bacterial strains and plasmids used and derived in Chapter 5

Strain	Species[1]	Plasmid	Genotype/Phenotype	Derivation
PPW2	P.p.	pUU2	Trp^-,$2Mcpa^+$,Sm^R,Ap^R,Km^R,Tc^R	PPW1 X PaW340
PAU2	P.a.	-	Leu^-,R^-,M^+,Rif^R	Mutant of PA01162
PAU9	P.a.	pUU28	Leu^-,R^-,M^+,$2Mcpa^+$,Rif^R,Ap^R, Km^R,Tc^R	PPW2 X PAU2
PAU10	P.a.	pUU29	"	"
PPW5	P.p.	-	$2Mcpa^+$,His^-	Mutant of PP3
ED8654	E.c.	-	*Sup*E,*Sup*F,*hsd*,R^-,M^+,S^+, Met^-,*trp*R	A.J. Weightman
HB101	E.c.	-	F^-,*hsd* S20 ($r_B^-m_B^-$), *rec*A13,*ara*14,*pro*A2, *lac*Y1,*gal*K2,*rsp*120 (Sm^R), *xyl*5, *mtl*-1,*Sup*E44λ^-	P.C. Gowland

1. Species: P.p. - *Pseudomonas putida*
P.a. - *Pseudomonas aeruginosa*
E.c. - *Escherichia coli*

PLATE 5.1

Digests of R68.45, pUU2, pUU28 and pUU29 with PstI and SmaI

16h
PstI
SmaI
R68·45
pUU2
pUU28
pUU29
λ
λHindIII
λEcoRI/HindIII
R68·45
pUU2
pUU28
pUU29
60h
PstI
SmaI
R68·45
pUU2
pUU28
pUU29
λ
λHindIII
λEcoRI/HindIII
R68·45
pUU2
pUU28
pUU29

16h
60h
PstI
SmaI
R68·45
pUU2
pUU28
pUU29
λ
λHindIII
λEcoRI/HindIII
R68·45
pUU2
pUU28
pUU29
PstI
SmaI
R68·45
pUU2
pUU28
pUU29
λ
λHindIII
λEcoRI/HindIII
R68·45
pUU2
pUU28
pUU29

TABLE 5.2

Sizes of restriction fragments of R68.45, pUU2, pUU28 and pUU29 with PstI and SmaI

a. PstI

Strain		PPW2	PAU9	PAU10
Plasmid	R68.45	pUU2	pUU28	pUU29
	32.67	32.67	32.67	32.67
	24.86	14.82	26.12	26.44
	6.52	12.38	6.52	6.52
	2.77	6.52	3.23*	4.37
	1.34	3.23*	2.77	3.23*
	0.77*	2.77	1.19*	2.77
	0.66	1.19*	0.87*	1.19*
		0.87*	0.77	0.87*
		0.77	0.66	0.77
		0.66	0.60*	0.66
		0.60*	?[1]	0.60*
		0.29*		?
Size of plasmid	70.34kb	82.95kb	81.86kb	86.55kb
Increase of size over R68		14.72	13.63	18.33

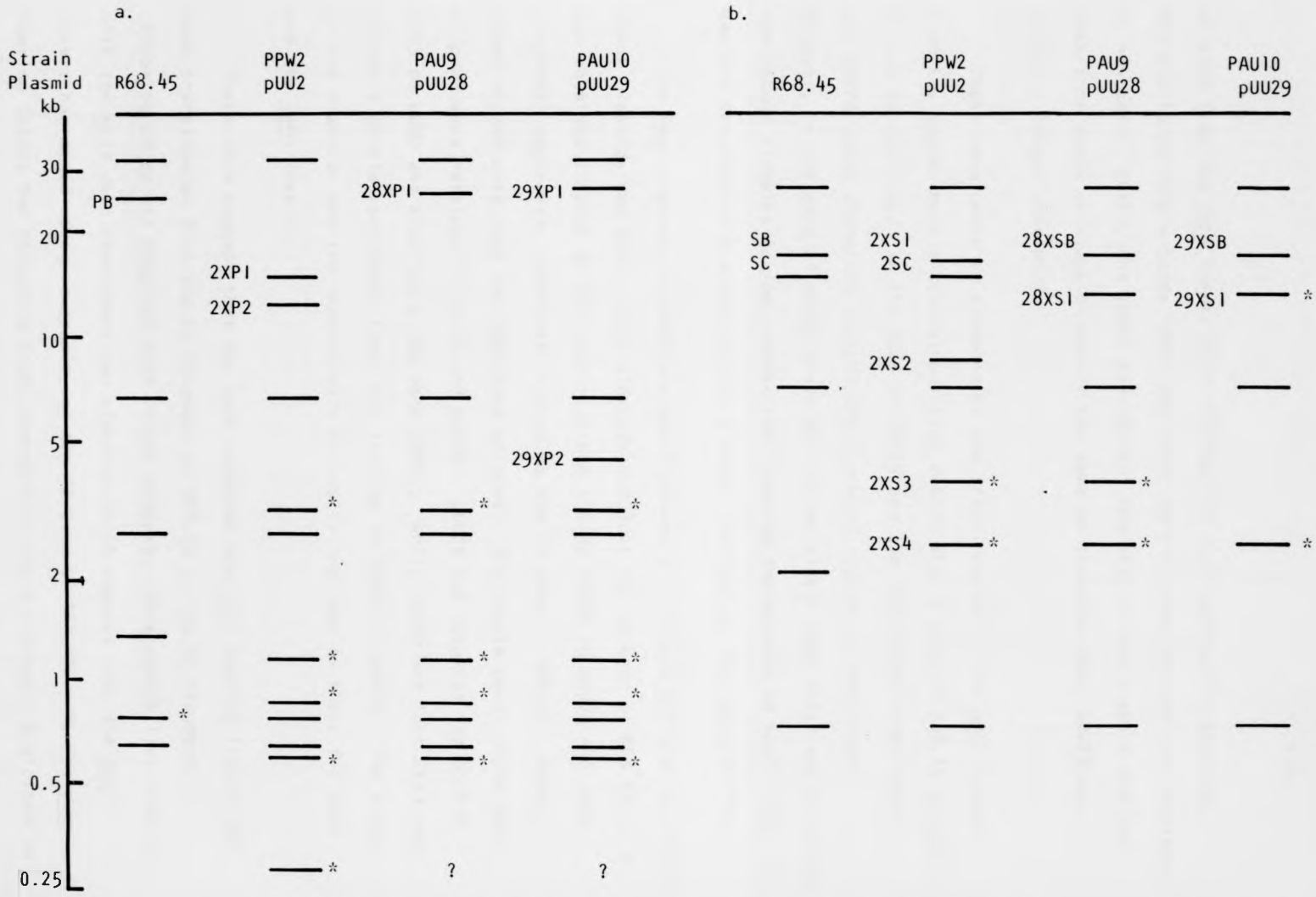
1. Because of the limitations of the rapid method for purifying plasmid DNA it is not possible to determine with any certainty the presence or absence of the smallest digest fragments. The sizes of the plasmids are calculated assuming the presence of the smallest bands of pUU2.

b. SmaI

Strain		PPW2	PAU9	PAU10
Plasmid	R68.45	pUU2	pUU28	pUU29
	26.52	26.52	26.52	26.52
	17.55	16.60	17.55	17.55
	15.10	15.10	13.30	13.30*
	7.15	8.45	9.14	7.15
	2.12	7.15	7.15	2.51*
	0.76	3.80*	3.80*	0.76
		2.51*	2.51*	
		0.76	0.76	
Size of plasmid	69.20kb	87.20kb	87.04kb	83.60kb
Increase in size over R68		20.12	19.96	16.52

FIGURE 5.1

Diagram of PstI and SmaI generated restriction fragments of R68.45, pUU2, pUU28, and pUU29


a.
Strain
Plasmid
kb
R68.45
PPW2
pUU2
PAU9
pUU28
PAU10
pUU29
30
20
10
5
2
1
0.5
0.25
PB
2XP1
2XP2
28XP1
29XP1
29XP2
*
?
b.
R68.45
PPW2
pUU2
PAU9
pUU28
PAU10
pUU29
SB
SC
2XS1
2SC
28XSB
28XS1
29XSB
29XS1
2XS2
2XS3
2XS4
*

of pUU2 from the dehI insert were retained in these derivative plasmids. But the large single bands (2XP1 and 2XP2) were missing and had been replaced by new bands, 28XP1, and 29XP1 and 29XP2. The size of the plasmid and the insert of pUU28 were approximately the same as those of pUU2; pUU29 was slightly larger than pUU2.

These data could be produced by the translocation of the dehI insert from its approximately central position within the PB band of R68.45 to one of its extremities. In the case of pUU29 the two new single bands 29XP1 and 29XP2 would therefore include the flanking regions of the insert. However, in the case of pUU28 there was no new single band which could include the second flanking region, unless the flanking region were so small that it was not detected in a digest of the plasmid isolated by the rapid method.

The SmaI digests of pUU28 and pUU29 showed significant differences between these plasmids and pUU2. Both plasmids had lost the SC band of R68.45 which was retained in pUU2 as 2SC, and regained a band (28SB in pUU28 and 29SB in pUU29) apparently identical in size to the SB band in R68.45. Both plasmids had also lost the 2XSC band of pUU2. The double novel bands 2XS4 of pUU2 were retained in pUU28 and pUU29. pUU28 had retained the double 2XS3 of pUU2 and acquired a new band 28XS1. While pUU29 had lost 2XS3 and gained a double band 29XS1 identical in size to 28XS1 in pUU28. The sizes of the plasmids and the inserts were virtually the same as those for pUU2 and the dehI insert.

These data suggest that for both plasmids the dehI bearing insert had been translocated from the SB fragment of R68.45 to the SC fragment, restoring SB to its original size in the process. This possibility ties in with the PstI data from these two plasmids which suggest that the dehI insert had moved to one extremity of the PB band of R68.45. 12, the dehI bearing insert had therefore translocated from the 3 o'clock - 4 o'clock to

the 2 o'clock - 3 o'clock region of the plasmid (Figure 5.2). Only two plasmids were analysed by restriction analysis, it was therefore not possible to determine how representative pUU28 and 29 were of this class of transconjugants. It cannot therefore be determined for certain whether the translocation of I2 was a necessary or coincidental adjunct to plasmid transfer.

5.3 Transformation of plasmid pUU2 into Pseudomonas aeruginosa PAO

Purified plasmid DNA from the R-prime pUU2 was transformed into Pseudomonas aeruginosa PAO1162 (see Section 3.4.2 for details). Transformants were selected for on either minimal medium supplemented with 2MCPA or nutrient agar supplemented with Ap ($50\mu gml^{-1}$), Km ($50\mu gml^{-1}$) and Tc ($150\mu gml^{-1}$). In all cases examined the transformants had coinherited the ability to utilise 2MCPA as sole carbon and energy source and resistance to the three antibiotics.

Plasmid DNA was purified by the rapid method from six of the transformants selected on nutrient agar and antibiotics and digested with PstI and SmaI. In all cases the plasmid digest patterns were indistinguishable from those of pUU2 (Plate 5.2).

These results contrast strongly with those obtained for the conjugal transfer of pUU2 into the PAO1162 derivative PAU2 (Section 5.2). In the two cases examined substantial reorganization of the plasmid had occurred involving the translocation of the dehI bearing insert. These two sets of results suggest that translocation of the dehI insert was required for the efficacy of the transfer genes in conjugal transfer while such translocation was not necessary solely for replication of the plasmid in the new host.

5.4 Mating of P. putida PPW2 with E. coli strains

PPW2 containing the R-prime pUU2 was mated with two strains of

FIGURE 5.2

Map of R68

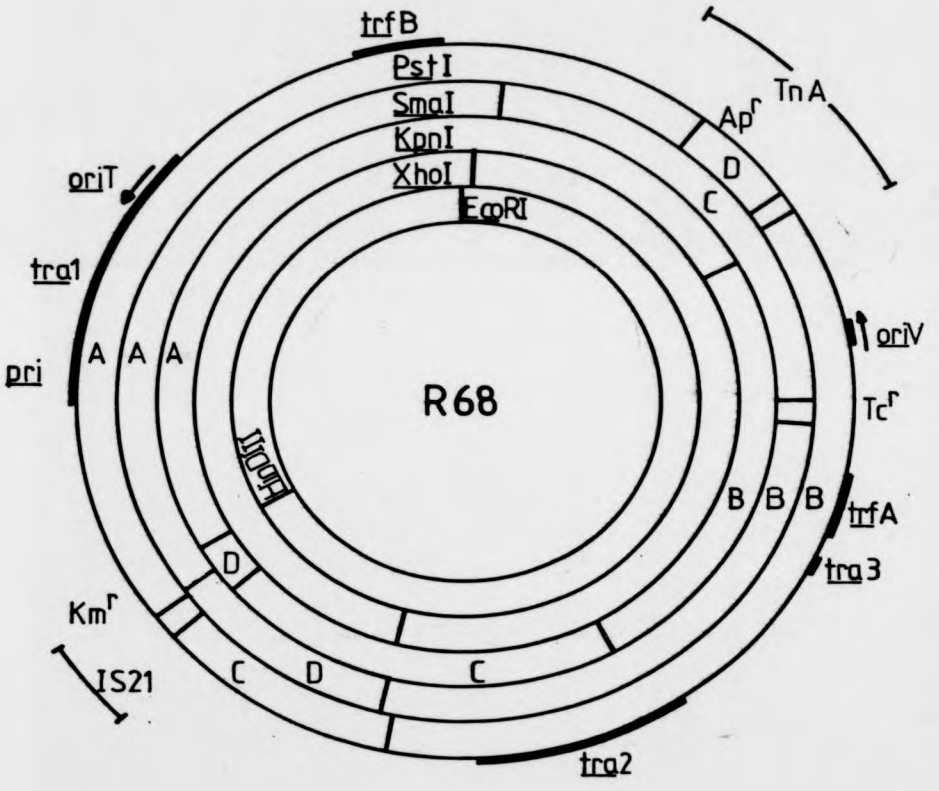
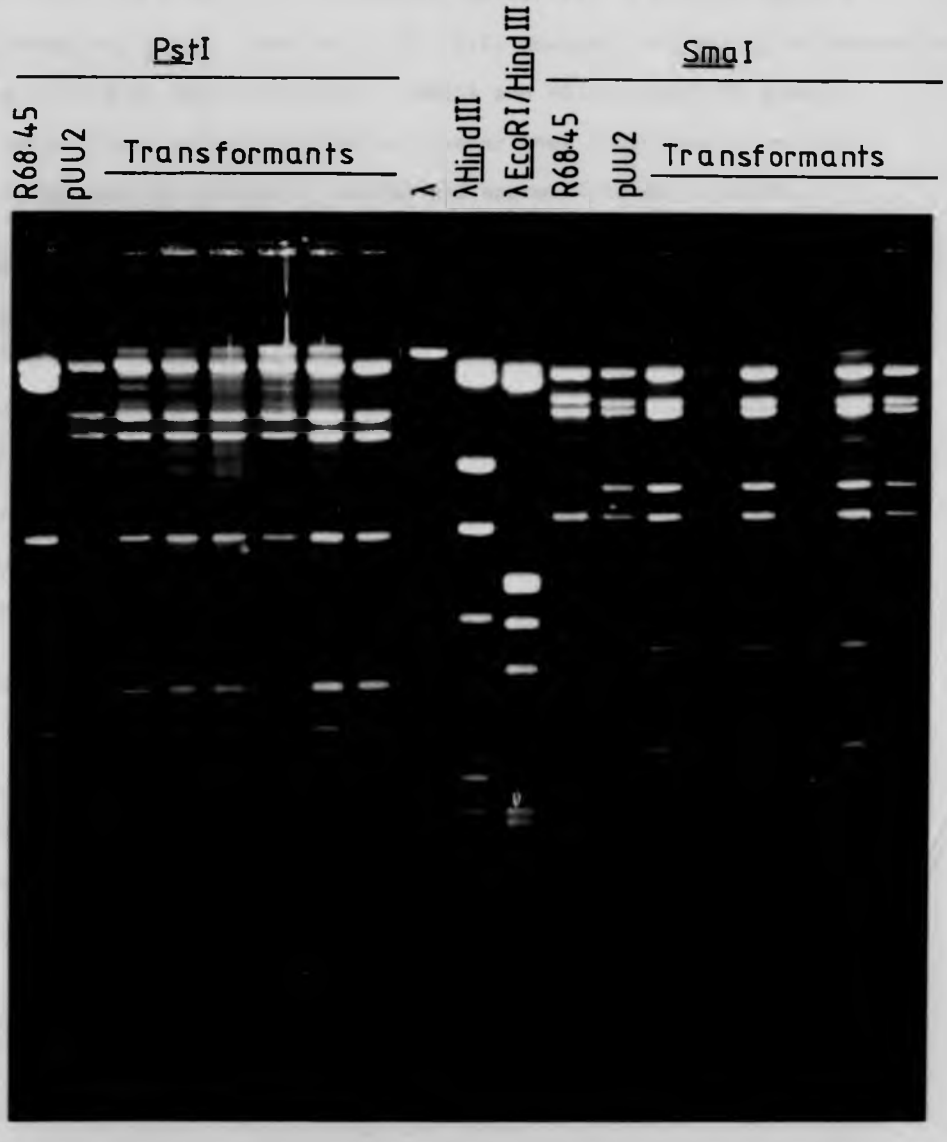
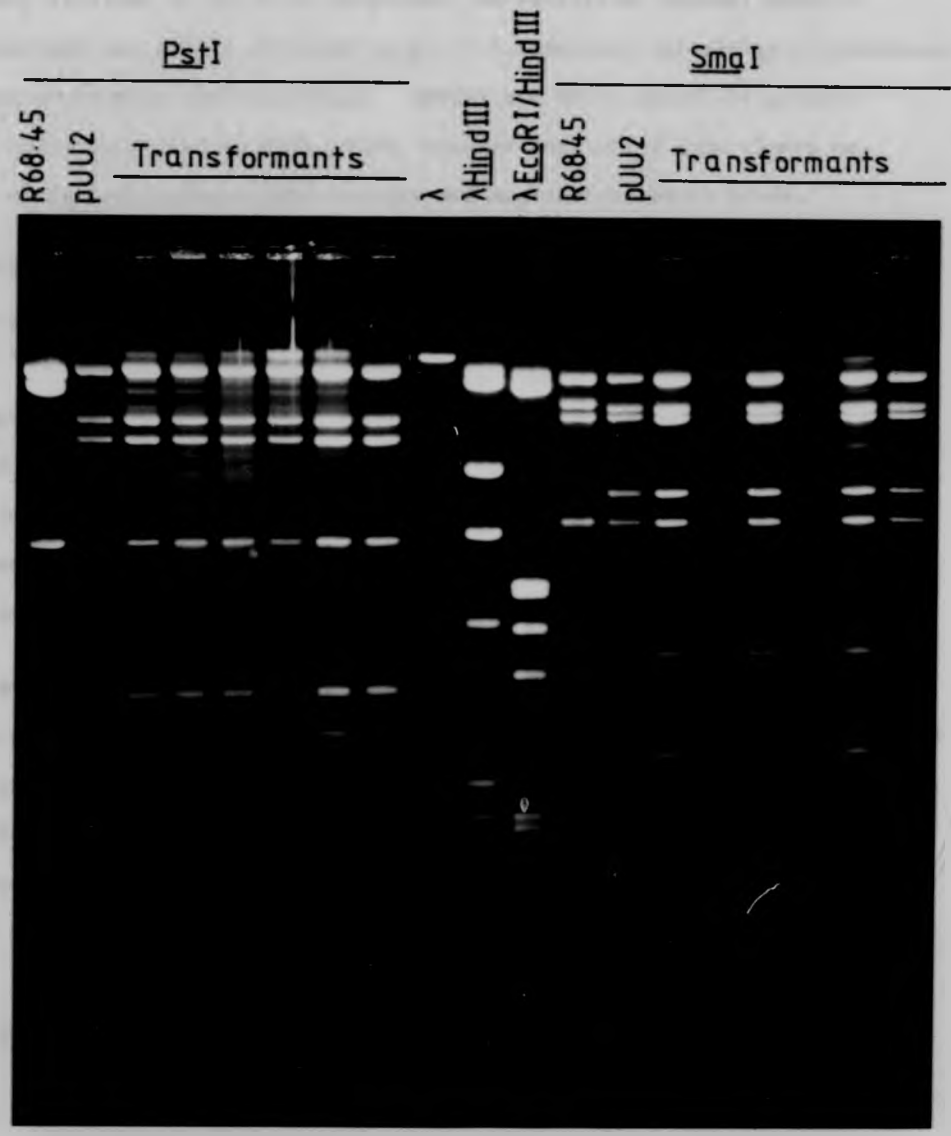

trfB
PstI
SmaI
KpnI
XhoI
EcoRI
HindIII
TnA
Ap^r
D
C
oriV
Tc^r
B B B
trfA
tra3
tra2
C D C
D
Km^r
IS21
oriT
tra1
pri
A A A
R68

PLATE 5.2

Digests of plasmids from transformants of PA01162 with pUU2 DNA, with PstI and SmaI


PstI
SmaI
R68.45
pUU2
Transformants
λ
λHindIII
λEcoRI/HindIII
R68.45
pUU2
Transformants


PstI
SmaI
R68.45
pUU2
Transformants
λ
λHindIII
λEcoRI/HindIII
R68.45
pUU2
Transformants

Escherichia coli, ED8654 and HB101 (Table 5.1). ED8654 a Rec^+ auxotroph was kindly provided by Dr. A.J. Weightman, University of Geneva; HB101 a recA auxotroph was kindly provided by Dr. P.C. Gowland, University of Manchester Institute of Science and Technology. ED8654 and HB101 would not grow on minimal media supplemented with 2MCPA, however they would grow slowly on minimal media supplemented with lactate and appropriate amino acids.

PPW2 was membrane mated separately with ED8654 and HB101. In both cases transconjugants were selected for on nutrient agar supplemented with Ap ($10\mu gml^{-1}$) and Km ($10\mu gml^{-1}$) and on minimal medium supplemented with 2MCPA. No colonies grew on the plates containing 2MCPA. Transconjugants were obtained on nutrient agar containing Ap and Km at $3x10^{-6}$ per donor cell for ED8654 and $1x10^{-5}$ for HB101. The auxotrophic markers of the transconjugants were checked to confirm that the strains were E. coli. They exhibited no growth on 2MCPA.

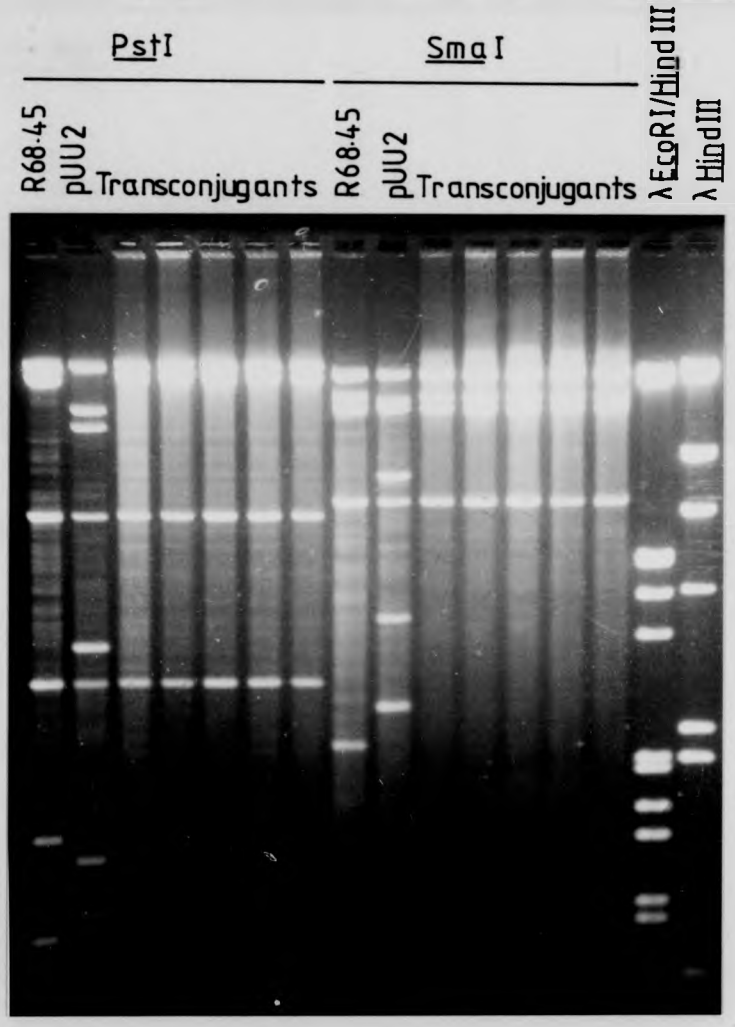
Plasmid DNA was extracted by the rapid method from five of the ED8654 derived transconjugants and from six of the HB101 derived strains and digested with PstI and SmaI (Plates 5.3; 5.4). In all eleven cases the digest patterns were indistinguishable from those of R68. pUU2 had lost the dehI insert, I2 and reverted to a form indistinguishable from R68, regardless of whether the E. coli was Rec^+ or Rec^-.

5.5 Transformation of plasmid pUU2 into E. coli strains

E. coli strains ED8654 and HB101 were transformed with purified pUU2 DNA. Transformants were selected for on minimal medium supplemented with 2MCPA and on nutrient agar supplemented with Ap ($10\mu gml^{-1}$), Km ($10\mu gml^{-1}$) and Tc ($15\mu gml^{-1}$). No transformants were obtained on minimal medium and 2MCPA. Transformants were obtained on nutrient agar and antibiotics. These transformants proved unable to grow on 2MCPA.

PLATE 5.3

Digests of plasmids from transconjugants from the mating PPW2 X ED8654, with *Pst*1 and *Sma*1


PstI
SmaI
R68.45
pUU2
Transconjugants
R68.45
pUU2
Transconjugants
λ EcoRI/HindIII
λ HindIII

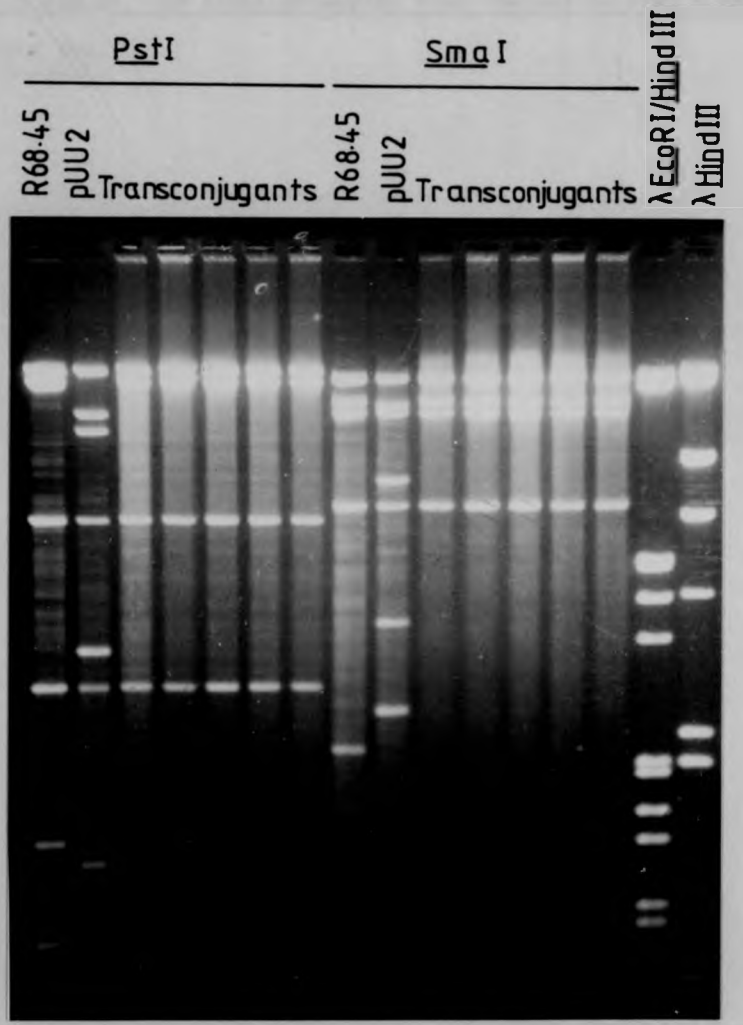
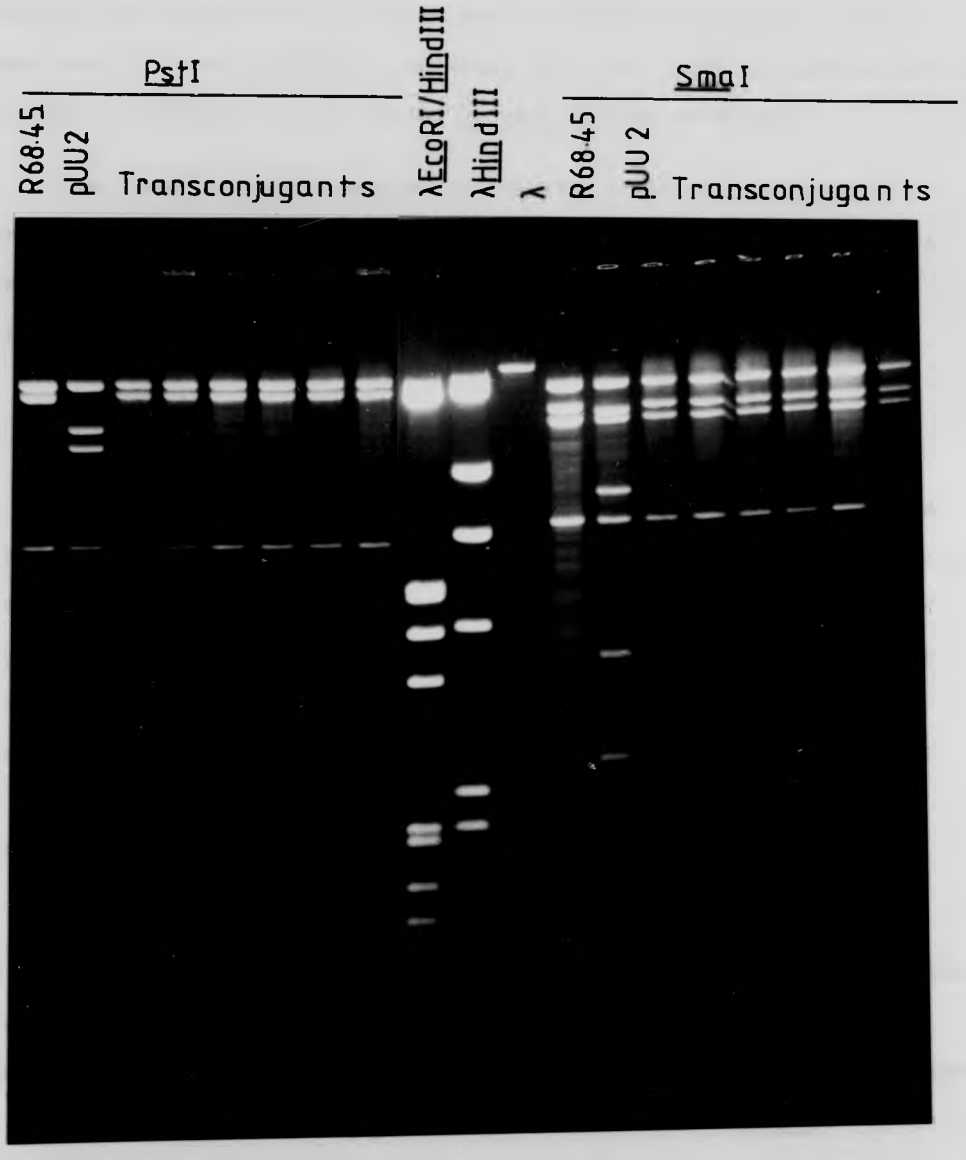
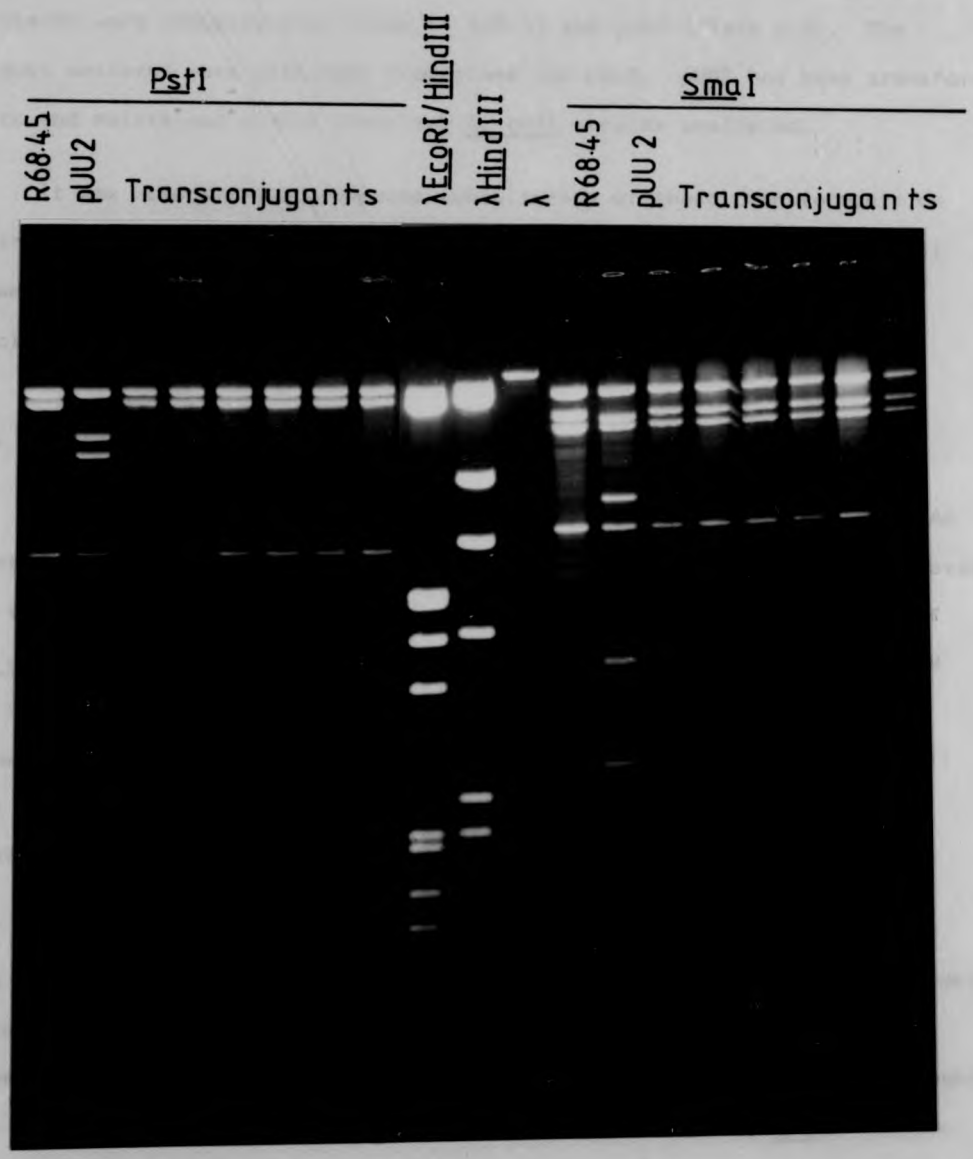

PstI
SmaI
R68.45
pUU2
Transconjugants
R68.45
pUU2
Transconjugants
λ EcoRI/HindIII
λ HindIII

PLATE 5.4

Digests of plasmids from transconjugants from the mating PPW2 X HB101, with *Pst*I and *Sma*I


PstI
SmaI
R68.45
pUU2
Transconjugants
λEcoRI/HindIII
λHindIII
λ
R68.45
pUU 2
Transconjugants


PstI
SmaI
R68.45
pUU2
Transconjugants
λEcoRI/HindIII
λHindIII
λ
R68.45
pUU2
Transconjugants

Plasmid DNA was purified by the rapid method from three ED8654 and three HB101 derived transformants and digested with PstI and SmaI. The digest patterns were compared with those of R68.45 and pUU2 (Plate 5.5). The digest patterns were unchanged from those for pUU2. pUU2 had been transformed into and maintained within these two E. coli strains unaltered.

It was observed that repeated subculturing of these transformants on nutrient agar plus antibiotics led to the loss from the plasmid of digest bands associated with the I2 insert of pUU2, and to the eventual total excision of I2 and the reversion of the plasmid to an R68-like form.

5.6 Discussion

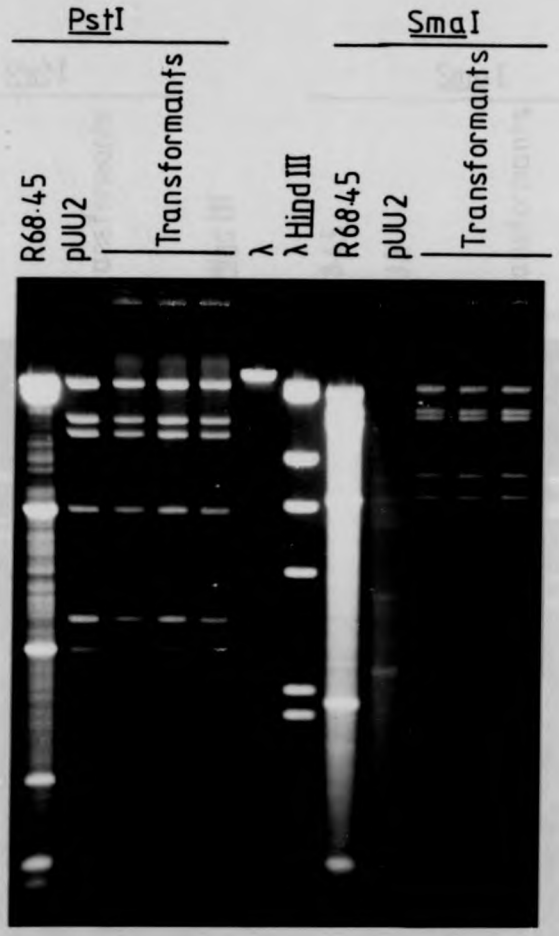
Transconjugants from matings of P. putida PPW2 with P. aeruginosa PAO contained plasmids in which the dehI bearing insert I2 had been translocated to the SC region of the plasmid. Yet when PPW2 was mated with strains of E. coli the transconjugants contained plasmids indistinguishable from R68 by restriction analysis. pUU2 was transformed into P. aeruginosa and remained unaltered. Yet when pUU2 was transformed into E. coli, though it was initially present as pUU2, on repeated subculturing the insert was excised and the plasmid reverted to an R68-like form.

In Section 3.5.3.6.2 it was suggested that I2 was located in or near the tra3 gene. This might inactivate or reduce the activity of tra3, in which case only plasmids in which I2 had translocated to other regions of the plasmid would be capable of efficient conjugal transfer. A similar phenomenon was observed in pUU1 in which I1 was closely associated with tra3. I1 was lost on transfer with selection for antibiotic resistance. Yet when pUU2 was mated and selected for by antibiotic resistance alone the ability to utilise 2MCPA as sole carbon and energy source was generally cotransferred. In other words the insert I2 bearing dehI was not lost in such matings.

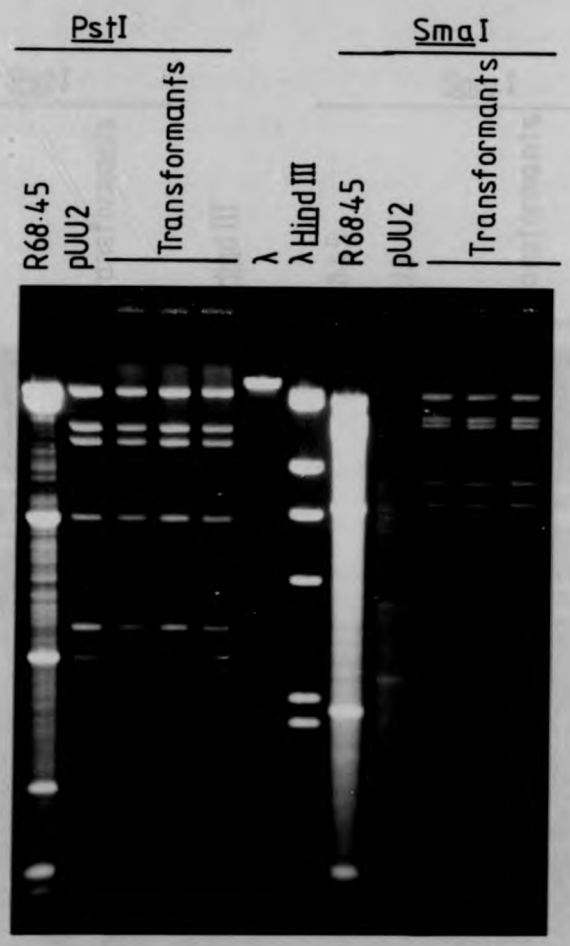
PLATE 5.5

Digests of plasmids from transformants of ED8654 and HB101 with pUU2 DNA, with PstI and SmaI

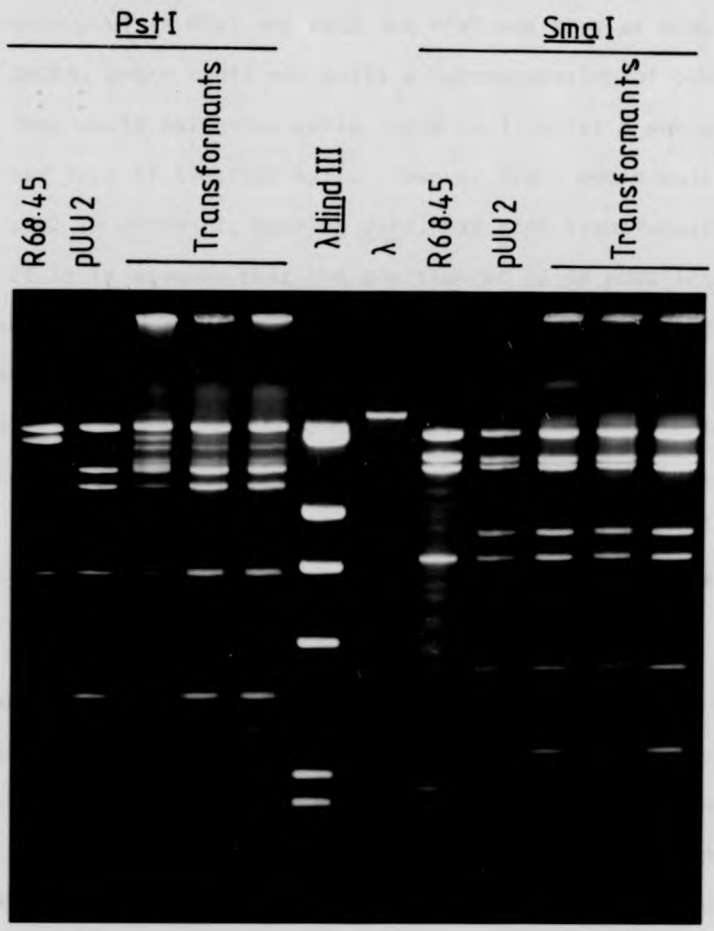
ED8654


PstI
SmaI
R68.45
pUU2
Transformants
λ
λ HindIII
R6845
pUU2
Transformants

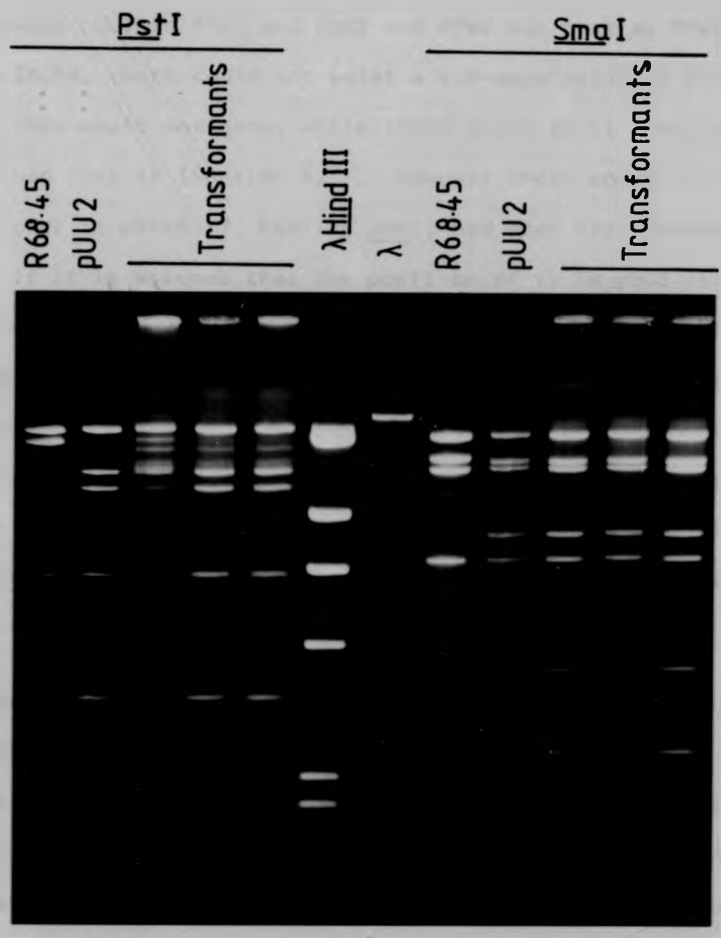
ED8654


PstI
SmaI
R68·45
pUU2
Transformants
λ
λ HindIII
R6845
pUU2
Transformants

HB101


PstI
SmaI
R68.45
pUU2
Transformants
λHind III
λ
R68.45
pUU2
Transformants

HB101


PstI
SmaI
R68.45
pUU2
Transformants
λHind III
λ
R68.45
pUU2
Transformants

Therefore though I2 may be translocated within the plasmid during matings it tended not to be lost even when it was not selected for. The crucial difference between pUU1 in PPW1 and pUU2 and PPW2 was that as PPW2 was maintained on 2MCPA, there could not exist a sub-population of pUU2 which had lost dehI they would not grow; while there could exist a sub-population of pUU1 which had lost I1 (Section 4.3). However there could exist a sub-population of pUU2 in which I2, bearing dehI, had been translocated within the plasmid. If it is assumed that the position of I2 in pUU2 interferes with the normal function of the tra3 gene, then the translocation of I2 elsewhere in the plasmid would render such plasmids tra-positive. Similarly the loss of I1 from pUU1 would render it tra-positive. pUU2 existed predominantly in its untranslocated form, as this was the form detected by restriction analysis. Certainly the translocation of I2 was required solely for conjugal transfer into P. aeruginosa PAO and not for replication in this new host, as transformed pUU2 was always recovered in its native form.

If the location of I2 in pUU2 rendered that plasmid transfer deficient the question arises as to how the R-prime was formed in the first place. R-prime formation required transfer of the plasmid. This leads to two possibilities: a.) due in some way to the mechanism of R-prime formation the inactivation of the transfer function by the insert was not important; or, b.) the insert into the plasmid though it reduced the activity of tra3 did not totally inactivate the gene. Though little is known about the mechanism of R-prime formation the former possibility seems highly improbable. The latter possibility therefore seems the most likely. However if that were the case non-translocated plasmids, i.e. original pUU2 plasmids, ought to have been detected in some matings of PPW2 with PAU2. They were not. Therefore, either the clones examined were unrepresentative of such matings, possibility b.), above, is incorrect, or the frequency of transfer of the plasmid due to the reduced expression of tra3 in pUU2 was lower than the

frequency of transfer due to the transfer-positive insert translocated sub-population of R-primes within PPW2. The last suggestion is probably the most likely.

It is interesting that the features of the insert which were conserved in these two derivative plasmids of pUU2 were the double bands, suggesting, perhaps, that there was some duplicated feature of the insert which was important to its function.

The results with *E. coli* as recipient suggest that the species of the host may have an effect on either the stability of an insert of foreign DNA within a plasmid or on the ability of such an R-prime plasmid to conjugally transfer into that host.

The reversion of pUU2 to the R68 form observed in the mating of pUU2 with *E. coli* strains was possibly due to the subculturing entailed in purifying the transconjugants. This was suggested by the instability of transformed pUU2 in *E. coli* on repeated subculturing. However the I2 insert must be more unstable in *E. coli* than in *P. aeruginosa* PAO, as 95% of the colonies subjected to similar purification in the absence of selection for growth on 2MCPA retained the *dehI* insert as evidenced by subsequent growth on 2MCPA minimal medium.

CHAPTER 6

THE GROWTH OF *P. PUTIDA* PPW2 IN A CHEMOSTAT ON 2MCPA UNDER PHOSPHATE LIMITATION

Chapter 6

The Growth of *P. putida* PPW2 in a Chemostat on 2MCPA under Phosphate Limitation

6.1 Introduction

Inorganic phosphate is an essential constituent of DNA. Therefore in a chemostat culture grown under phosphate limitation there ought to exist a selective advantage to a mutant which could economise on its use of phosphate; for example by losing non-essential portions of DNA. It has been shown that bacteria harbouring non-essential plasmids were outcompeted by plasmid minus strains in phosphate limited chemostats (Melling *et al.*, 1977; Godwin and Slater, 1979; Dale and Smith, 1979; Klemperer *et al.*, 1979; Adams *et al.*, 1979; Wouters and Van Andell, 1979; Wouters *et al.*, 1980; Jones and Primrose, 1979; and Jones *et al.*, 1980). In most cases members of the bacterial population lost the plasmid in one step, but Godwin and Slater (1979) observed the loss of individual plasmid markers but the retention of the plasmid; in other words, plasmid fragmentation. Dwiveda *et al.* (1982) observed the loss of a cloned insert from pSC101 and the retention of the plasmid under non-selective conditions.

pUU2 possessed, in addition to the antibiotic resistance markers, I2 bearing *dehI* conferring the ability to grow on 2MCPA as sole carbon and energy source. Therefore in the growth of PPW2 containing pUU2 under phosphate limitation on 2MCPA, though there would be no selection for the retention of the antibiotic resistance markers there would be active selection for the retention of a functional *dehI*. In which case though fragmentation and modifications of the plasmid may occur, the plasmid as a whole cannot be lost without the prior translocation of *dehI* to the bacterial chromosome.

6.2 Conditions of chemostat culture and sampling routine

P. putida PPW2 was grown in a chemostat on the medium of Hershey (1955) containing phosphate as KH_2PO_4 at a concentration of $0.005gl^{-1}$, tryptophan at $50\mu ml^{-1}$ and 2MCPA at $0.5gCl^{-1}$. The dilution rate was $0.1h^{-1}$. The experiment lasted for 2600 hours. Once or twice a week the chemostat was sampled and the absorbance at 600nm taken. Appropriate serial dilutions were made and the culture plated out on the following media:

2MCPA + trp

2MCPA + trp + Ap ($50\mu gml^{-1}$)

2MCPA + trp + Km ($50\mu gml^{-1}$)

2MCPA + trp + Tc ($150\mu gml^{-1}$)

2MCPA + trp + Ap ($50\mu gml^{-1}$) + Km ($50\mu gml^{-1}$) + Tc ($150\mu gml^{-1}$)

The plasmids from about six of the colonies isolated from the 2MCPA + trp plates were purified by the rapid method. Digests of these plasmids with *PstI* were compared with digests of pUU2. In this way strains with plasmids yielding novel digest patterns could be identified.

6.3 Changes in Antibiotic Resistance of the Chemostat Populations

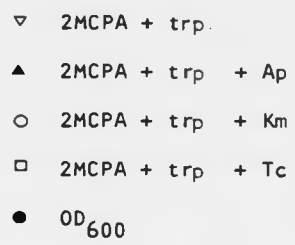
The changes in absorbance of the culture at 600nm and the number of colony forming units (c.f.u.) on the different antibiotic containing media were plotted (Figure 6.1).

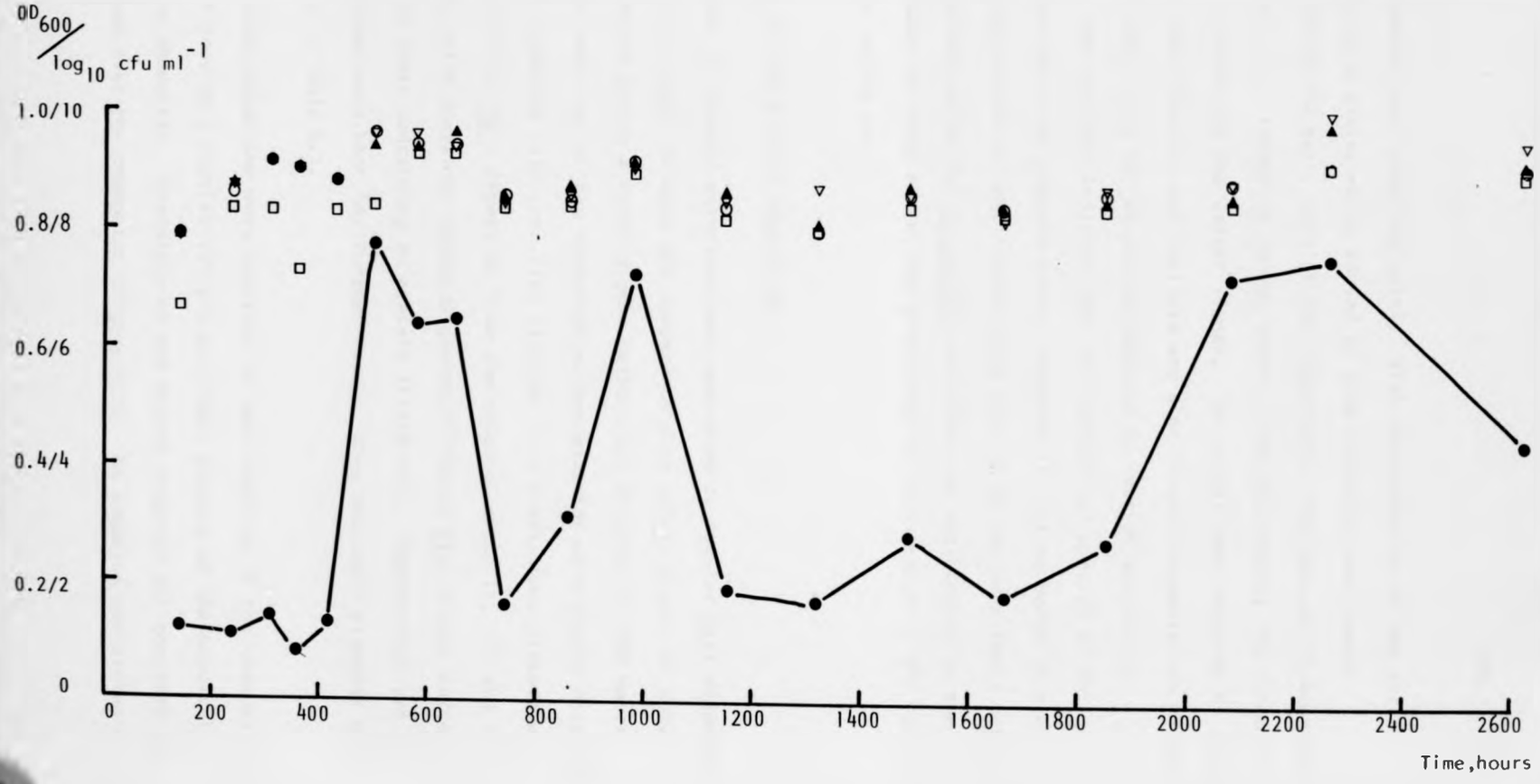
The bacterial population as measured by the absorbance showed considerable fluctuations throughout the 2600 hours of the experiment. These fluctuations are not uncommon especially in long term chemostat experiments. They can often be traced to the age of the medium being fed into the culture vessel and the phenomenon of grow-back in the inlet pipe. In the latter case the offending pipe often has to be replaced with a fresh sterile one.

The number of c.f.u. on the different antibiotic containing media were

FIGURE 6.1

Changes in absorbance and number of c.f.u. on different media during chemostat experiment




OD600
log10 cfu ml-1
1.0/10
0.8/8
0.6/6
0.4/4
0.2/2
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
Time, hours

nearly the same at each sampling point. With the exception of the tetracycline containing plates which tended to give slightly lower counts especially during the early part of the experiment. The general fluctuations in numbers of c.f.u. tended to follow those of the absorbance; the former is a logarithmic scale and the latter linear. The significant features of these results were that they did not indicate any significant fragmentation of the plasmid, at least in so far as can be measured by loss of antibiotic resistances, nor did they indicate that any substantial portion of the chemostat population was plasmid-minus. However it must be borne in mind that the concentrations of antibiotics used were in no way near their minimum inhibitory concentration for P. putida, and often the antibiotics in the agar plates were not very fresh, the plates having been stored at 4°C for up to several weeks before use.

6.4 Changes in the plasmid population

Variations in plasmid structure were monitored by regular PstI digestion of plasmids individual isolates and comparison with a PstI digest of pUU2. The first plasmid giving a novel digest pattern was observed at 430 hours. On subsequent samplings of the chemostat mutant plasmids were always observed. The isolated organisms fell into five classes: I, a class whose plasmid was indistinguishable by PstI digestion from the original pUU2; II, III and IV, three classes which contained plasmids giving different PstI digest patterns, and V, a fifth class containing no plasmid (Plate 6.1). Representatives of each mutant class were kept for further study. They and their plasmids were designated as in Table 6.1.

Insufficient organisms were analysed on each sampling of the chemostat to be able to provide a statistically significant picture of the population changes in the chemostat. Certainly no one mutant organism out competed the others and took over the chemostat (Figure 6.2). An organism containing a

PLATE 6.1

Example PstI digest from routine monitoring of the chemostat, showing examples of all 5 classes of organisms

λ HindIII
pUU2
V
I
III
I
I
I
II
I
IV
λ HindIII

λ HindIII
pUU2
V
I
III
I
I
I
II
I
IV
λ HindIII

TABLE 6.1

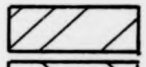
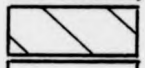
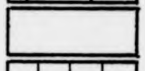
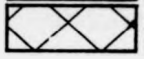
Classes of isolates from phosphate limited chemostat

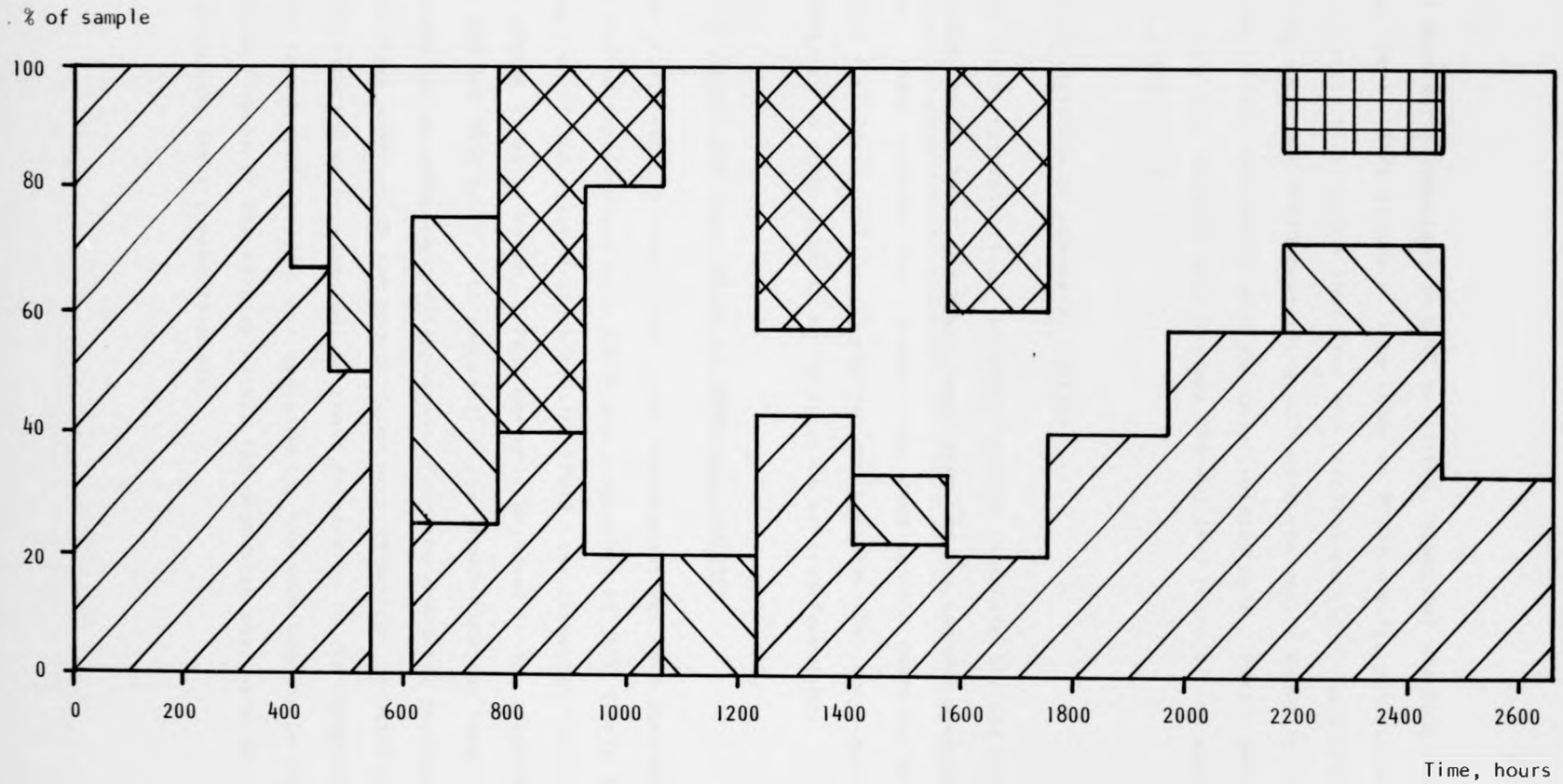
Class	Strain	Plasmid	Number of isolates[1]
I	PPW2*	pUU2*	27
II	PPW9	pUU3	7
III	PPW10	pUU4	33
IV	PPW11	pUU5	1
V	PPW12	-	9

1. Number of isolates. This figure refers to the number of times representatives of that class were isolated from the detection of the first mutant plasmid, i.e. from 430 hours.

FIGURE 6.2

Frequency of isolation of mutant classes during chemostat experiment

	Representative Strain	Plasmid
 Class I	PPW2*	pUU2*
 Class II	PPW9	pUU3
 Class III	PPW10	pUU4
 Class IV	PPW11	pUU5
 Class V	PPW12	-


% of sample
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
Time, hours

plasmid apparently identical to pUU2 persisted throughout the 2600 hours of the experiment. This strain will be known as PPW2* and its plasmid as pUU2*, so as to distinguish them from the original strain and plasmid. PPW2* containing pUU2* and PPW10 containing pUU4 were the most frequently isolated organisms. PPW11 containing pUU5 was only isolated once. PPW12, the plasmid-minus strain, was isolated only between 850 and 1650 hours of the experiment (Table 6.1 and Figure 6.2).

6.5 Characteristics of chemostat isolates

The class representatives isolated from the phosphate limited chemostat, PPW2*, PPW9, PPW10, PPW11 and PPW12, were examined for various phenotypic features. These included their growth rate, dehalogenase characteristics, antibiotic resistance profile and whether any plasmids they contained could be transferred to other species by conjugation or transformation.

6.5.1 Growth characteristics of chemostat isolates

The growth rates of the mutant class representatives were determined on minimal medium supplemented with 2MCPA and tryptophan at 30°C (Table 6.2). With the exception of the plasmid-minus strain, PPW12, the chemostat isolates had similar growth rates in batch. PPW12 was only detected in the chemostat between 800 and 1650 hours, its comparative low growth rate may have contributed to its subsequent disappearance from the chemostat population. The conditions under which the growth rates were determined in batch cultures cannot be expected to be identical to those pertaining in the chemostat. Therefore though these growth rate measurements had some bearing on the relative competitive fitness of the strains within the chemostat they were but one factor affecting their competitiveness.

TABLE 6.2

Specific growth rates of chemostat isolates on 2MCPA

Strain	μ
PPW2	0.36
PPW2*	0.40
PPW9	0.43
PPW10	0.43
PPW11	0.42
PPW12	0.29

6.5.2 Dehalogenase activity of chemostat isolates

Crude cell free extracts were prepared from batch cultures of the chemostat isolates grown on minimal medium supplemented with 2MCPA. These extracts were assayed for their dehalogenase activity towards a range of chlorinated alkanoic acids (Table 6.3). The substrate activity profiles of the chemostat isolates, PPW9, PPW10, PPW11 and PPW12, closely resembled that of the original organism PPW2 which contained only dehalogenase I. This evidence suggested that the chemostat isolates, including the plasmid-minus strain PPW12, still possessed dehalogenase I and had not acquired any new dehalogenase.

The crude cell-free extracts of the isolates were run on a non-denaturing polyacrylamide gel and stained to reveal dehalogenases (Plate 6.2). The gel revealed the presence of only one dehalogenase in the chemostat isolates, possessing the same mobility as dehalogenase I from *P. putida* PP3. This result confirmed the presence of only dehalogenase I in the chemostat isolates.

The chemostat isolates were tested for the constitutivity of dehalogenase I. Crude cell-free extracts from cultures grown on minimal medium supplemented with succinate were assayed for their dehalogenase activity. No significant dehalogenase activity was detected in any of the strains with any of the substrates. This indicated that in none of the chemostat isolates was dehalogenase I constitutive.

6.5.3 Antibiotic resistance profiles of chemostat isolates

The chemostat isolates were tested for their antibiotic resistances. In the case of the plasmid borne resistances concentrations of antibiotics were used which were above the minimum inhibitory concentration for *P. putida* PaW340 but below that for *P. putida* PPW2. The concentrations used were

TABLE 6.3

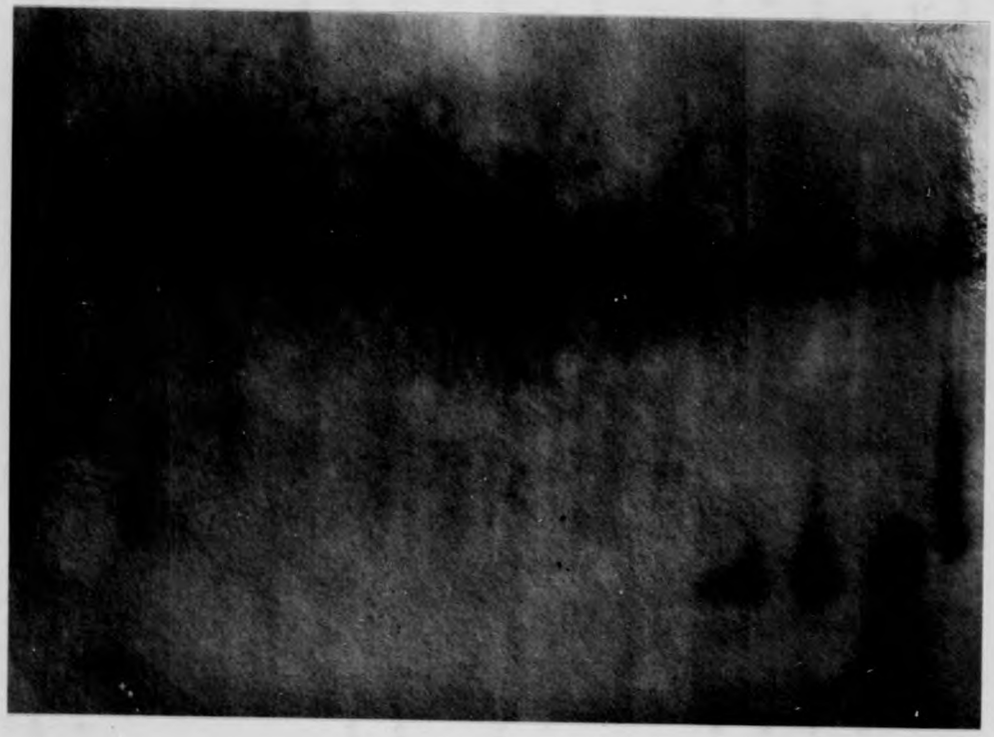
Dehalogenase specific activities of chemostat isolates

Strain	Dehalogenase specific activities {μmol. substrate min^{-1} (mg protein)$^{-1}$}[1]			
	MCA	DCA	2MCPA	22DCPA
P. putida PP3	0.45	0.63	0.15	0.08
	(1.00)	(1.40)	(0.33)	(0.18)
P. putida PPW2	0.66	0.08	0.28	0.14
	(1.00)	(0.12)	(0.42)	(0.21)
P. putida PPW9	0.38	0.07	0.14	0.09
	(1.00)	(0.18)	(0.37)	(0.25)
P. putida PPW10	0.52	0.09	0.27	0.16
	(1.00)	(0.18)	(0.52)	(0.31)
P. putida PPW11	0.55	0.11	0.37	0.17
	(1.00)	(0.20)	(0.67)	(0.31)
P. putida PPW12	0.38	0.10	0.20	0.11
	(1.00)	(0.27)	(0.53)	(0.30)

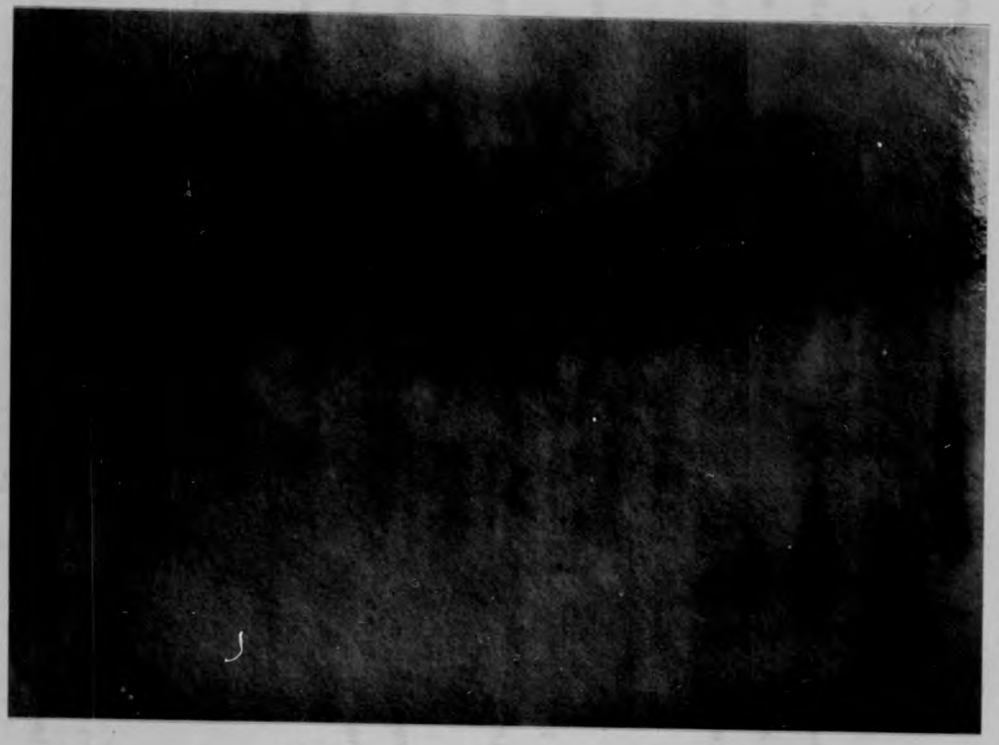
1. Numbers in parenthesis are dehalogenase substrate activity ratios with respect to MCA.

PLATE 6.2

Discontinuous PAGE of crude cell-free extracts of PP3, PPW1, PPW2, PPW9, PPW10, PPW11 and PPW12


+
PPW12
PPW11
PPW10
PPW9
PPW2
PPW1
PP3
Dehalogenase I
Dehalogenase II
-

+ PPW12 PPW11 PPW10 PPW9 PPW2 PPW1 PP3



Dehalogenase I

Dehalogenase II

-

Ap (200μgml^{-1}), Km (200μgml^{-1}), Tc (250μgml^{-1}) and Sm (1000μgml^{-1}) in minimal medium agar plates. In addition the requirement for tryptophan was tested. All the isolates from the chemostat experiment as well as the class representatives were tested. The antibiotic resistance profile of the class representatives were typical of the class as a whole (Table 6.4).

The chemostat isolates were all streptomycin resistant and tryptophan requiring confirming that they were derived from P. putida PaW340 via P. putida PPW2 and were not contaminants. PPW2* and PPW9 showed the same resistance pattern as PPW2. PPW10 and PPW11 were both tetracycline sensitive but remained resistant to the other antibiotics. PPW12, the strain from which no plasmid could be isolated, had lost its resistance to those antibiotics to which R68 gives resistance. This evidence confirmed the plasmid-minus status of PPW12.

Routine monitoring of the chemostat population had not detected any significant change in antibiotic resistance; except possibly, for a slight decrease in tetracycline resistance (Section 6.3). This decrease was probably due to the tetracycline sensitive strains identified above. However Pseudomonads are notoriously resistant to antibiotics and only fresh plates containing high concentrations of antibiotics are likely to positively identify antibiotic sensitive strains.

6.5.4 Transfer ability of the plasmids from chemostat isolates

The chemostat isolates PPW9, PPW10, PPW11 and PPW12 were mated with P. aeruginosa PAU2 (Table 6.5). PPW2 was mated in parallel as a control. Selection for transconjugants was on minimal media supplemented with either 2MCPA and leucine; or succinate, leucine and Km 100μgml^{-1}. Transconjugants were obtained from the mating of PPW2 with PAU2, however no transconjugants were obtained from the matings involving the chemostat isolates. These matings

TABLE 6.4

Antibiotic resistance profiles and tryptophan requirements of chemostat isolates[1]

Class	Strain	Ap	Km	Tc	Sm	Tryptophan requirement
	PaW340	-	-	-	+	+
	PPW2	+	+	+	+	+
I	PPW2*	+	+	+	+	+
II	PPW9	+	+	+	+	+
III	PPW10	+	+	-	+	+
IV	PPW11	+	+	-	+	+
V	PPW12	-	-	-	+	+

1. + indicates antibiotic resistance or requirement for tryptophan
   - indicates antibiotic sensitivity

TABLE 6.5

Bacterial strains and plasmids used or derived in Chapter 6

Strain	Species[1]	Plasmid	Genotype/Phenotype	Derivation/Source
PPW2	P.p.	pUU2	$2Mcpa^{+1}, Trp^{-}, Sm^{R}, Ap^{R}, Km^{R}, Tc^{R}$	PPW1 x PaW340
PPW2*	P.p.	pUU2*	$2Mcpa^{+}, Trp^{-}, Sm^{R}; Ap^{R}, Km^{R}, Tc^{R}$	PPW2 in chemostat
PPW9	P.p.	pUU3	$2Mcpa^{+}, Trp^{-}, Sm^{R}, Ap^{R}, Km^{R}, Tc^{R}$	PPW2 in chemostat
PPW10	P.p.	pUU4	$2Mcpa^{+}, Trp^{-}, Sm^{R}, Ap^{R}, Km^{R}$	PPW2 in chemostat
PPW11	P.p.	pUU5	$2Mcpa^{+}, Trp^{-}, Sm^{R}, Ap^{R}, Km^{R}$	PPW2 in chemostat
PPW12	P.p.	-	$2Mcpa^{+}, Trp^{-}, Sm^{R}$	PPW2 in chemostat
PAU2	P.a.	-	Leu-38, R^{-}, M^{+}, Rif^{R}	Mutant of PA01162
PA01162	P.a.	-	Leu-38, R^{-}, M^{+}	K.N. Timmis

1. Species: P.p. Pseudomonas putida

P.a. Pseudomonas aeruginosa

were repeated on three more occasions with similar lack of success. These results suggest that the plasmids in PPW9, PPW10 and PPW11 were transfer deficient.

6.5.5 Transformation of plasmids from chemostat isolates

Purified plasmid DNA from PPW9 (pUU3), PPW10 (pUU4) and PPW11 (pUU5) was transformed into P. aeruginosa PA01162. pUU2 from PPW2 was transformed as a control. Selection for transformants was on minimal medium supplemented with 2MCPA and leucine, or nutrient agar containing Km ($100\mu gml^{-1}$). Transformants were obtained on the control plates treated with pUU2 DNA but not on those treated with pUU3, pUU4 or pUU5. These transformations were repeated three times but without success. The transformation of large plasmids is fraught with difficulties. On less than half the experiments did pUU2 transform successfully. It was difficult to pinpoint the factors which influence the success of these transformations. Therefore these results cannot be taken to imply that genes for Kanamycin resistance and utilization of 2MCPA were no longer carried on pUU3, pUU4 and pUU5. Unfortunately these results did not provide evidence for the converse. They did not prove that pUU3, pUU4 and pUU5 were still R-primes bearing dehI. Also, as the plasmids were transfer deficient (see Section 6.5.4) no evidence could be gained from that quarter as to the R-prime status of the plasmids.

6.6 Restriction analysis of the plasmids pUU3, pUU4 and pUU5

Plasmid DNA was purified from the chemostat isolates by the crude lysate caesium chloride method. Digests of these plasmids with the restriction endonucleases PstI, SmaI, KpnI, XhoI, HindIII and EcoRI were compared with those from R68.45 and pUU2 so as to determine the physical relationships between those plasmids. The molecular structure of the plasmids was, where possible, related to the phenotype of the strains from which they were obtained.

6.6.1 Comparison of restriction digests of R68.45, pUU2 and pUU3

Restriction digests of R68.45, pUU2 and pUU3 were run on agarose gels of different percentage agarose (0.5 - 1.4%) at various voltages (1 - 3Vcm^{-1}) and for different lengths of time (3 - 60h). These different conditions were in order to examine different aspects of the digest patterns.

6.6.1.1 Digestion of R68.45, pUU2, and pUU3 with PstI

R68.45, pUU2 and pUU3 were digested with the restriction endonuclease PstI and the restriction fragments sized (Plate 6.3; Figure 6.3; Table 6.6). All the bands from R68.45 present in pUU2 were retained in pUU3 with the exception of 2PA which was missing. All the bands in pUU2 associated with the dehI bearing insert, I2, were present in pUU3. pUU3 had acquired 9 new bands not present in either R68.45 or pUU2. The PstI digests suggested that pUU3 has increased in size over pUU2 by about 10kb to 92.93kb.

These data suggest that pUU3 differed from pUU2 by some modification in the 2PA region, this is between 8 o'clock and 1 o'clock in R68(Figure 6.4). This modification involved the acquisition of about 10kb of DNA bearing eight PstI sites. As they were larger than 10kb, fragments 3XP1 (19.45kb) and 3XP3 (12.60kb) probably contained the portions flanking this acquired piece of DNA. The rest of the plasmid appeared to be unaltered. The part of the plasmid which was modified contained the traI genes, which could explain why this plasmid was Tra$^-$.

6.6.1.2 Digestion of R68.45, pUU2 and pUU3 with SmaI

R68.45, pUU2 and pUU3 were digested with the restriction endonuclease SmaI and the restriction fragments sized (Plate 6.4; Figure 6.5; Table 6.7). All the bands from R68.45 present in pUU2 were also present in pUU3, except for 2SA which was absent. All the bands in pUU2 associated with I2 were still

PLATE 6.3

Restriction digests of plasmids R68.45, pUU2, pUU3, pUU4 and pUU5 with PstI

a. 0.5% gel, $1Vcm^{-1}$, 16h

b. 0.5% gel, $1Vcm^{-1}$, 60h

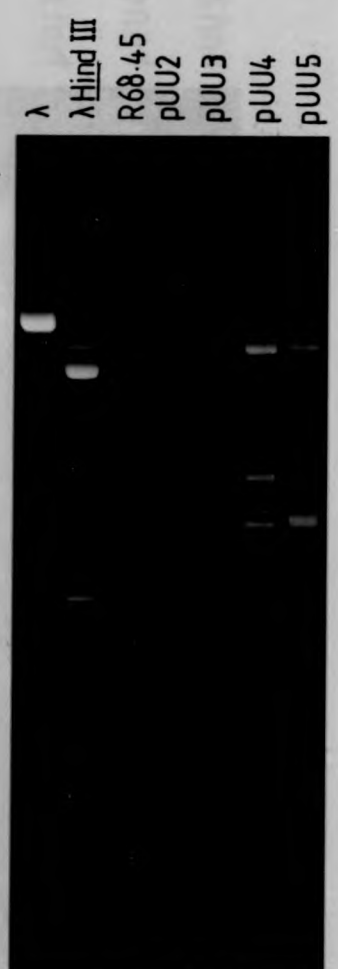
c. 1.4% gel, $3Vcm^{-1}$, 5h

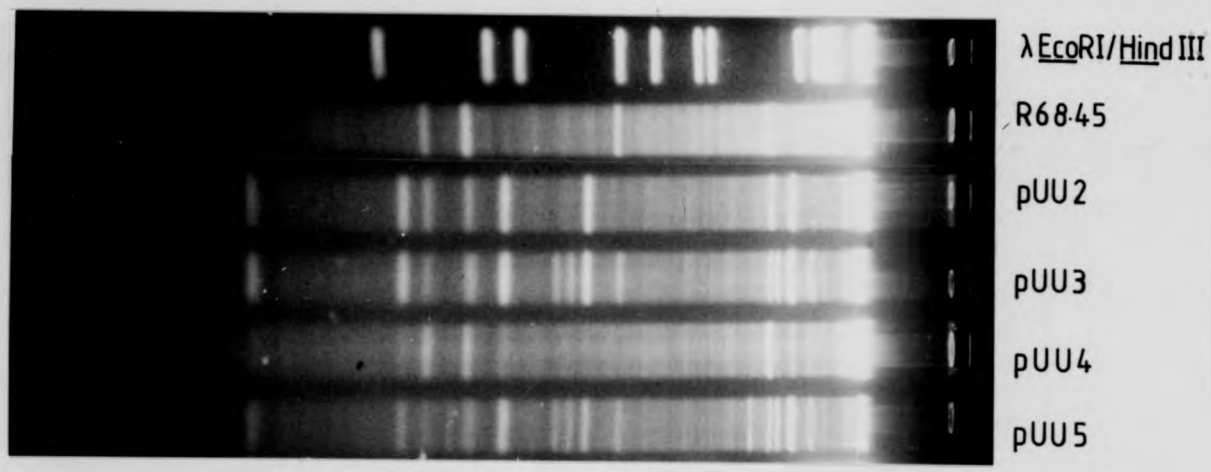
d.


λ
λ EcoRI/Hind III
R68.45
pUU2
pUU3
pUU4
pUU5

b.

b.




λ EcoRI/Hind III
R68·45
pUU2
pUU3
pUU4
pUU5

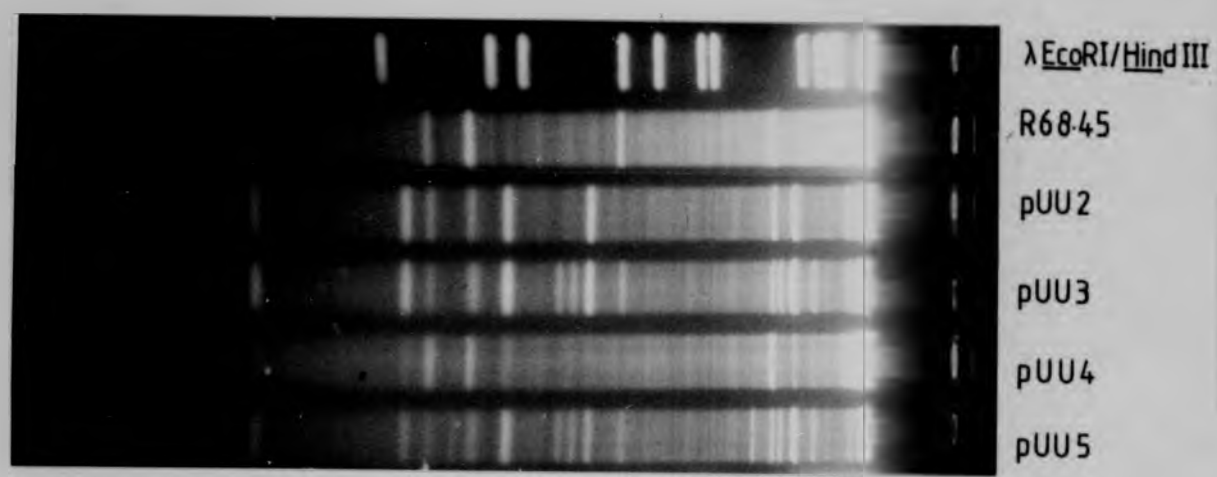

λ EcoRI/Hind III
R68·45
pUU2
pUU3
pUU4
pUU5

FIGURE 6.3

Diagram of PstI digests of R68.45, pUU2 and pUU3

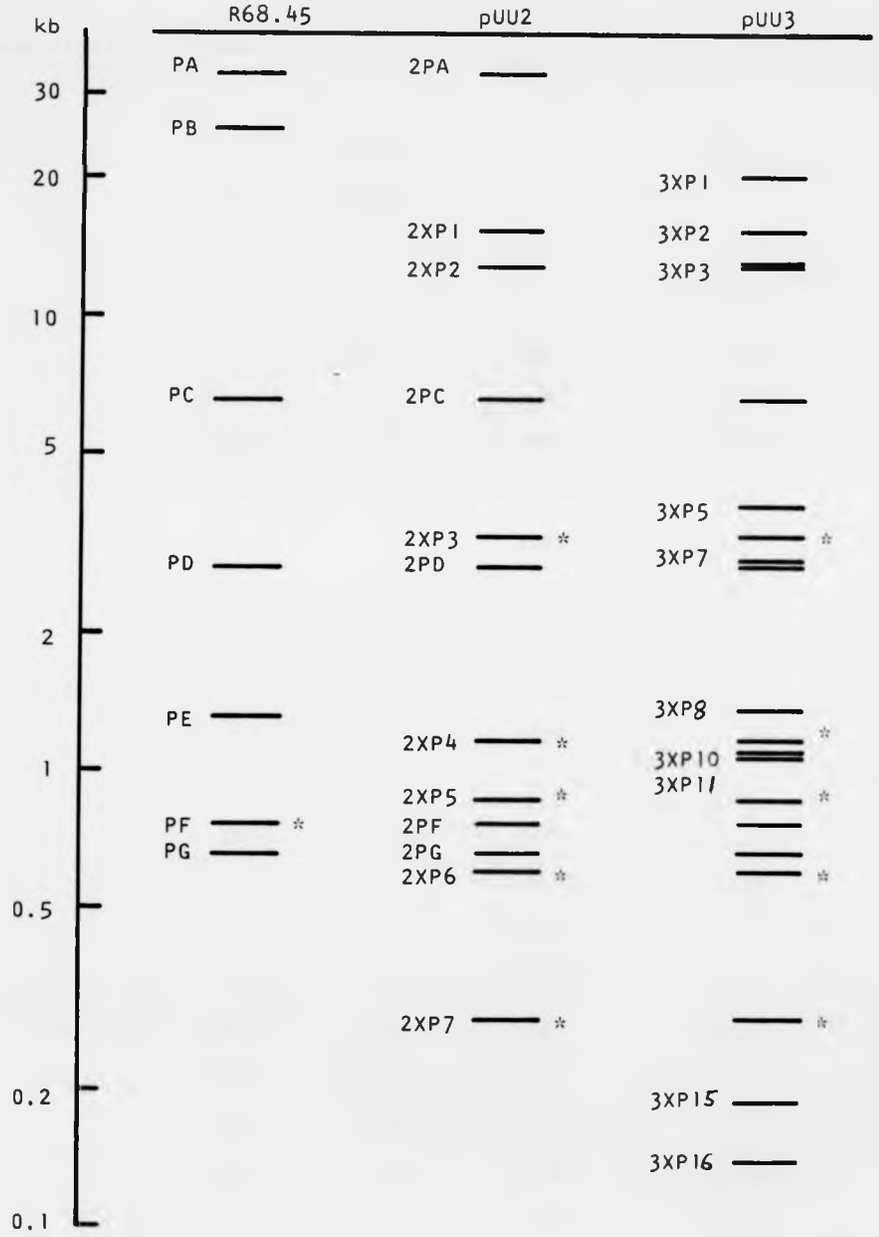

kb
R68.45
pUU2
pUU3
30
20
10
5
2
1
0.5
0.2
0.1
PA
PB
PC
PD
PE
PF
PG
2PA
2XP1
2XP2
2PC
2XP3
2PD
2XP4
2XP5
2PF
2PG
2XP6
2XP7
3XP1
3XP2
3XP3
3XP5
3XP7
3XP8
3XP10
3XP11
3XP15
3XP16

TABLE 6.6

Sizes of restriction fragments of the plasmids R68.45, pUU2 and pUU3 with PstI

	R68.45		pUU2		pUU3	
PA	32.67	2PA	32.67			
PB	24.84					
				3XP1	19.45	
		2XP1	14.82	3XP2	14.82	
				3XP3	12.60	
		2XP2	12.38	3XP4	12.38	
PC	6.52	2PC	6.52	3PC	6.52	
				3XP5	3.81	
		3XP3	3.23 *	3XP6	3.23 *	
				3XP7	2.91	
PD	2.77	2PD	2.77	3PD	2.77	
				3XP8	1.38	
PE	1.34					
		2XP4	1.19 *	3XP9	1.19 *	
				3XP10	1.11	
				3XP11	1.06	
		2XP5	0.87 *	3XP12	0.87 *	
PF	0.77 *	2PF	0.77	3PF	0.77	
PG	0.66	2PG	0.66	3PG	0.66	
		2XP6	0.60 *	3XP13	0.60 *	
		2XP7	0.29 *	3XP14	0.29 *	
				3XP15	0.19	
				3XP16	0.14	
Size	70.34kb		82.95kb		92.43kb	

FIGURE 6.4

Map of R68

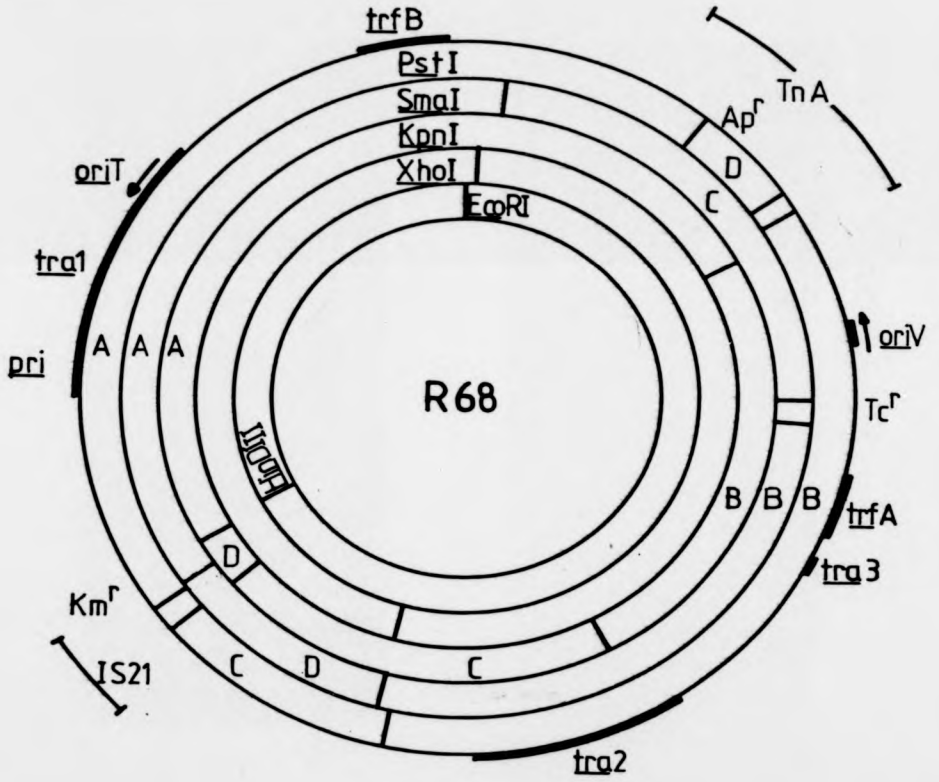

trfB
PstI
SmaI
KpnI
XhoI
EcoRI
HindIII
TnA
Ap^r
D
C
oriV
Tc^r
B B B
trfA
tra3
tra2
C D C
Km^r
IS21
D
A A A
pri
tra1
oriT
R68

PLATE 6.4

Restriction digests of plasmids R68.45, pUU2, pUU3, pUU4 and pUU5 with SmaI

a. 0.5% gel, $1Vcm^{-1}$, 16h

b. 0.5% gel, $1Vcm^{-1}$, 60h

a.

R68.45
pUU2
pUU3
pUU4
pUU5
λEcoRI/HindIII
λ

b.

R68.45
pUU2
pUU3
pUU4
pUU5
λ
λHindIII

a.

R68.45
pUU2
pUU3
pUU4
pUU5
λEcoRI/HindIII
λ

b.

R68.45
pUU2
pUU3
pUU4
pUU5
λ
λHindIII

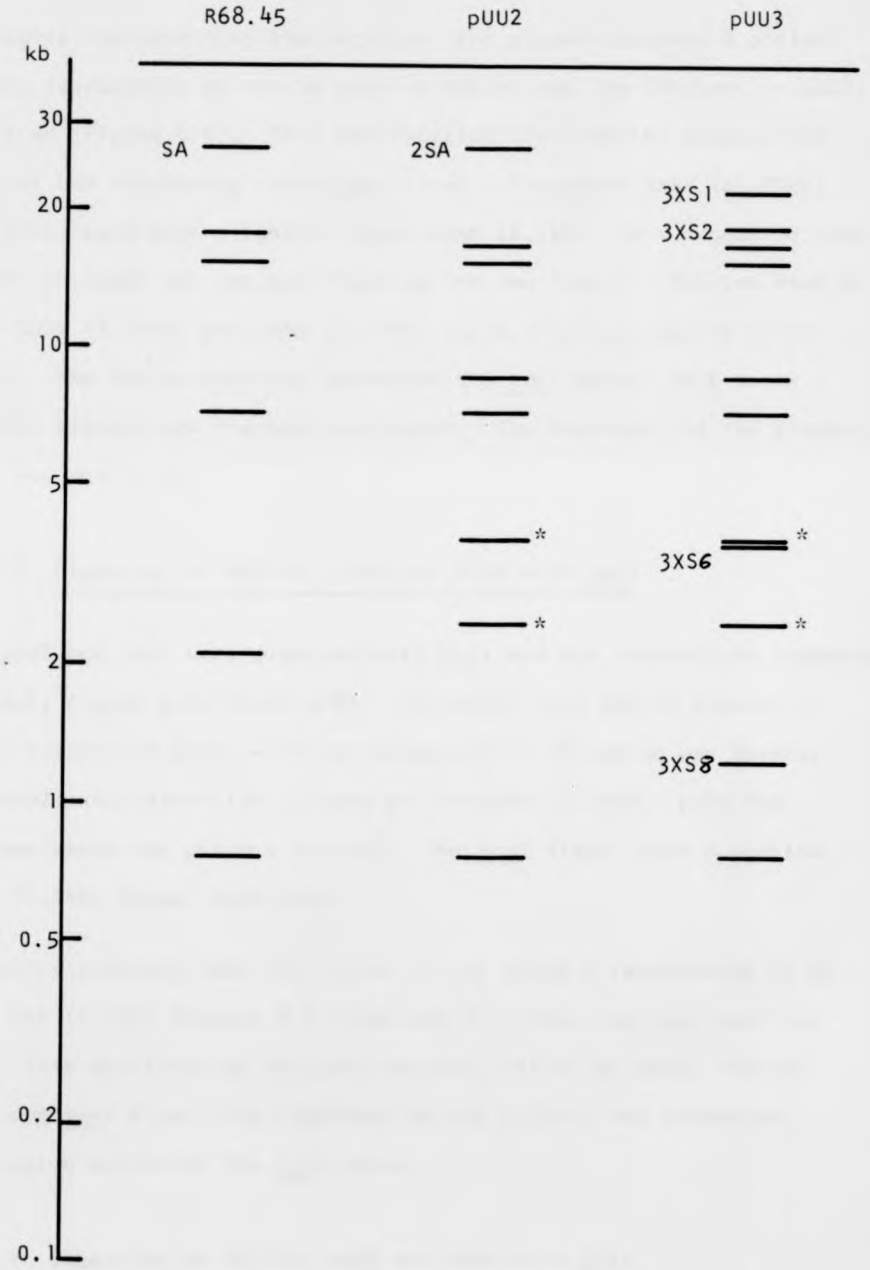
TABLE 6.7

Sizes of the restriction fragments of the plasmids R68.45, pUU2 and pUU3 with SmaI

	R68.45		pUU2		pUU3	
SA	26.52	2SA	26.52			
				3XS1	21.50	
				3XS2	18.21	
SB	17.55					
		2XS1	16.60	3XS3	16.60	
SC	15.10	2SC	15.10	3SC	15.10	
		2XS2	8.45	3XS4	8.45	
SD	7.15	2SD	7.15	3SD	7.15	
		2XS3	3.80	* 3XS5	3.80	*
				3XS6	3.64	
		2XS4	2.51	* 3XS7	2.51	*
SE	2.12					
				3XS8	1.23	
SF	0.76	2SF	0.76	3SF	0.76	
	69.20kb		87.20kb		105.30kb	

FIGURE 6.5

Diagram of *Sma*I digests of R68.45, pUU2 and pUU3


R68.45
pUU2
pUU3
kb
30
20
10
5
2
1
0.5
0.2
0.1
SA
2SA
3XS1
3XS2
3XS6
3XS8
*
*
*
*

present in pUU2. The SmaI digest data suggest that pUU3 had increased in size over pUU2 by 18.1kb.

These results indicate that the region of the plasmid between 8 o'clock and 12 o'clock, represented by the SA band in R68.45 and the 2SA band in pUU2, had been modified (Figure 6.4). This modification involved the acquisition of about 18kb of DNA containing three SmaI sites. Fragments 3XS1 (21.50kb) and 3XS2 (18.21kb) were both slightly larger than 18.1kb. This suggested that these fragments included the regions flanking the new insert. The two smaller new fragments 3XS6 (3.64kb) and 3XS8 (1.23kb) would originate wholly within the new insert. The region modified contained the traI genes; this could explain why this plasmid was transfer deficient. The remainder of the plasmid, including I2, was unaltered.

6.6.1.3 Digestion of R68.45, pUU2 and pUU3 with KpnI

R68.45, pUU2 and pUU3 were digested with KpnI and the restriction fragments sized (Plate 6.5; Figure 6.6; Table 6.8). The bands from R68.45 present in pUU2 were also present in pUU3, with the exception of 2KA which was absent. The bands in pUU2 associated with I2 were all present in pUU3. pUU3 had acquired two new bands not present in pUU2. The KpnI digest data suggested that pUU3 was 21.24kb larger than pUU2.

These results indicate that the region of the plasmid represented by KA in R68.45 and 2KA in pUU2 between 8 o'clock and 2 o'clock had been modified (Figure 6.4). This modification included the acquisition of about 21kb of DNA including one KpnI site. The remainder of the plasmid was unchanged. The modified region contained the traI genes.

6.6.1.4 Digestion of R68.45, pUU2 and pUU3 with XhoI

R68.45, pUU2 and pUU3 were digested with the restriction endonuclease

PLATE 6.5

Restriction digests of plasmids R68.45, pUU2, pUU3, pUU4 and pUU5 with KpnI

a. 0.5% gel, $1Vcm^{-1}$, 16h.

b. 0.5% gel, $1Vcm^{-1}$, 60h.

PLATE 6.5

Restriction digests of plasmids R68.45, pUU2, pUU3, pUU4 and pUU5 with KpnI

a. 0.5% gel, $1Vcm^{-1}$, 16h.

b. 0.5% gel, $1Vcm^{-1}$, 60h.

16h

λ
λHind III
λEcoRI/Hind III
R68.45
pUU 2
pUU 3
pUU 4
pUU 5

60h

λ
λHind III
λEcoRI/Hind III
R68.45
pUU2
pUU3
pUU4
pUU 5

16h
λ
λHind III
λEcoRI/Hind III
R68·45
pUU 2
pUU 3
pUU 4
pUU 5
60h
λ
λHind III
λEcoRI/Hind III
R68·45
pUU2
pUU3
pUU4
pUU5

FIGURE 6.6

Diagram of restriction digests of R68.45, pUU2 and pUU3 with KpnI

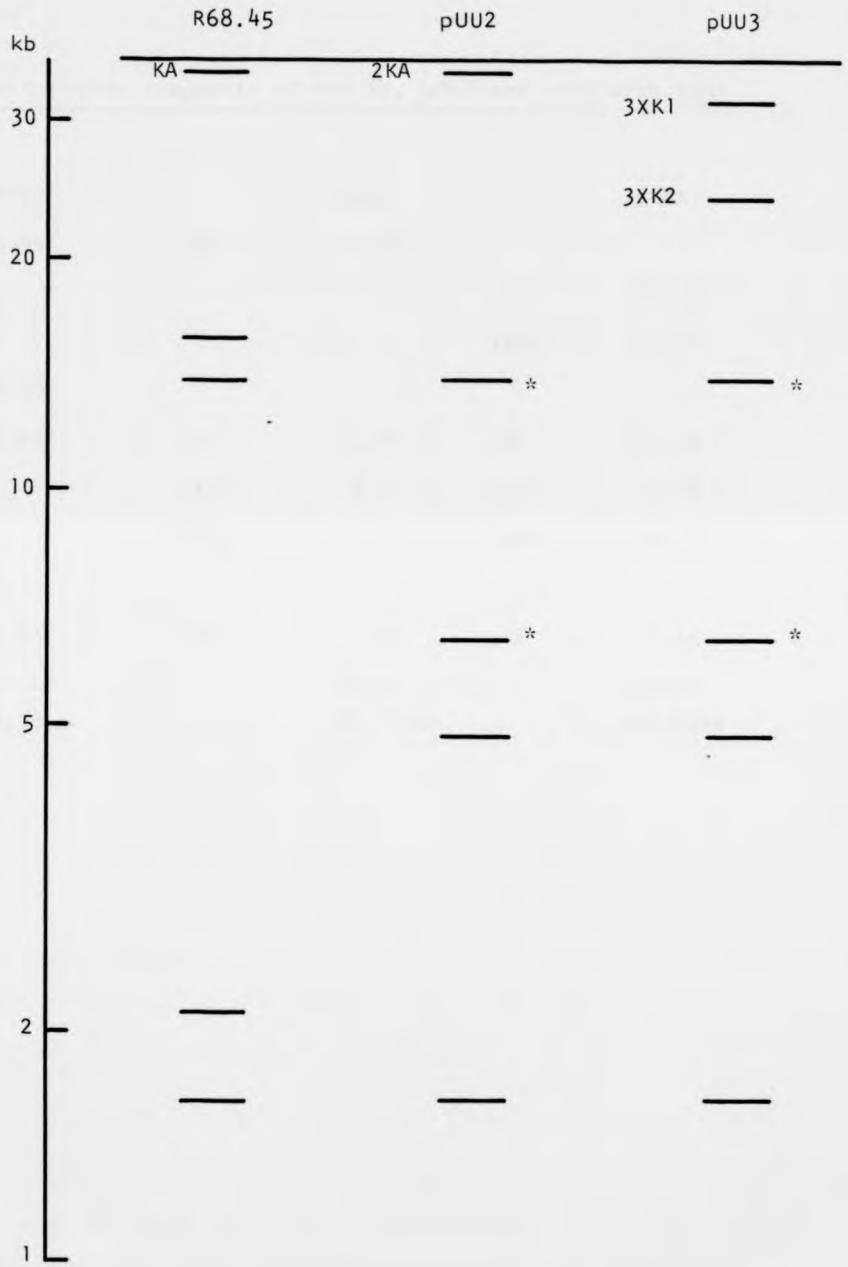

R68.45
pUU2
pUU3
kb
KA
2KA
3XK1
3XK2
30
20
10
5
2
1
*
*
*
*

TABLE 6.8

Sizes of the restriction fragments of R68.45, pUU2 and pUU3 with KpnI

	R68.45		pUU2		pUU3
KA	34.44	2KA	34.44		
				3XK1	31.73
				3XK2	23.95
KB	15.66				
KC	13.96	2KC	13.96 *	3KC	13.96 *
		2XK1	6.49 *	3XK3	6.49 *
		2XK2	4.79	3XK4	4.79
KD	2.11				
KE	1.61	2KE	1.61	3KE	1.61
	67.78kb		81.74kb		102.98kb

XhoI and the restriction fragments sized (Plate 6.6; Figure 6.7; Table 6.9). The new XhoI bands in pUU2 associated with I2 were present in pUU3. However 2XA from pUU2 which was equivalent to one of the XA bands in R68.45 was missing and had been replaced by two new bands 3XX1 (45.48kb) and 3XX5 (2.86kb).

These results indicated that pUU3 had acquired about 14kb of DNA containing one XhoI site somewhere between 6 o'clock and 12 o'clock on the plasmid (Figure 6.4). One of the two new fragments, 3XX5, was small (2.86kb) indicating that the acquired DNA had inserted near one of the ends of this region.

6.6.1.5 Digestion of R68.45, pUU2 and pUU3 with EcoRI and HindIII

R68.45, pUU2 and pUU3 were digested with the restriction endonucleases EcoRI and HindIII separately and together and the restriction fragments sized (Plate 6.7; Figure 6.8; Table 6.10).

The EcoRI digests indicated that pUU3 had acquired three EcoRI sites compared to pUU2 and had increased in size by about 13.55kb.

Three of the HindIII generated bands, including the pair of double bands, which were present in pUU2 were also present in pUU3. The largest HindIII generated band, 2XH1 (44.79kb), from pUU2 was missing and had been replaced by four new bands indicating the acquisition of three HindIII sites. pUU3 was 20.91kb larger than pUU2 according to the HindIII data.

The double digest with EcoRI and HindIII indicated that pUU3 had conserved all the bands from pUU2 which had been associated with the dehI bearing insert I2. However the 23.93kb fragment E/HB from R68.45 which was conserved in pUU2 was absent in pUU3. This band had been replaced by seven smaller bands. pUU3 had increased in size by 18.1kb over pUU2. The E/HB band from R68.45 was derived from the 8 o'clock - 12 o'clock region of the plasmid (Figure 6.4). Therefore these results indicate that the region of

PLATE 6.6

Restriction digests of plasmids R68.45, pUU2, pUU3, pUU4 and pUU5 with XhoI

a. 0.5% gel, $1Vcm^{-1}$, 16h.

b. 0.5% gel, $1Vcm^{-1}$, 60h.

a. 16h.

R68.45
pUU2
pUU3
pUU 4
pUU 5
λEcoRI/HindIII
λ Hind III
λ

b. 60h.

R6845
pUU2
pUU3
pUU 4
pUU5
λEcoRI/HindIII
λ Hind III
λ

a. 16h.

R68.45
pUU2
pUU3
pUU 4
pUU 5
λEcoRI/HindIII
λ Hind III
λ

b. 60h.

R68.45
pUU2
pUU3
pUU 4
pUU5
λEcoRI/HindIII
λ Hind III
λ

FIGURE 6.7

Diagram of the XhoI digests of R68.45, pUU2 and pUU3

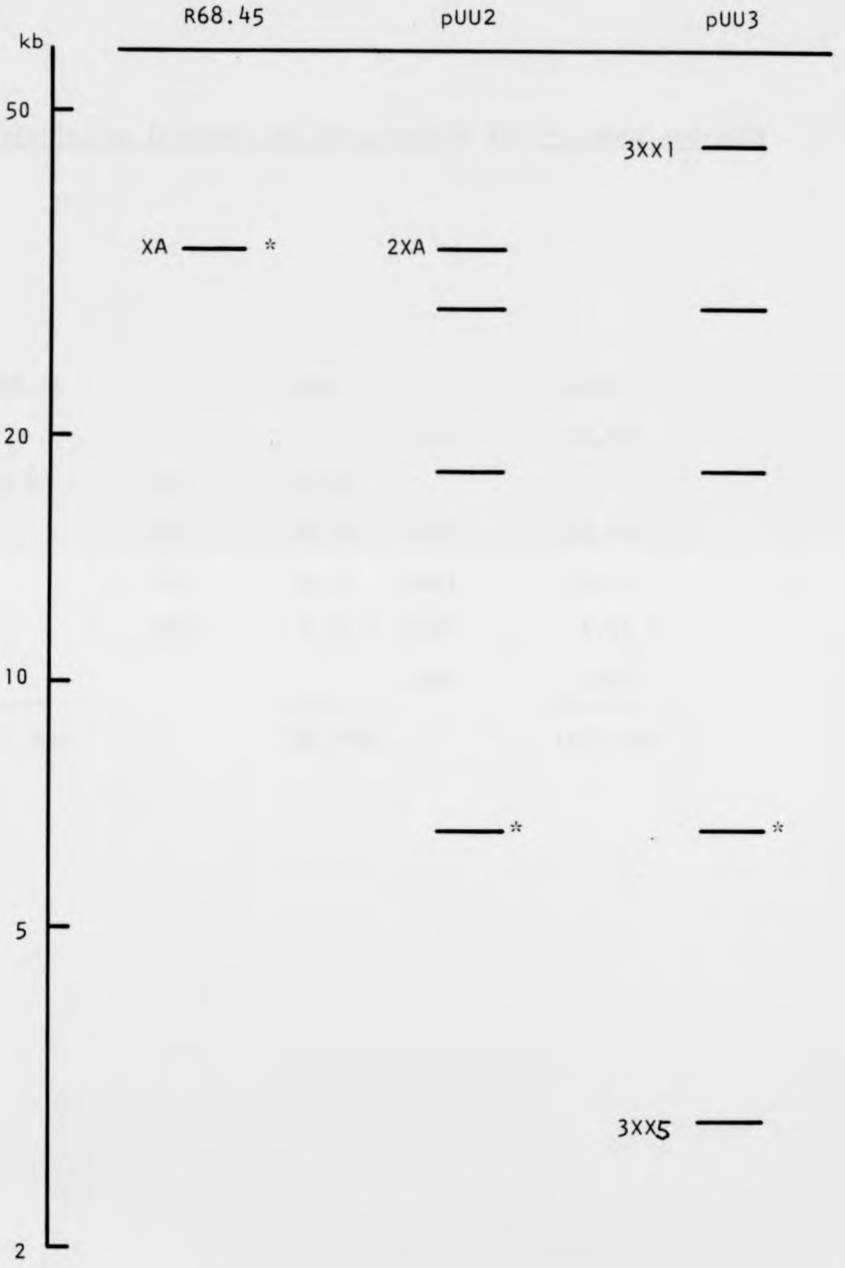

R68.45
pUU2
pUU3
kb
50
20
10
5
2
3XX1
XA
*
2XA
*
*
3XX5

TABLE 6.9

Sizes of the restriction fragments of the plasmids R68.45, pUU2 and pUU3 with XhoI

	R68.45		pUU2		pUU3
				3XX1	45.48
XA	33.98 *	2XA	33.98		
		2XX1	28.46	3XX2	28.46
		2XX2	18.20	3XX3	18.20
		2XX3	6.55 *	3XX4	6.55 *
				3XX5	2.86
	67.96kb		93.74kb		108.10kb

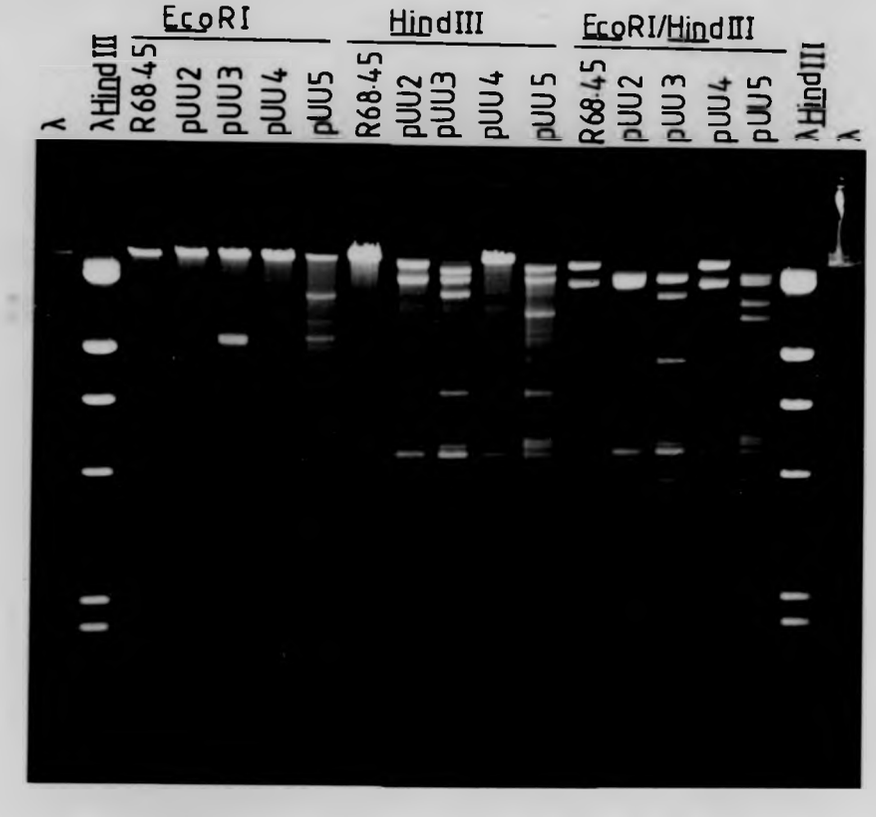
PLATE 6.7

Restriction digests of plasmids R68.45, pUU2, pUU3, pUU4 and pUU5 with EcoRI, HindIII and EcoRI/HindIII

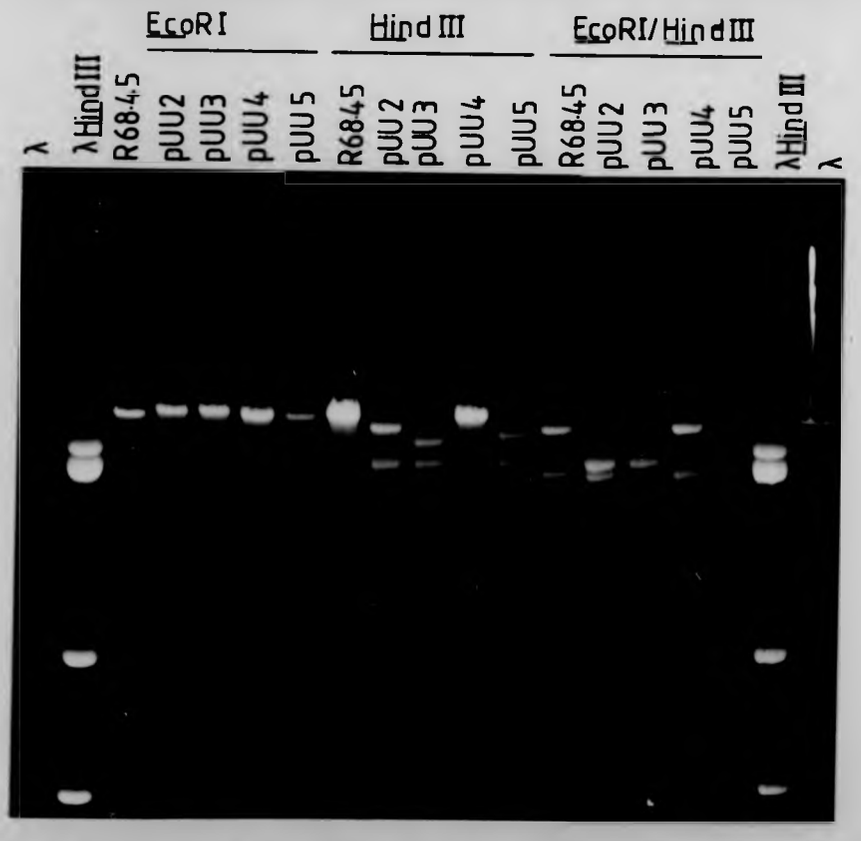
a. 0.5% gel, $1Vcm^{-1}$, 16 h

b. 0.5% gel, $1Vcm^{-1}$, 60 h

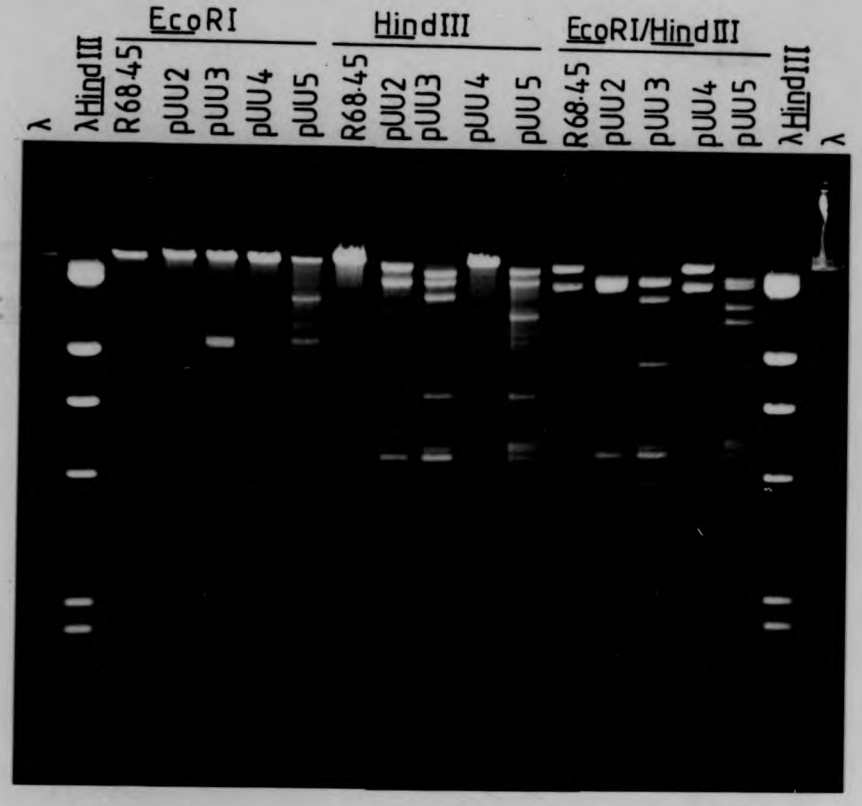
a.16h.


EcoRI
HindIII
EcoRI/HindIII
λ
λHindIII
R68.45
pUU2
pUU3
pUU4
pUU5
R68.45
pUU2
pUU3
pUU4
pUU5
R68.45
pUU2
pUU3
pUU4
pUU5
λHindIII
λ

b.60h.


EcoRI
HindIII
EcoRI/HindIII
λ
λHindIII
R68.45
pUU2
pUU3
pUU4
pUU5
R6845
pUU2
pUU3
pUU4
pUU5
R6845
pUU2
pUU3
pUU4
pUU5
λHindIII
λ

a.16h.


EcoRI
HindIII
EcoRI/HindIII
λ
λHindIII
R68-45
pUU2
pUU3
pUU4
pUU5
R68-45
pUU2
pUU3
pUU4
pUU5
R68-45
pUU2
pUU3
pUU4
pUU5
λHindIII
λ

b.60h.

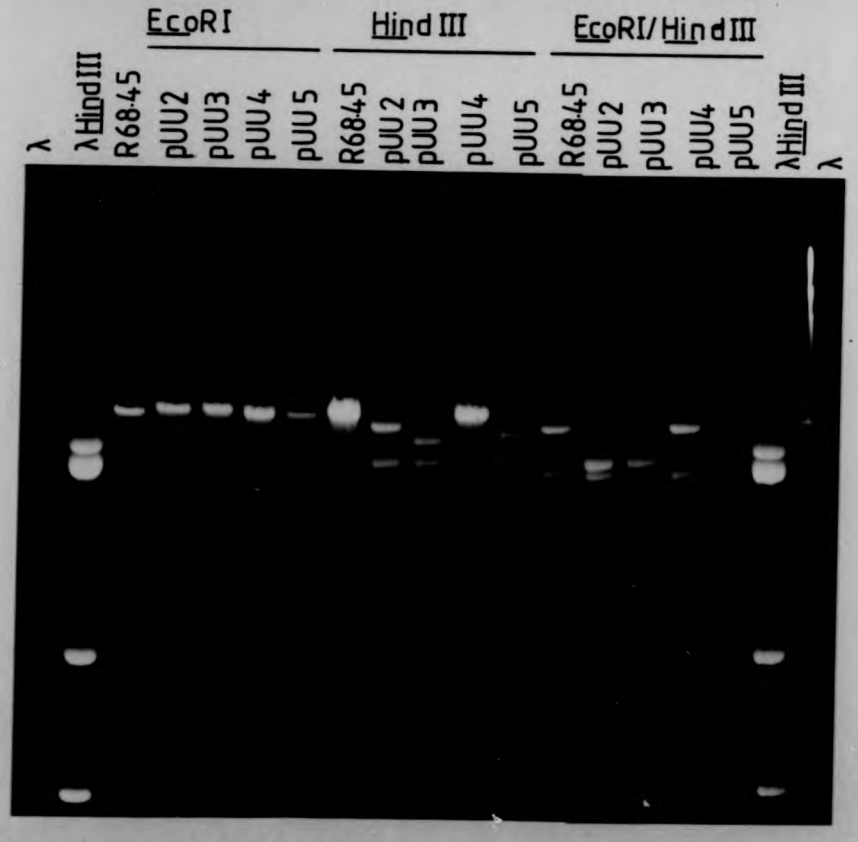

EcoRI
HindIII
EcoRI/HindIII
λ
λHindIII
R68-45
pUU2
pUU3
pUU4
pUU5
R6845
pUU2
pUU3
pUU4
pUU5
R68-45
pUU2
pUU3
pUU4
pUU5
λHindIII
λ

FIGURE 6.8

Diagram of restriction digests of R68.45, pUU2 and pUU3 with EcoRI, HindIII and EcoRI/HindIII

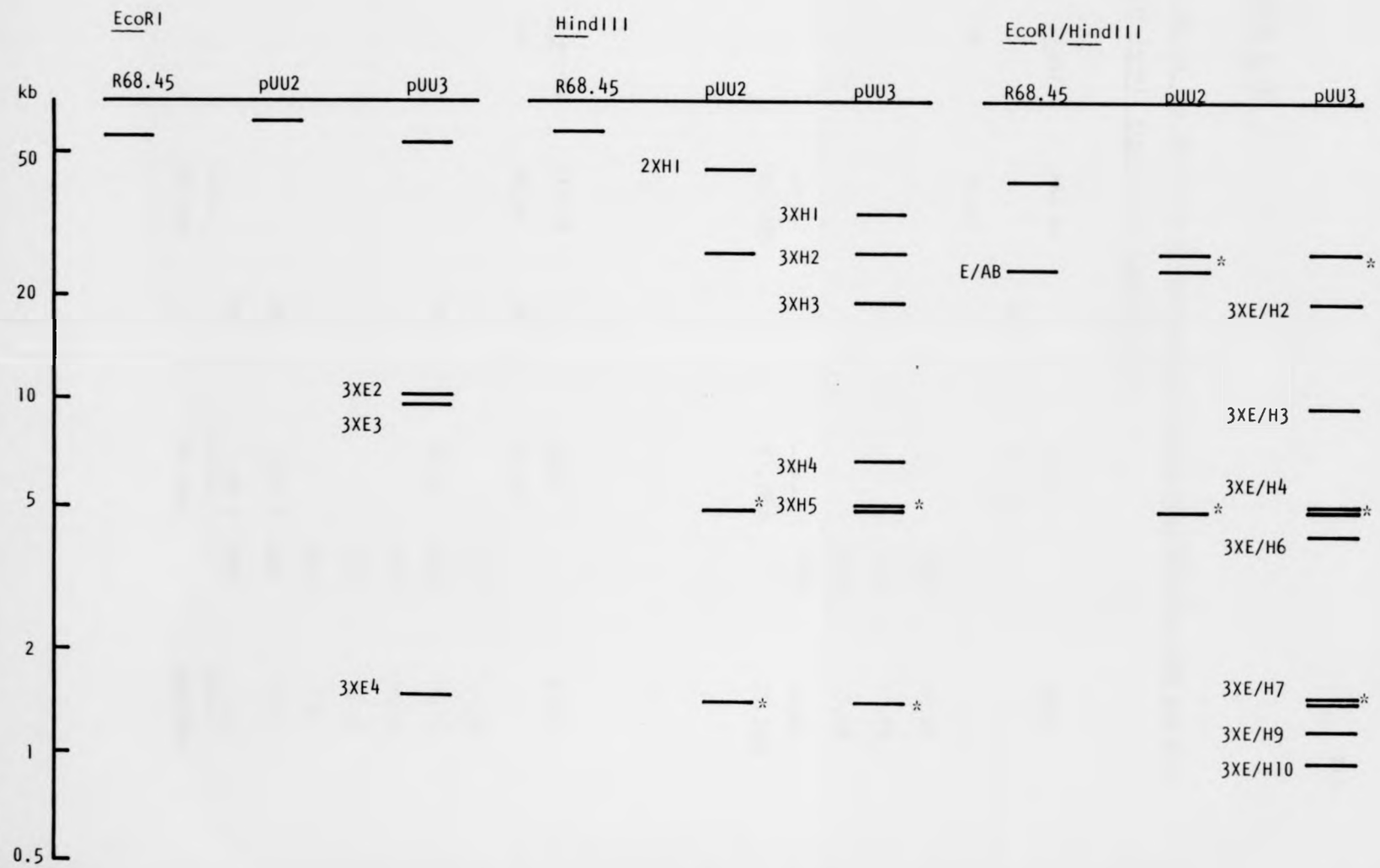

EcoRI
HindIII
EcoRI/HindIII
kb
R68.45
pUU2
pUU3
50
20
10
5
2
1
0.5
2XH1
3XH1
3XH2
3XH3
3XH4
3XH5
3XE2
3XE3
3XE4
E/AB
3XE/H2
3XE/H3
3XE/H4
3XE/H6
3XE/H7
3XE/H9
3XE/H10

TABLE 6.10

Sizes of the restriction fragments of the plasmids R68.45, pUU2 and pUU3 with EcoRI, HindIII and EcoRI/HindIII.

a. EcoRI	R68.45		pUU2		pUU3
		2E1	61.78		
EA	55.61				
				3XE1	53.63
				3XE2	10.49
				3XE3	9.69
				3XE4	1.52
	55.61kb		61.78kb		75.33kb

b. HindIII	R68.45		pUU2		pUU3
HA	58.30	2H1	44.79		
				3XH1	33.98
		2H2	26.70	3XH2	26.70
				3XH3	18.99
				3XH4	6.92
				3XH5	5.21
		2H3	5.04 *	3XHS	5.04 *
		2H4	1.43 *	3XH6	1.43 *
	58.30kb		83.83kb		104.74kb

c. EcoRI/HindIII

	R68.45		pUU2		pUU3
E/HA	42.02				
		2XE/H1	26.70 *	3XE/H1	26.70 *
E/HB	23.93	2E/HB	23.93		
				3XE/H2	18.99
				3XE/H3	9.69
				3XE/H4	5.21
		2XE/H2	5.04 *	3XE/H5	5.04 *
				3XE/H6	4.37
				3XE/H7	1.52
		2XE/H3	1.43 *	3XE/H8	1.43 *
				3XE/H9	1.25
				3XE/H10	0.98
	65.95kb		90.27kb		108.37kb

the plasmid corresponding to E/HB of R68.45 had increased in size by about 18.1kb and had acquired three EcoRI and three HindIII sites.

6.6.1.6 Interpretation of restriction digest data for pUU3

Restriction digests with a range of endonucleases confirmed that the region bearing the dehI insert I2 had not been physically altered in pUU3 compared with pUU2. The remainder of the plasmid was unaltered except for the region opposite I2 which had increased in size and gained a range of restriction sites. The estimation of the size of the increase varied, depending on the digest from which it was calculated, between 9.99kb for PstI to 21.24kb for KpnI. Smaller restriction fragments are more accurately sized than larger ones. Therefore the restriction digest which gave the smallest maximum restriction fragment from this modified region ought to have produced the most accurate sizing of the extra DNA. This digest was the EcoRI/HindIII double digest which gave a size of 18.1kb to the extra DNA.

The bulk of the restriction digests enabled the approximate position of the extra DNA to be located in the plasmid. However the single and double digests with EcoRI or HindIII enabled the location of some of the new restriction sites to be mapped with a reasonable amount of accuracy.

The extra portion of DNA in pUU3 was designated I3. The EcoRI/HindIII double digests allowed the most precise localization of I3 within pUU3. It had inserted into the region corresponding to E/HB of R68.45 (Figure 6.4; Table 6.10c.). E/HB was 23.93kb, I3 was 18.1kb, therefore this region had increased in size to 42.03kb.

The EcoRI single digest of pUU3 gave three fragments associated with I3: 3XE2 (10.49kb), 3XE3 (9.69kb) and 3XE4 (1.52kb) (Table 6.10a, Figure 6.8). One of these fragments must include the region flanking I3, anticlockwise from the original EcoRI site of R68.45 (Figure 6.4). This fragment is probably

one of the larger two as otherwise the size of I3 would be larger than the estimated 18.1kb. The remaining two fragments must be located within I3.

The HindIII single digest gave four fragments associated with I3: 3XHI (33.98kb), 3XH3 (18.99kb), 3XH4 (6.92kb) and 3XH5 (5.21kb) (Table 6.10b; Figure 6.8). 3XHI (33.98kb) presumably corresponded to the part of the plasmid anticlockwise from the most closewise HindIII site of I2, it therefore contained the clockwise extremity of I3. Because of its size 3XH3 (18.99kb) was probably not located within I3, but presumably was the other flanking region. It would therefore be located clockwise from the original HindIII site of R68.45 and contained part of the original E/HB band of R68.45 and the anticlockwise end of I3. The remaining two fragments must be located within I3.

A comparison of the EcoRI/HindIII double digest with the EcoRI and HindIII single digests enable the identification of the restriction sites at the ends of fragments and the determination as to whether or not fragments produced by one enzyme contain sites for the other enzyme within it (Table 6.11).

The HindIII single digest fragment 3XH4 (6.92kb) must contain one or more EcoRI sites. These must generate with the double digest two EcoRI/HindIII produced fragments plus, possibly, one or more EcoRI/EcoRI produced fragment. The EcoRI/EcoRI produced fragment 3XE/H3 (9.69kb) is too large. The two EcoRI/HindIII produced fragments 3XE/H9 (1.25kb) and 3XE/H10 (0.98kb) could not make the required 6.92kb even with the remaining EcoRI/EcoRI produced fragment 3XE/H7 (1.52kb). Therefore 3XE/H6 (4.37kb) must be within the fragment 3XH4 (6.92kb). But 3XE/H6 (4.37kb) plus either 3XE/H9 (1.25kb) or 3XE/H10 (0.98kb) equals 5.97kb and 5.71kb respectively, both of which are short of the required 6.92kb of 3XH4. Therefore the EcoRI/EcoRI produced fragment 3XE/H7 (1.52kb) must be included. This then gives (Table 6.12):

TABLE 6.11

A comparison of restriction fragments produced by single and double digests of pUU3 with EcoRI and HindIII

EcoRI/HindIII Fragments	Size, kb	Equivalent to	Produced by
3XE/H2	18.99	3XH3	HindIII/HindIII
3XE/H3	9.69	3XE3	EcoRI/EcoRI
3XE/H4	5.21	3XH5	HindIII/HindIII
3XE/H6	4.37	-	EcoRI/HindIII
3XE/H7	1.52	3XE4	EcoRI/EcoRI
3XE/H9	1.25	-	EcoRI/HindIII
3XE/H10	0.99	-	EcoRI/HindIII

TABLE 6.12

Sum of different EcoRI/HindIII produced restriction fragments

	A	B
3XE/H6	4.37	4.37
3XE/H7	1.52	1.52
3XE/H9	1.25	
3XE/H10	____	0.98
	7.14kb	6.87kb

Both results are near the required 6.92kb for 3XH4. The combination of fragments B was nearer the required size and was therefore the most probable combination of fragments. This yielded the arrangement of fragments (Figure 6.9c).

The E/HB region of R68.45 which contains I3 in pUU3 was 42.03kb (18.1 + 23.93). If 3XH3 (= 3XE/H2, 18.99kb) was the most anticlockwise fragment of this region, the internal HindIII sites of I3 must lie in the next 12.13kb (3XH4, 6.92kb + 3XH5, 5.21kb); and the remaining 10.91kb of this region only contained EcoRI sites, if any. This enables the position of two HindIII sites and the alternatives for the third site to be mapped (Figure 6.9b). The EcoRI/EcoRI produced fragment 3XE/H3 (9.69kb) is probably the piece of DNA within this 10.91kb region, and would there be positioned just anticlockwise of the original EcoRI site of E/HB of R68.45. The remaining two EcoRI produced fragments 3XE2 (10.49kb) and 3XE4 (1.52kb) must be anticlockwise of this EcoRI site. This enables the position of two EcoRI sites within I3 to be mapped, and two alternative positions for the third site to be given Figure 6.9b).

FIGURE 6.9

Mapping of EcoRI and HindIII sites of I3 in pUU3

a. The E/HB fragment of R68.45.

b. The E/HB fragment plus I3 of pUU3. H' and E'; alternative positions for HindIII and EcoRI sites.

c. Probable arrangement of restriction sites within 3XH4.

d. Probable arrangement of restriction sites within 3XE2.

e. Arrangement of restriction sites within I3.

f. Restriction map of the region of pUU3 bearing I3 for EcoRI and HindIII.

g. Restriction map of the I3 region of pUU3 with possible sites for other enzymes.

S^1, S^2 and K^1 are alternative locations for SmaI and KpnI restriction sites.

E : EcoRI

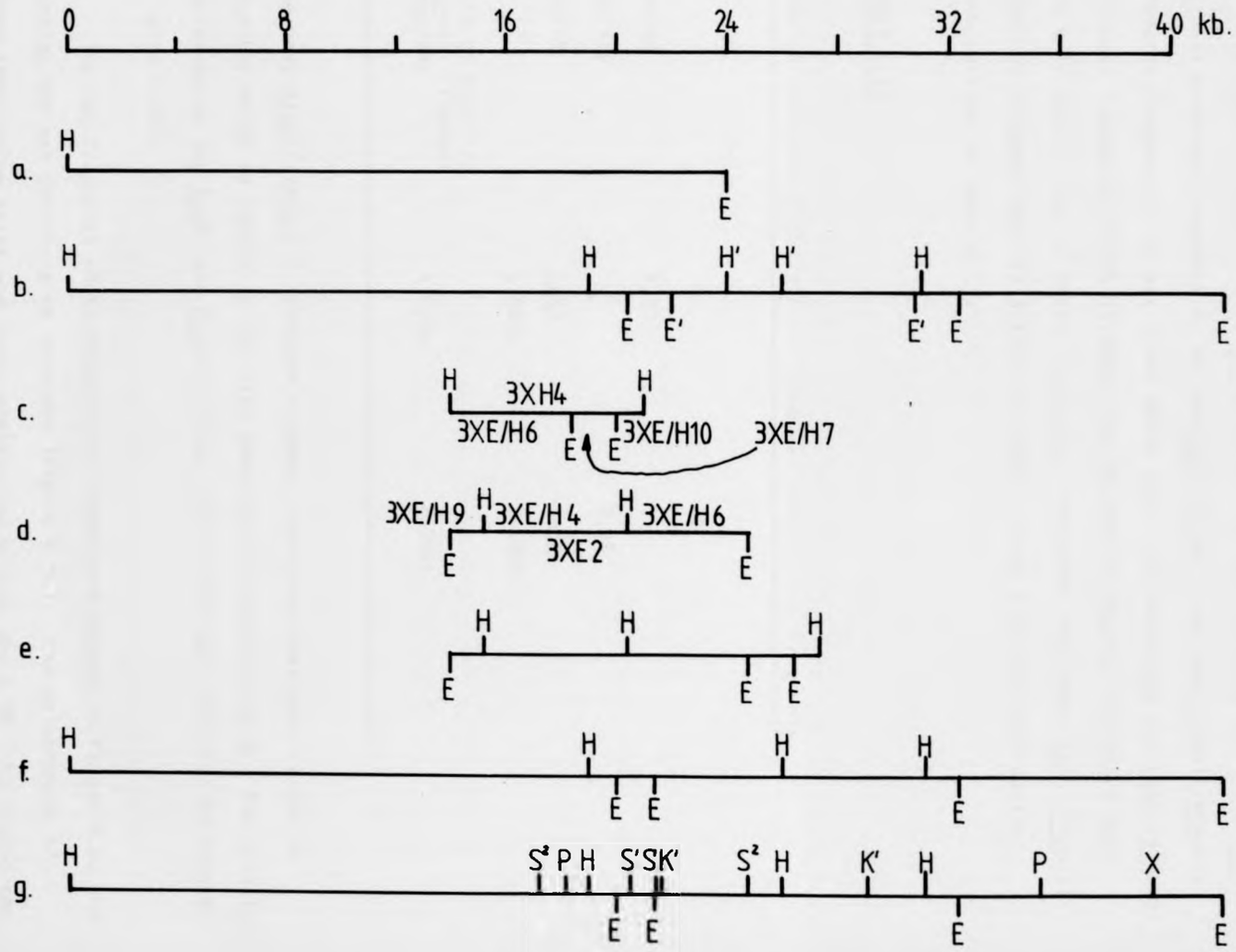
H : HindIII

P : PstI

S : SmaI

K : KpnI

X : XhoI


0
8
16
24
32
40 kb.
a.
H
E
b.
H
H
H'
H'
H
E
E'
E'
E
E
c.
H
3XH4
H
3XE/H6
E
E
3XE/H10
3XE/H7
d.
3XE/H9
H
3XE/H4
H
3XE/H6
E
3XE2
E
e.
H
H
H
E
E
E
f.
H
H
H
H
E
E
E
E
g.
H
S²
P
H
S'
S
K'
S²
H
K'
H
P
X
E
E
E
E

The EcoRI generated fragment 3XE2 (10.49kb) must contain within it one or more HindIII sites, but no EcoRI sites. It must be made up of two EcoRI/HindIII produced fragments or of two EcoRI/HindIII and one HindIII/HindIII produced fragments. It was shown above that 3XH4 contained the EcoRI/HindIII produced fragments 3XE/H6 (4.37kb) and 3XE/H10 (0.98kb). Therefore 3XE2 can only contain one of these fragments. Therefore the other EcoRI/HindIII produced fragment must be 3XE/H9 (1.25kb). These give the alternative combinations in Table 6.13:

TABLE 6.13

Sums of different EcoRI/HindIII produced restriction fragments

	A	B
3XE/H6	4.37	
3XE/H10		0.98
3XE/H9	1.25	1.25
	5.62kb	2.23kb
Size of HindIII/HindIII fragment required	4.87kb	8.26kb

The only HindIII/HindIII produced fragment remaining available is 3XE/H4 (5.21kb) which is nearer to the size required for possibility A. The relative positions of the EcoRI and HindIII sites within 3XE2 can therefore be mapped (Figure 6.9d).

The two fragments whose restriction sites were mapped in Figure 6.9c and overlap and can therefore be combined (Figure 6.9e). The orientation of these restriction sites and their positioning within the E/HB - 13 region can

be determined by comparing Figures 6.9b. and 6.9e. If the fragment mapped in Figure 6.9e. is inserted one set of the alternative positions for the EcoRI and HindIII sites can be accepted and the other rejected (Figure 6.9f.).

If it is assumed that the largest new restriction fragments from pUU3 contain the regions flanking I3, it is possible through comparison with the published map of R68 (Nayudu and Holloway, 1980), to tentatively add a few more restriction sites to the map derived above.

The two largest new restriction fragments from the digest of pUU3 with PstI were 3XP1 (19.45kb) and 3XP3 (12.60kb) (Table 6.6; Figure 6.3). These fragments would give PstI restriction sites at about 18.2kb clockwise from the original HindIII site and 6.60kb anticlockwise from the original EcoRI site (Figure 6.9g.).

In the SmaI digest of pUU3 the two largest new fragments were 3XS1 (21.5kb) (Table 6.7; Figure 6.5). Because of their similar lengths, these fragments give two alternative sets of SmaI sites in the I3 region (Figure 6.9g.).

The KpnI digest data indicated that I3 contained only one KpnI site (Table 6.8; Figure 6.6). Unfortunately due to inaccuracies in measuring large restriction fragments, the position of the KpnI site as determined from one of the nearest KpnI sites of R68, does not correspond exactly with the position as determined from the other nearest KpnI site of R68. However if the average of these two sites is taken two alternative positions for the KpnI site of pUU3 can be determined (Figure 6.9f.).

The XhoI digest data indicated the presence of one XhoI site in the I3 region of pUU3 (Table 6.9; Figure 6.7). This XhoI site was located 2.86kb from one extremity of the 6 o'clock - 12 o'clock original XhoI fragment of R68. In order to coincide with the probable location of I3 as determined from

the other restriction digests the new XhoI would have to be positioned about 2.5kb anticlockwise from the original EcoRI site (Figure 6.9f.). This location of the XhoI site is at the clockwise extreme of the proposed location for I3.

Several genetic functions have been mapped in the region of R68 which contains I3 in pUU3 (Figure 6.4). These include the traI gene. PPW9 was unable to transfer pUU3 conjugatively indicating that this plasmid had suffered inactivation of its transfer functions. This observation ties in with the molecular evidence obtained through restriction digests of the plasmid. PPW9 was still resistant to Ap, Km and Tc. Restriction digests showed that the regions of the plasmid coding for these resistances were unmodified.

6.6.2 Comparison of restriction digests of R68.45, pUU2 and pUU4

Restriction digests of R68.45, pUU2 and pUU4 were electrophoresed on agarose gels under varying conditions so as to reveal different aspects of the digest patterns (see Section 6.6.1 for details).

6.6.2.1 Digestion of R68.45, pUU2 and pUU4 with PstI

R68.45, pUU2 and pUU4 were digested with the restriction endonuclease PstI and the restriction fragments sized (Plate 6.3; Figure 6.10; Table 6.14).

All the bands from R68.45 present in pUU2 were conserved in pUU4. All the double bands in pUU2 indicative of I2 were missing in pUU4. The only bands associated with I2 in pUU2 which were conserved in pUU4 were the two large single bands 2XP1 (14.82kb) and 2XP2 (12.38kb). These two bands had been identified in Chapter 3 as corresponding to the flanking regions of I2. pUU4 would have appeared to have lost I2 except for about 2.36kb containing one PstI site.

FIGURE 6.10

Diagram of PstI digests of R68.45, pUU2 and pUU4

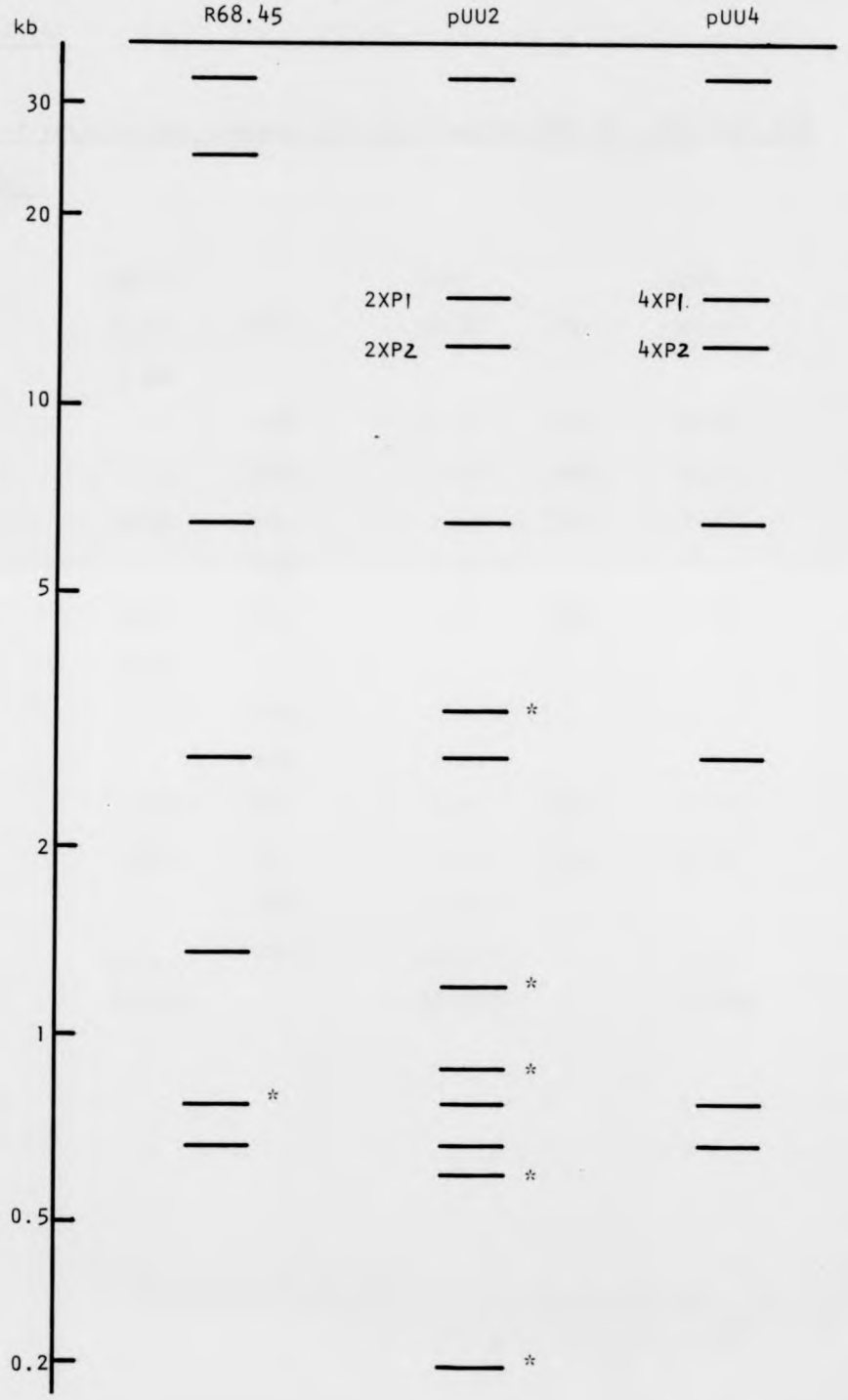

kb
R68.45
pUU2
pUU4
30
20
10
5
2
1
0.5
0.2
2XP1
2XP2
4XP1
4XP2
*

TABLE 6.14

Sizes of restriction fragments of the plasmids R68.45, pUU2 and pUU4 with PstI

	R68.45		pUU2		pUU4
PA	32.67	2PA	32.67	4PA	32.67
PB	24.84				
		2XP1	14.82	4XP1	14.82
		2XP2	12.38	4XP2	12.38
PC	6.52	2PC	6.52	4PC	6.52
		2XP3	3.23 *		
PD	2.77	2PD	2.77	4PD	2.77
PE	1.34				
		2XP4	1.19 *		
		2XP5	0.87 *		
PF	0.77 *	2PF	0.77	4PF	0.77
PG	0.66	2PG	0.66	4PG	0.66
		2XP6	0.60 *		
		2XF7	0.29 *		
	70.34kb		82.95kb		70.59kb

6.6.2.2 Digestion of R68.45, pUU2 and pUU4 with SmaI

R68.45, pUU2 and pUU4 were digested with the restriction endonuclease SmaI and the restriction fragments sized (Plate 6.4; Figure 6.11; Table 6.15). The R68.45 derived bands in pUU2 remained unchanged in pUU4. The two sets of double bands in pUU2 associated with I2 were missing in pUU4. The two new single bands in pUU2, 2XS1 (16.60kb) and 2XS2 (8.45kb) were also missing in pUU4. pUU4 had acquired a new band not present in either R68.45 or pUU2, 4XS1 (21.50kb).

As with the PstI results, discussed in 6.6.2.1, these SmaI data indicate that pUU4 had lost the bulk of I2 from pUU2, retaining about 3.95kb of DNA which contained no SmaI sites. The remainder of the plasmid would appear to be unaltered.

6.6.2.3 Digestion of R68.45, pUU2 and pUU4 with KpnI

R68.45, pUU2 and pUU4 were digested with restriction endonuclease KpnI and the restriction fragments sized (Plate 6.5; Table 6.16; Figure 6.12). The R68.45 bands present in pUU2 were conserved in pUU4. The double band 2XK1 (6.49kb) internal to I2 was missing in pUU4. The two single bands, $2KC^{1}$ (13.96kb) and 2XK2, which were new in pUU2 were still present in pUU4.

These results indicate that the bulk of the duplicated part of I2 was missing in pUU2. A small portion, 3.09kb in size, of I2 bearing two KpnI sites has been retained in pUU4. The remainder of the plasmid would appear to be unaltered.

6.6.2.4 Digestion of R68.45, pUU2 and pUU4 with XhoI

R68.45, pUU2 and pUU4 were digested with the restriction endonuclease XhoI and the restriction fragments sized (Plate 6.6; Table 6.17). Band 2XA

FIGURE 6.11

Diagram of *Sma*I digest of R68.45, pUU2 and pUU4

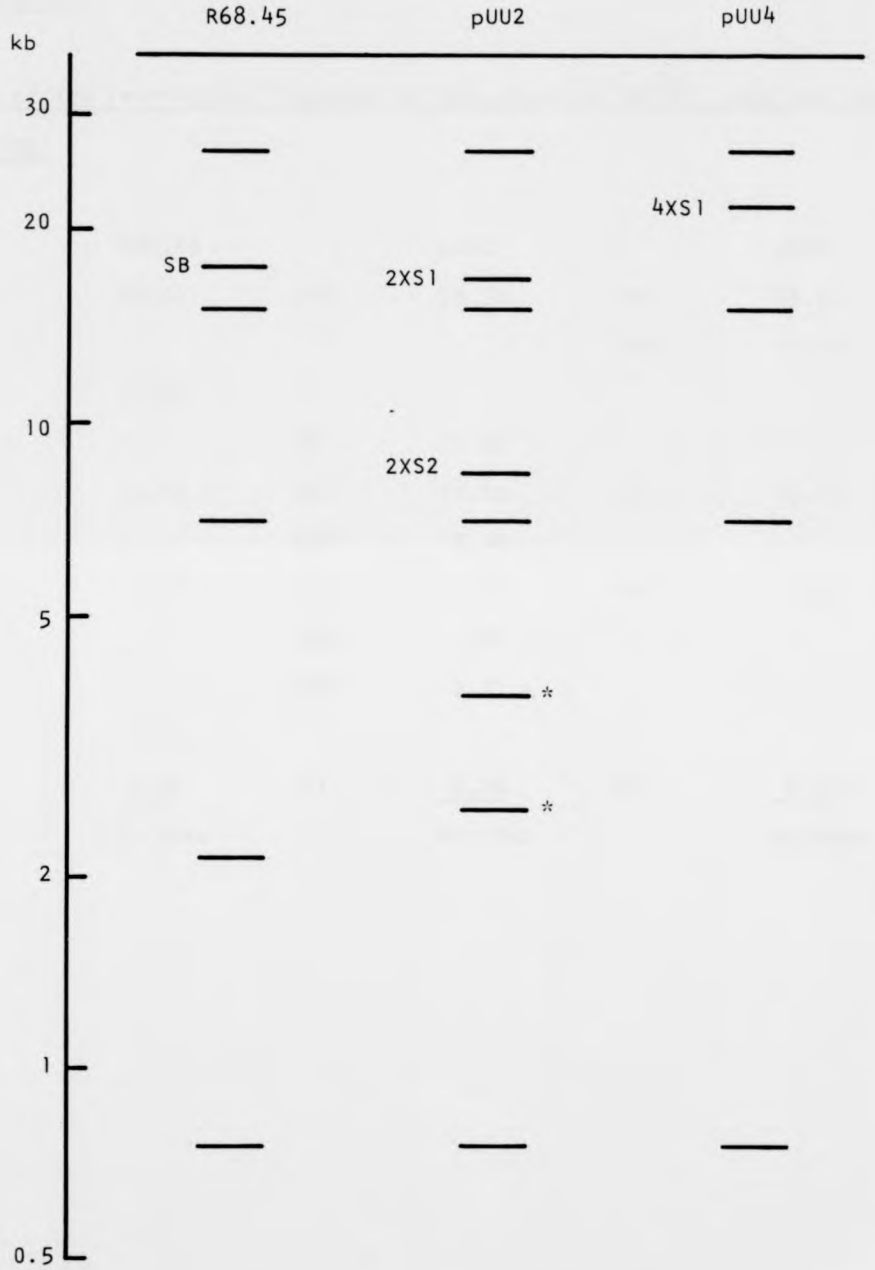

R68.45
pUU2
pUU4
kb
30
20
10
5
2
1
0.5
SB
2XS1
2XS2
4XS1
*
*

TABLE 6.15

Sizes of the restriction fragments of the plasmids R68.45, pUU2 and pUU4 with SmaI

	R68.45		pUU2		pUU4
SA	26.52	2SA	26.52	4SA	26.52
				4XS1	21.50
SB	17.55				
		2XS1	16.60		
SC	15.10	2SC	15.10	4SC	15.10
		2XS2	8.45		
SD	7.15	2SD	7.15	4SD	7.15
		2XS3	3.80 *		
		2XS4	2.51 *		
SE	2.12				
SF	0.76	2SF	0.76	4SF	0.76
	69.20kb		87.20kb		69.03kb

TABLE 6.16

Sizes of restriction fragments of the plasmids R68.45, pUU2 and pUU4 with KpnI

	R68.45		pUU2		pUU4
KA	34.44	2KA	34.44	4KA	34.44
KB	15.66				
KC	13.96	2KC	13.96 *	4KC	13.96 *
		2XK1	6.49 *		
		2XK2	4.79	4XK1	4.79
KD	2.11				
KE	1.61	2KE	1.61	4KE	1.61
	67.78kb		81.74kb		68.76kb

FIGURE 6.12

Diagram of KpnI digests of R68.45, pUU2 and pUU4

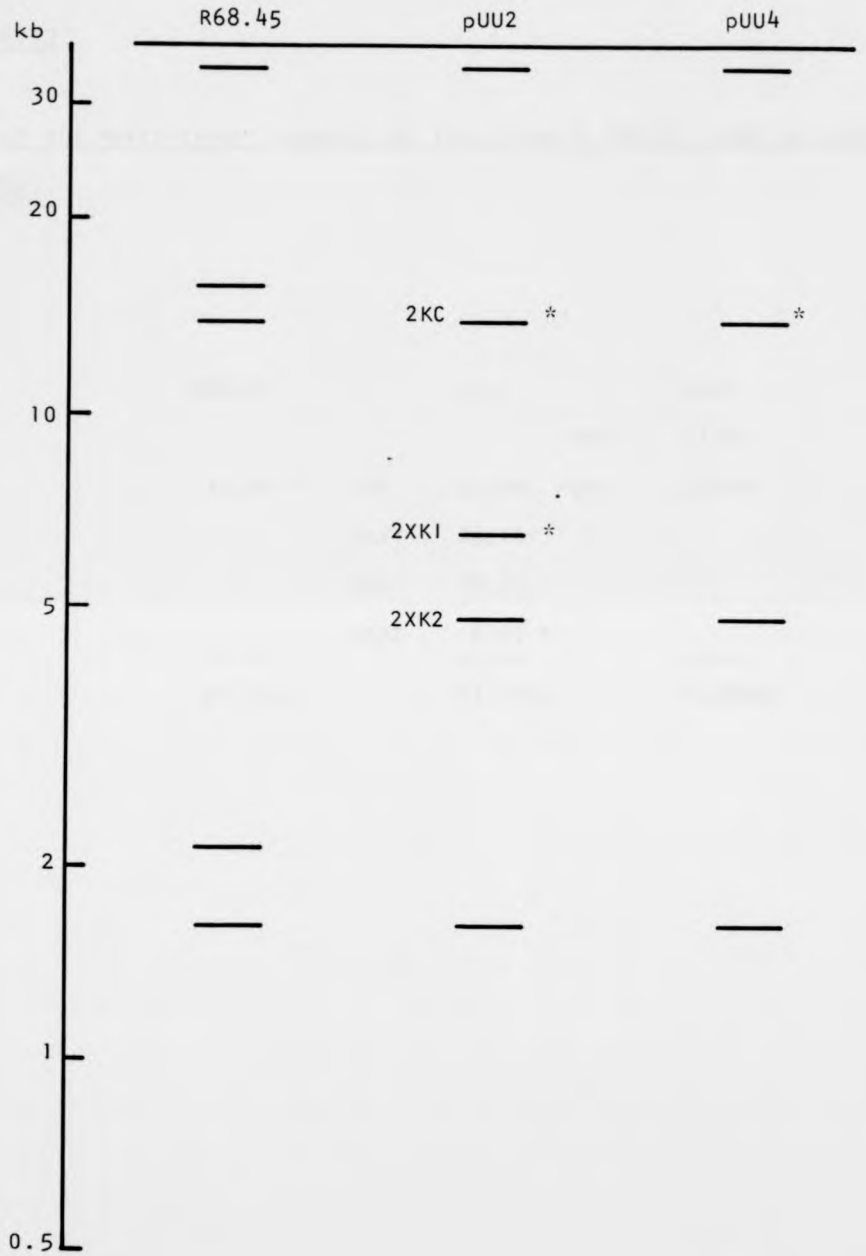

kb
R68.45
pUU2
pUU4
30
20
10
5
2
1
0.5
2KC
*
*
2XK1
*
2XK2

TABLE 6.17

Sizes of the restriction fragments of the plasmids R68.45, pUU2 and pUU4 with XhoI

	R68.45		pUU2		pUU4
				4XX1	41.98
XA	33.98 *	2XA	33.98	4XA	33.98
		2XX1	28.46		
		2XX2	18.20		
		2XX2	6.55 *		
	67.96kb		93.74kb		75.96kb

in pUU2 which was derived from XA in R68.45 was conserved in pUU4. The double band 2XX2 (6.55kb) and the two large single bands 2XX1 (28.46kb) and 2XX2 (18.20kb) from pUU2 which were associated with I2 were absent in pUU4. pUU4 had acquired a large band of about 41.98kb which was not present in either R68.45 or pUU2.

These results indicate that pUU4 had lost the bulk of I2, retaining a small portion containing no XhoI sites. This retained portion was estimated at 8kb, however this is probably an inaccurate measurement being based on the sizing of a very long fragment.

6.6.2.5 Digestion of R68.45, pUU2 and pUU4 with EcoRI and HindIII

R68.45, pUU2 and pUU4 were digested with the restriction endonucleases EcoRI and HindIII, singly and together. The restriction fragments were sized (Plate 6.7; Table 6.18; Figure 6.13). These digests indicated that pUU4 had lost one HindIII double band 2XH4 (1.4kb) which in pUU2 was associated with I2. pUU4 retained a single HindIII band equivalent in size to the double band 2XH3 (5.04kb) present in pUU2.

A puzzling feature of these digests was that to produce the digest pattern of pUU4 with HindIII or with EcoRI/HindIII, pUU4 would have to have a HindIII site 5.04kb from the original single HindIII site of R68. This position is not within the area modified by I2 in pUU2. Certainly the digests of pUU4 with other enzymes did not indicate any modification outside the area associated with I2.

6.6.2.6 Interpretation of restriction digest data for pUU4

With the exception of the HindIII digest data all the digests confirmed that pUU4 had lost the bulk of I2. The bands lost were for all enzymes, except HindIII, the double bands from I2. pUU4 had retained between 2.36 -

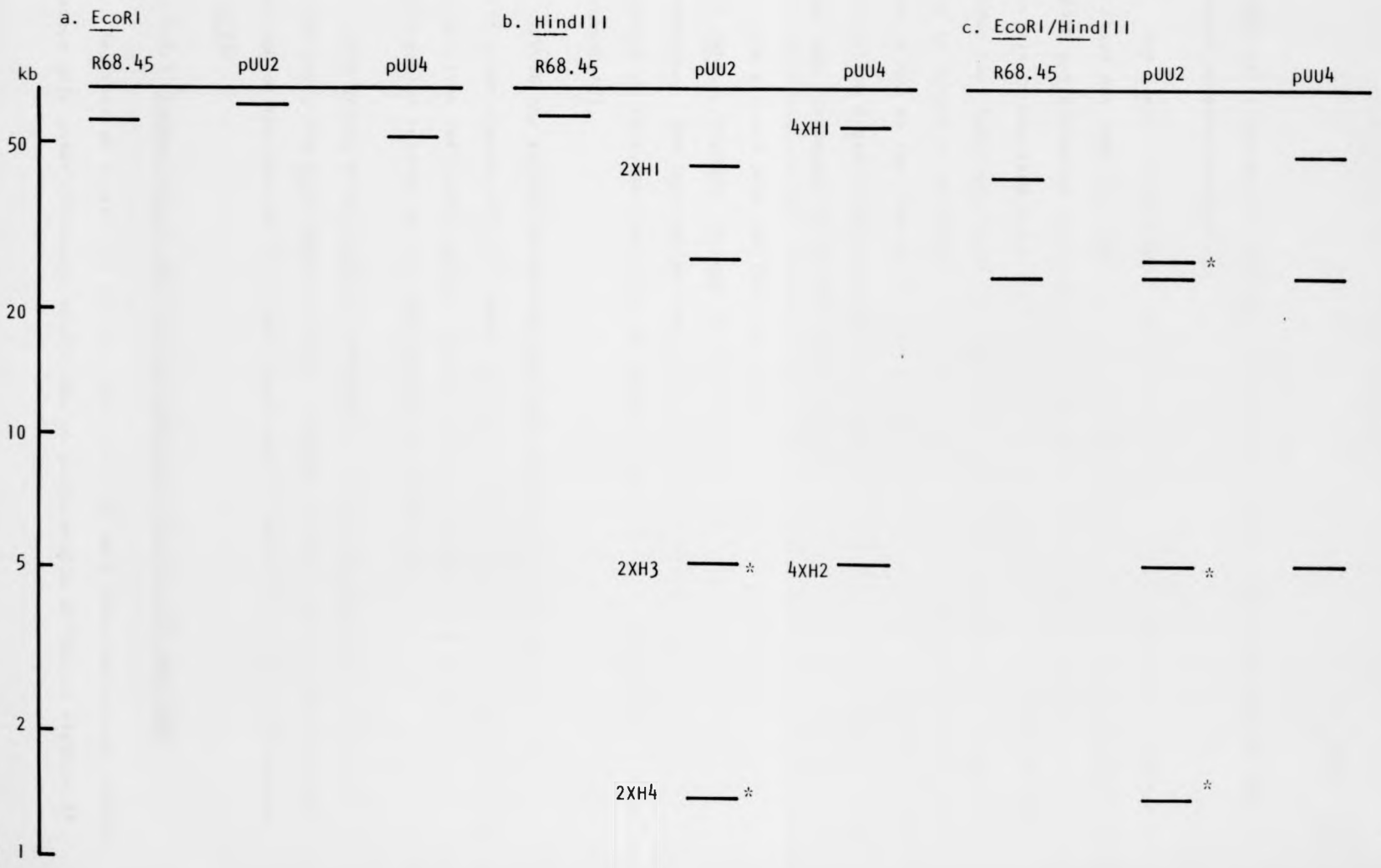
TABLE 6.18

Sizes of the restriction fragments of the plasmids R68.45, pUU2 and pUU4 with EcoRI, HindIII and EcoRI/HindIII

a. EcoRI		R68.45		pUU2		pUU4
			2XE1	61.78		
	EA	55.61				
					4XE1	51.97
b. HindIII		R68.45		pUU2		pUU4
	HA	58.30				
					4XH1	54.25
			2XH1	44.79		
			2XH2	26.70		
			2XH3	5.04 *	4XH2	5.04
			2XH4	1.43 *		
		58.30kb		83.83kb		59.29kb
c. EcoRI/HindIII		R68.45		pUU2		pUU4
					4XE/H1	47.79
	E/HA	42.02				
			2XE/H1	26.70 *		
	E/HB	23.93	2E/HB	23.93	4XE/H2	23.93
			2XE/H2	5.04 *	4XE/H3	5.04
			2XE/H3	1.43 *		
		65.95kb		57.10kb		76.76kb

FIGURE 6.13

Diagram of restriction digests of R68.45, pUU2 and pUU4 with EcoRI HindIII and EcoRI/HindIII


a. EcoRI
R68.45
pUU2
pUU4
kb
50
20
10
5
2
1
b. HindIII
R68.45
pUU2
pUU4
4XH1
2XH1
2XH3
4XH2
2XH4
c. EcoRI/HindIII
R68.45
pUU2
pUU4

3.09kb of I2 containing one PstI and two KpnI sites. The remainder of the plasmid appeared unaltered.

The HindIII digest data indicated the loss of the bulk of I2. However it stood out from the other digests by indicating the presence of a HindIII site 5.04kb anticlockwise from the original HindIII site of R68. Whilst it is possible to envisage a new restriction site appearing in the plasmid through random base mutation, it would seem to be highly unlikely for the restriction site so formed to be exactly the same distance away from the original HindIII site of R68 as the size of one of the double bands from I2. None of the other restriction digests indicated any change in this region of the plasmid. These data seem inadequate to resolve this dilemma.

The plasmid pUU4 had lost the bulk of I2. This suggested that it has lost dehI, presumably through the insertion of these genes into the host's chromosome. The implication that pUU4 was dehI$^-$ could not be tested as attempts to mate and transform the plasmid into another host proved unsuccessful.

PPW10 the strain harbouring pUU4 was tetracycline sensitive. The restriction digests did not reveal any gross alterations in the region of the tetracycline resistance genes. However this by no means rules out any modification leading to the inactivation of these genes.

pUU4 proved to be transfer deficient. I2 was inserted very close to, if not into, the tra3 region in pUU2. Changes in this region caused by the excision of the bulk of I2 in pUU2 could easily have led to the inactivation of tra3.

6.6.3 Comparison of restriction digests of R68.45, pUU2 and pUU5

Restriction digests of R68.45, pUU2 and pUU5 were electrophoresed through agarose gels under different conditions so as to enable different aspects of

the digest patterns to be compared.

6.6.3.1 Digestion of R68.45, pUU2 and pUU5 with PstI

R68.45, pUU2 and pUU5 were digested with PstI and the restriction fragments sized (Plate 6.3; Table 6.19; Figure 6.14). All the bands from R68.45, present in pUU2 were conserved in pUU5. All the new bands in pUU2 associated with I2 were present in pUU5 except 2XP1 (14.82kb) which was missing. pUU5 had acquired ten new bands not present in pUU2. Eight of these new bands corresponded to bands present in pUU3 (Figure 6.14). pUU5 had increased in size over pUU2 by 11.54kb.

These data suggest that the bulk if not all of I2 was conserved in pUU5. pUU5 had acquired an extra portion of DNA of about 11.54kb inserted into band 2XP1 from pUU2, which had been identified as a region flanking I2. This extra portion of DNA was very similar in its digest pattern to what was designated I3 in pUU3. The extra portion of DNA in pUU5 was designated I5.

6.6.3.2 Digestion of R68.45, pUU2 and pUU5 with SmaI

R68.45, pUU2 and pUU5 were digested with SmaI and the restriction fragments sized (Plate 6.4; Table 6.20; Figure 6.15). All the bands from R68.45 present in pUU2 were conserved in pUU5 except 2SC which was missing. All the bands in pUU2 associated with I2 were also present in pUU5 except 5XS5 which appeared as a single instead of double. pUU5 possessed four bands, including one double band, which were not present in pUU2. Two of these new bands in pUU5 were the same size as new bands in pUU3 which were identified with I3 (Figure 6.15). The SmaI data indicated that pUU5 was 21.00kb larger than pUU2.

These results suggest that in pUU5 the bulk of I2 was present unaltered and that the plasmid had acquired an extra portion of DNA of about 24.80kb

TABLE 6.19

Sizes of restriction fragments of the plasmids R68.45, pUU2 and pUU5 with PstI

	R68.45		pUU2		pUU5
PA	32.67	2PA	32.67	5PA	32.67
PB	24.84				
		2XP1	14.82		
				5XP1	12.60
		2XP2	12.38	5XP2	12.38
PC	6.52	2PC	6.52	5PC	6.52
				5XP3	3.81
		2XP3	3.23 *	5XP4	3.23 *
				5XP5	2.91
PD	2.77	2PD	2.77	SPD	2.77
				5XP6	2.44
				5XP7	1.38
PE	1.34				
		2XP4	1.19 *	5XP8	1.19 *
				5XP9	1.11
				5XP10	1.06
		2XP5	0.87 *	5XP11	0.87 *
PF	0.77 *	2PF	0.77	5PF	0.77
				5XP12	0.72
PG	0.66	2PG	0.66	5PG	0.66
		2XP6	0.60 *	5XP12	0.60 *
		2XP7	0.29 *	5XP13	0.29 *
				5XP14	0.19
				5XP15	0.14
	70.34kb		82.95kb		94.49kb

FIGURE 6.14

Diagram of PstI digests of R68.45, pUU2, pUU5 and pUU3

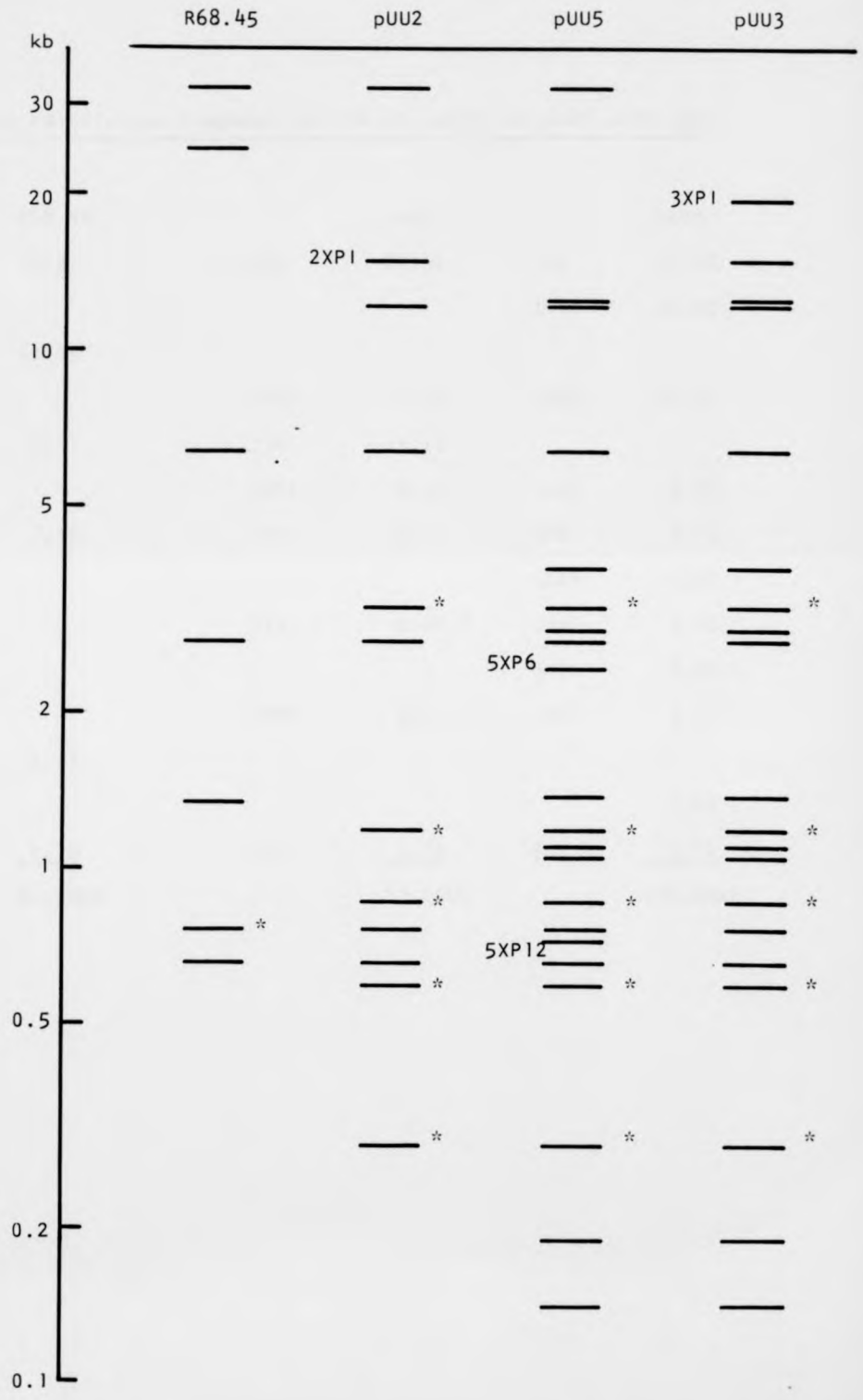

R68.45
pUU2
pUU5
pUU3
kb
30
20
10
5
2
1
0.5
0.2
0.1
2XP1
3XP1
5XP6
5XP12

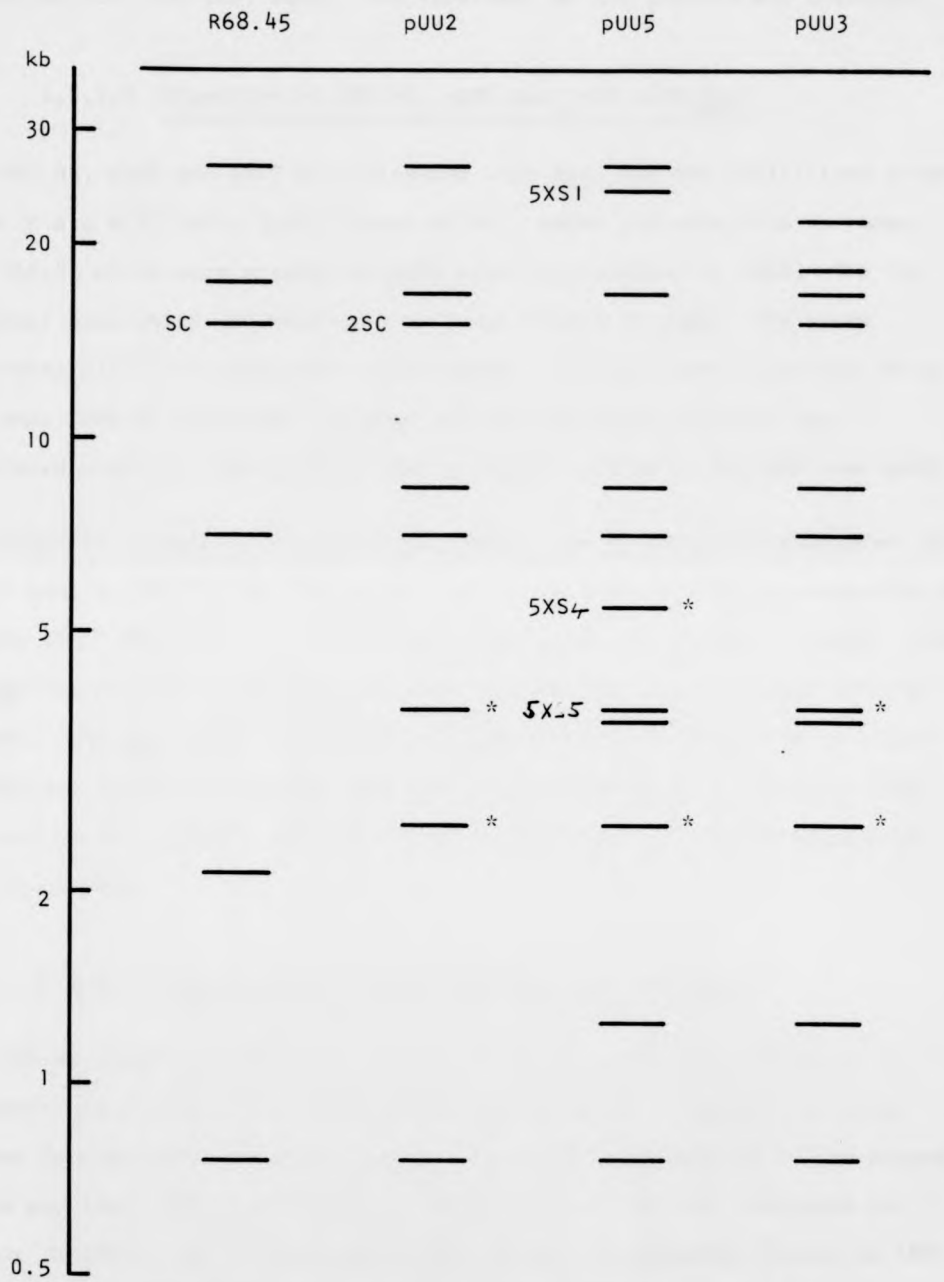
TABLE 6.20

Sizes of the restriction fragments of R68.45, pUU2 and pUU5 with SmaI

	R68.45		pUU2		pUU5
SA	26.52	2SA	26.52	5SA	26.52
				5XS1	23.99
SB	17.55				
		2XS1	16.60	5XS2	16.60
SC	15.10	2SC	15.10		
		2XS2	8.45	5XS3	8.45
SD	7.15	2SD	7.15	5SD	7.15
				5XS4	5.52 *
		2XS3	3.80 *	5XS5	3.80
				5XS6	3.64
		2XS4	2.51 *	5XS7	2.51 *
SE	2.12				
					1.23
SF	0.76	2SF	0.76	5SF	0.76
	96.20kb		87.20kb		108.20kb

FIGURE 6.15

Diagram of SmaI digests of R68.45, pUU2, pUU5 and pUU3


R68.45
pUU2
pUU5
pUU3
kb
30
20
10
5
2
1
0.5
SC
2SC
5XS1
5XS4
5XS5
*

within the 2SC (15.10kb) band. The remainder of the plasmid was unchanged.

6.6.3.3 Digestion of R68.45, pUU2 and pUU5 with KpnI

R68.45, pUU2 and pUU5 were digested with KpnI and the restriction fragments sized (Plate 6.5; Table 6.21; Figure 6.16). Bands the same size as those from R68.45 which were present in pUU2 were also present in pUU5. The 2KC (13.96kb) band which was double in pUU2 was single in pUU5. The bands associated with I2 in pUU2 were also present in pUU5. pUU5 possessed three new bands, one of which was the same size as the band 2XK2 from pUU3 associated with I3. The plasmid had increased in size by 24.12kb over pUU2.

Band 2KC in pUU2 consisted of two bands, one of which was equivalent to the KC band in R68.45, and the other, 2KC', was derived from the insertion of I2 into KB of R68.45. Only one of these two bands was present in pUU5. From the KpnI data alone it was not possible to determine which one was missing in pUU5. The KpnI data did however indicate that the bulk of the I2 region of pUU2 was conserved in pUU5, and that pUU5 had acquired a portion of DNA, one restriction fragment from which was of the same size as one associated with I3 in pUU3.

6.6.3.4 Digestion of R68.45, pUU2 and pUU5 with XhoI

R68.45, pUU2 and pUU5 were digested with XhoI and their restriction fragments sized (Plate 6.6; Table 6.22; Figure 6.17). Most of the bands present in pUU2 were conserved, except the double band 2XX3 which had become a single and 2XX1, which was missing. pUU5 had acquired four new bands not present in pUU2. One of these new bands, 5XX6, corresponded in size to 3XX5, a band present in pUU3 but not in pUU2. pUU5 had increased in size by 16.13kb over pUU2.

XhoI digests of these plasmids gave some fragments of very large size.

TABLE 6.21

Sizes of the restriction fragments of R68.45, pUU2 and pUU5 with KpnI

	R68.45		pUU2		pUU5
KA	34.44	2KA	34.44	5KA	34.44
				5XK1	23.95
KB	15.66				
KC	13.96	2KC	13.96 *	5KC	13.96
				5XK2	8.78
		2XK1	6.49 *	5XK3	6.49 *
				5XK4	5.35
		2XK2	4.79	5XK5	4.79
KD	2.11				
KE	1.61	2KE	1.61	5KE	1.61
	67.78kb		81.74kb		105.86kb

FIGURE 6.16

Diagram of KpnI digests of R68.45, pUU2, pUU5 and pUU3

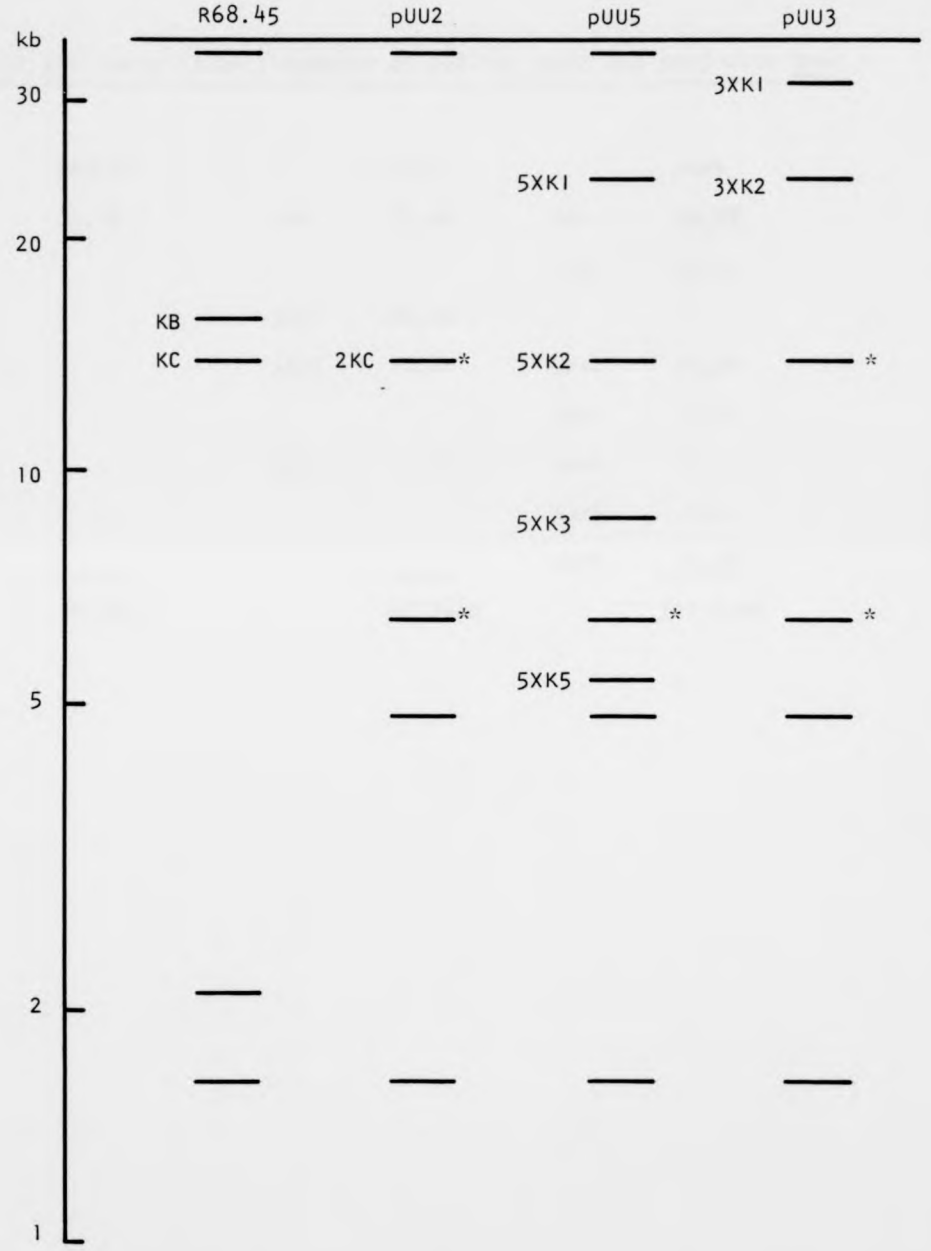

R68.45
pUU2
pUU5
pUU3
kb
30
20
10
5
2
1
3XK1
5XK1
3XK2
KB
KC
2KC
5XK2
5XK3
5XK5
*

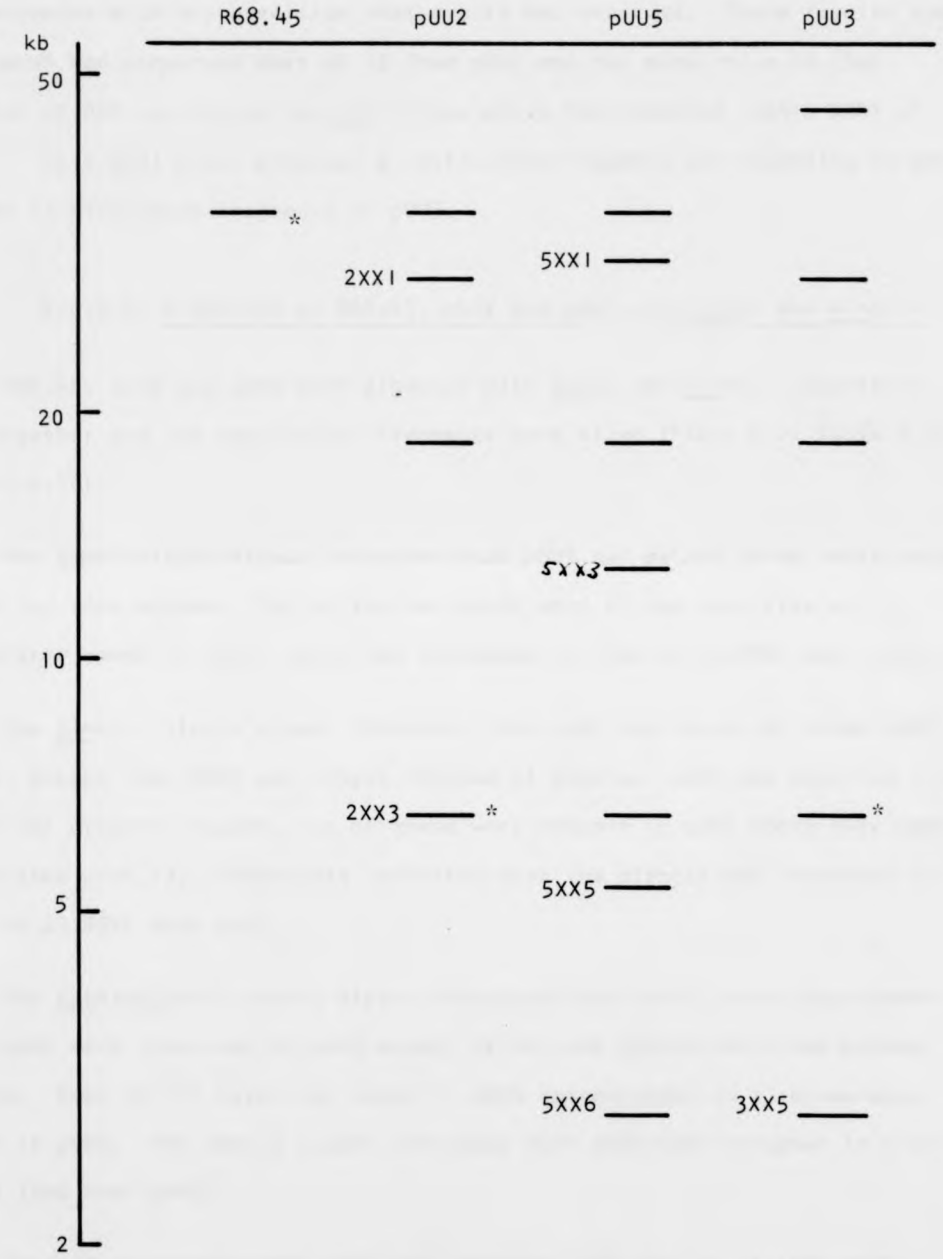
TABLE 6.22

Sizes of the restriction fragments of R68.45, pUU2 and pUU5 with XhoI

	R68.45		pUU2		pUU5
XA	33.98 *	2XA	33.98	5XA	33.98
				5XX1	30.22
		2XX1	28.46		
		2XX2	18.20	5XX2	18.20
				5XX3	12.81
		2XX3	6.55 *	5XX4	6.55
				5XX5	5.41
				5XX6	2.86
	67.96kb		93.74kb		110.03kb

FIGURE 6.17

Diagram of XhoI digests of R68.45, pUU2, pUU5 and pUU3


R68.45
pUU2
pUU5
pUU3
kb
50
20
10
5
2
*
2XX1
5XX1
5XX3
2XX3
*
*
5XX5
5XX6
3XX5

These were difficult to size accurately; therefore it was often difficult to determine with any precision what events had occurred. These results suggest that pUU5 had conserved most of I2 from pUU2 and had acquired a 16.13kb portion of DNA containing two XhoI sites which had inserted within 2XX1 of pUU2. These XhoI sites produced a restriction fragment corresponding to one of the I3 associated fragments in pUU3.

6.6.3.5 Digestion of R68.45, pUU2 and pUU5 with EcoRI and HindIII

R68.45, pUU2 and pUU5 were digested with EcoRI and HindIII separately and together and the restriction fragments were sized (Plate 6.7; Table 6.23; Figure 6.18).

The EcoRI single digest indicated that pUU5 had gained three restriction sites for this enzyme. Two of the new bands were of the same size as I3 associated bands in pUU3. pUU5 had increased in size by 20.09kb over pUU2.

The HindIII single digest indicated that pUU5 had conserved three pUU2 bands, except that 5XH7 was single instead of double. pUU5 had acquired five bands not present in pUU2, two of these were present in pUU3 where they were associated with I3. These data indicated that the plasmid had increased in size by 21.95kb over pUU2.

The EcoRI/HindIII double digest indicated that the I2 associated bands from pUU2 were conserved in pUU5 except 5XE/H1 and 5XE/H6 which had become single. Four of the seven new bands in pUU5 corresponded to I3 associated bands in pUU5. The double digest indicated that pUU5 had increased in size by 17.17kb over pUU2.

These data suggest that pUU5 had conserved the bulk of I2 from pUU2, and that pUU5 had acquired a piece of DNA about 20kb in size which gave digest patterns similar to I3 in pUU3.

TABLE 6.23

Sizes of the restriction fragments of R68.45, pUU2 and pUU5 with EcoRI, HindIII and EcoRI/HindIII

a. EcoRI		R68.45		pUU2		pUU5
			2XE1	61.78		
	EA	55.61				
					5XE1	52.56
					5XE2	17.30
					5XE3	10.49
					5XE4	1.52
		55.61kb		61.78kb		81.87kb

b. HindIII		R68.45		pUU2		pUU5
	HA	58.30				
			2XH1	44.79		
					5XH1	39.04
			2XH2	26.70	5XH2	26.70
					5XH3	14.56
					5XH4	6.92
					5XH5	5.45
					5XH6	5.21
			2XH3	5.04*	5XH7	5.04
			2XH4	1.43*	5XH8	1.43*
		58.50kb		83.83kb		105.78kb

c. EcoRI/HindIII

	R68.45		pUU2		pUU5
E/HA	42.02				
		2XE/H1	26.70 *	5XE/H1	26.70
E/HB	23.93	2E/HB	23.93	5E/HB	23.93
				5XE/H2	16.50
				5XE/H3	13.63
				5XE/H4	5.45
				5XE/H5	5.21
		2XE/H2	5.04 *	5XE/H6	5.04
				5XE/H7	4.37
				5XE/H8	1.52
		2XE/H3	1.43 *	5XE/H9	1.43 *
				5XE/H10	1.25
				5XE/H11	0.98
	65.95kb		90.27kb		107.44kb

FIGURE 6.18

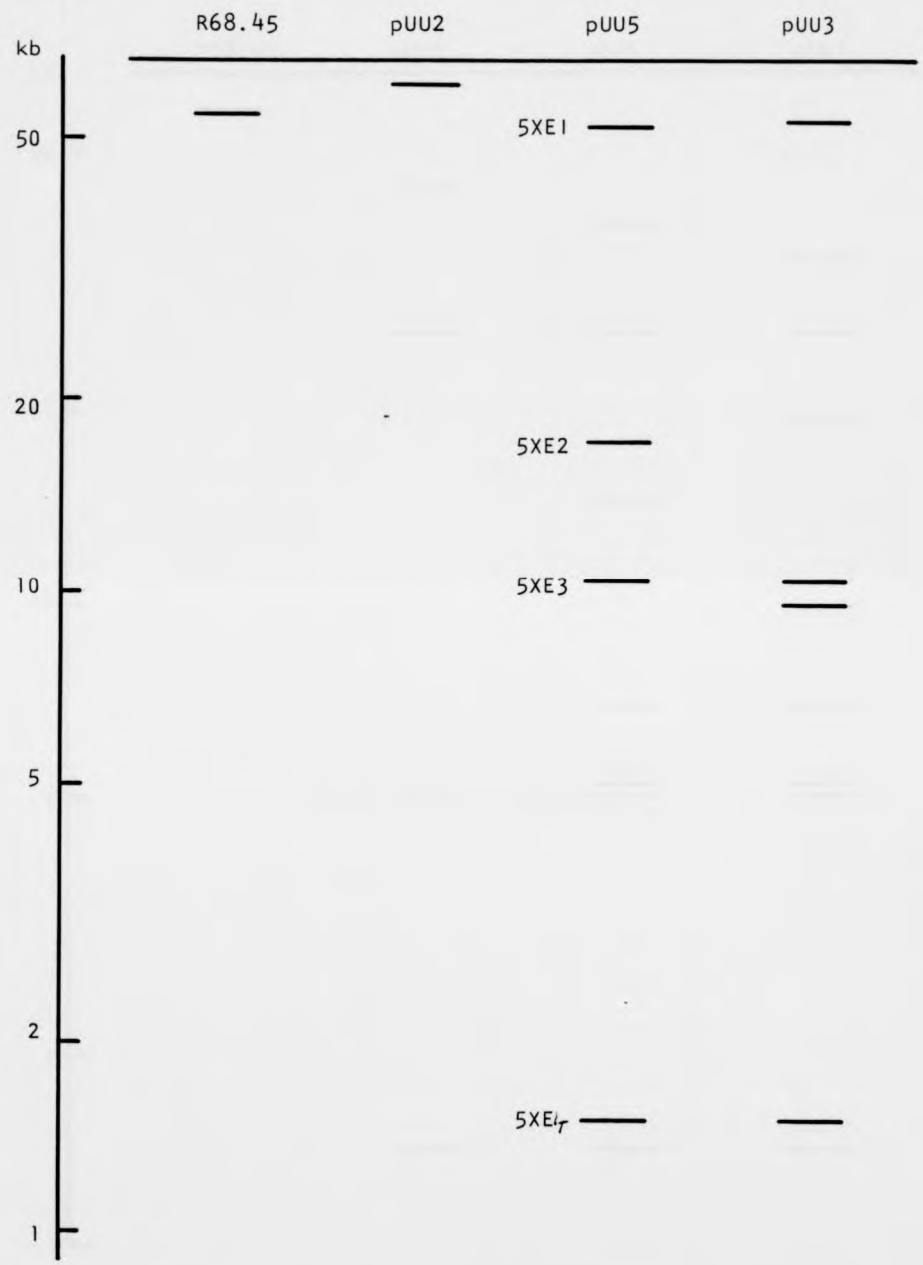
Diagram of EcoRI, HindIII and EcoRI/HindIII digests of R68.45, pUU2, pUU5 and pUU3

a. EcoRI

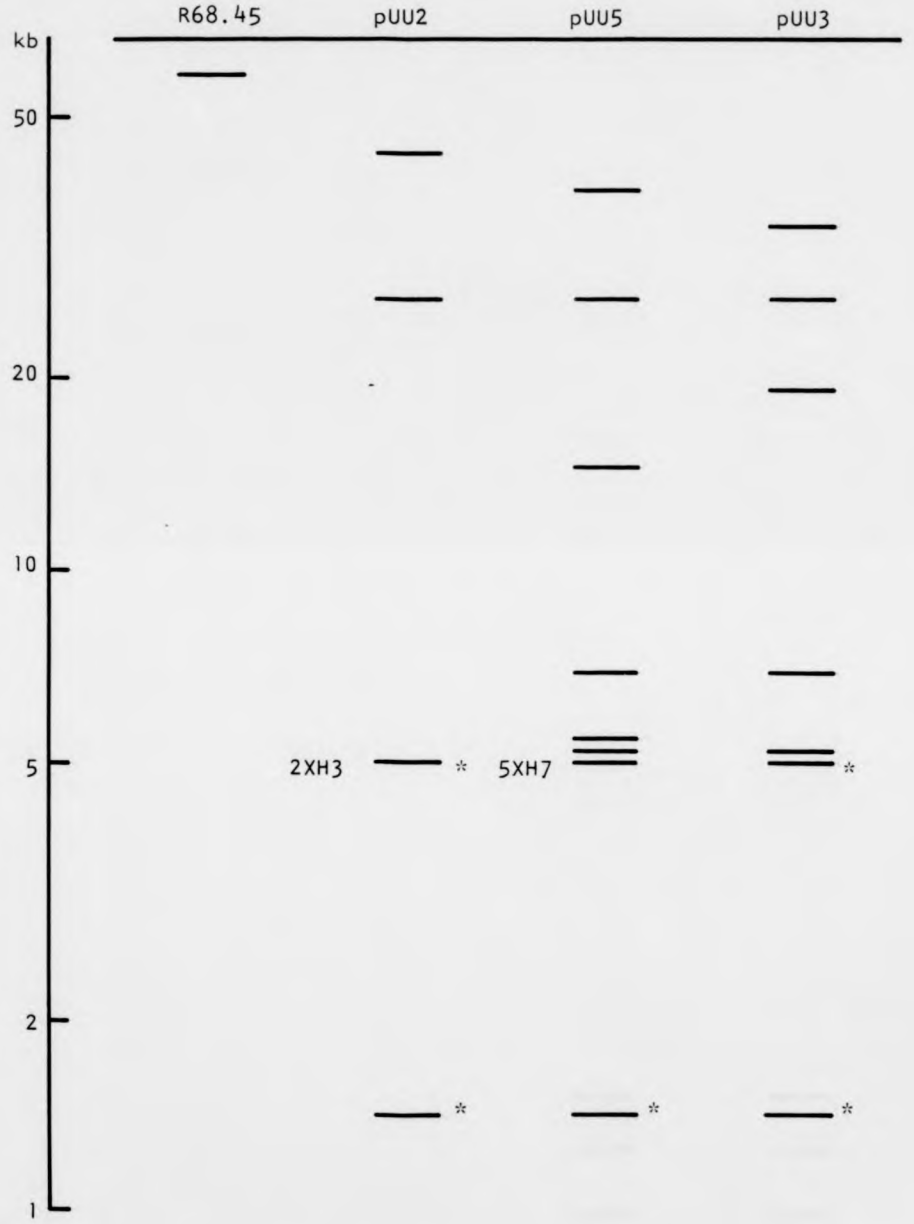
b. HindIII

c. EcoRI/HindIII

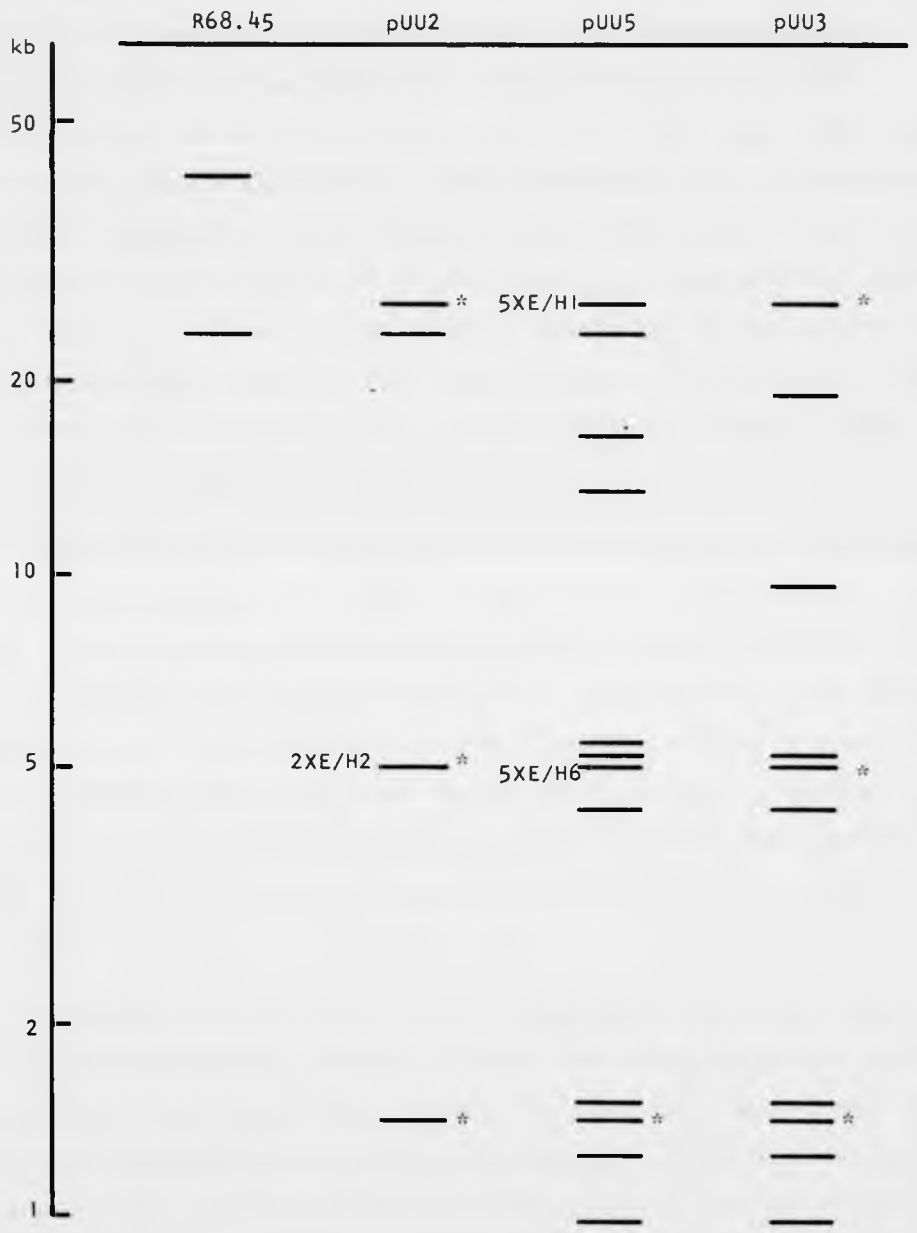
a. EcoR1



b. Hind III



c. EcoRI/HindIII



6.6.3.6 Interpretation of restriction digest data for pUU5

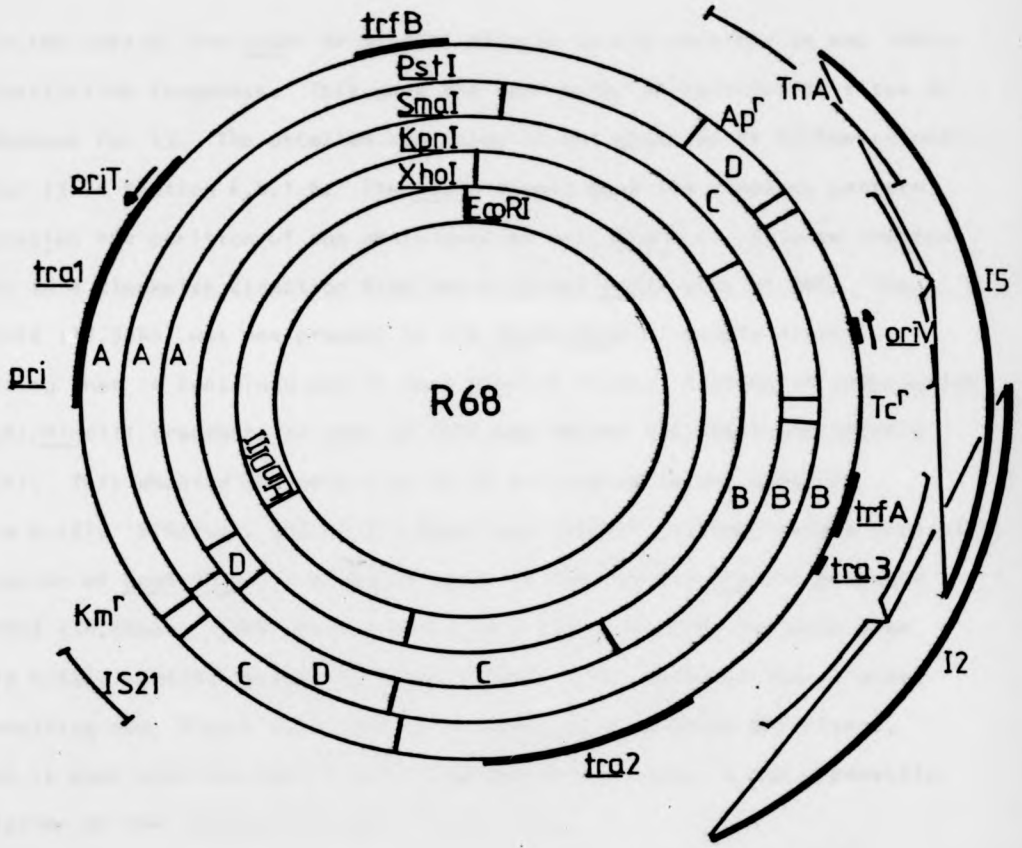
Restriction digests with a range of enzymes confirmed that pUU5 had conserved the bulk of I2 from pUU2. pUU5 had acquired a portion of DNA containing various restriction sites which gave restriction digest bands, many of which were the same size as bands in pUU3 associated with I3. Preliminary interpretation suggested that this acquired piece of DNA, known in pUU5 as I5, had inserted in the vicinity of I2 rather than at the opposite side of the plasmid as had been the case with I3 in pUU3. Estimations of the increase in size of pUU5 over pUU2 associated with these changes in the plasmid depended on the restriction enzyme used, from 11.54kb with PstI to 24.12kb with KpnI. The bulk of the estimates were around 20kb.

The SmaI digest data indicated that band 2SC from pUU2 (= SC in R68.45) was missing in pUU5 suggesting that I5 had inserted into this region of the plasmid. In the PstI and KpnI digests of pUU5 all the bands from R68.45 present in pUU2 were also present in pUU5. This indicated that I5 had inserted into that region of the plasmid derived from PB and KB in R68.45 into which I2 had also inserted. These three bands, SC, PB and KB, overlap in the two o'clock - three o'clock region of R68.45, suggesting that it was the region derived from this which was modified by the acquired portion of DNA, I5, in pUU5 (Figure 6.19).

The digest data indicated that I2 was slightly modified in most cases by the conversion of a double band to a single. No internal modification of I2 was detected from the PstI data. However the identification of a band as a double or a single was rarely unambiguous, especially if the band was not in close proximity to others with which to compare it. In the case of the PstI and KpnI digests bands which had been identified as flanking I2 were also missing, 2XPI and 2KC' respectively. Probably it was these bands into which the newly acquired DNA, I5, was inserted. Because of the slight

FIGURE 6.19

Map of R68 indicating region of insertion of I2 in pUU2 and I5 in pUU5


trfB
PstI
SmaI
KpnI
XhoI
EcoRI
HindIII
oriT
tra1
pri
A
A
A
D
C
B
B
B
C
D
C
D
Apr
TnA
I5
oriV
Tcr
trfA
tra3
I2
tra2
Kmr
IS21
R68

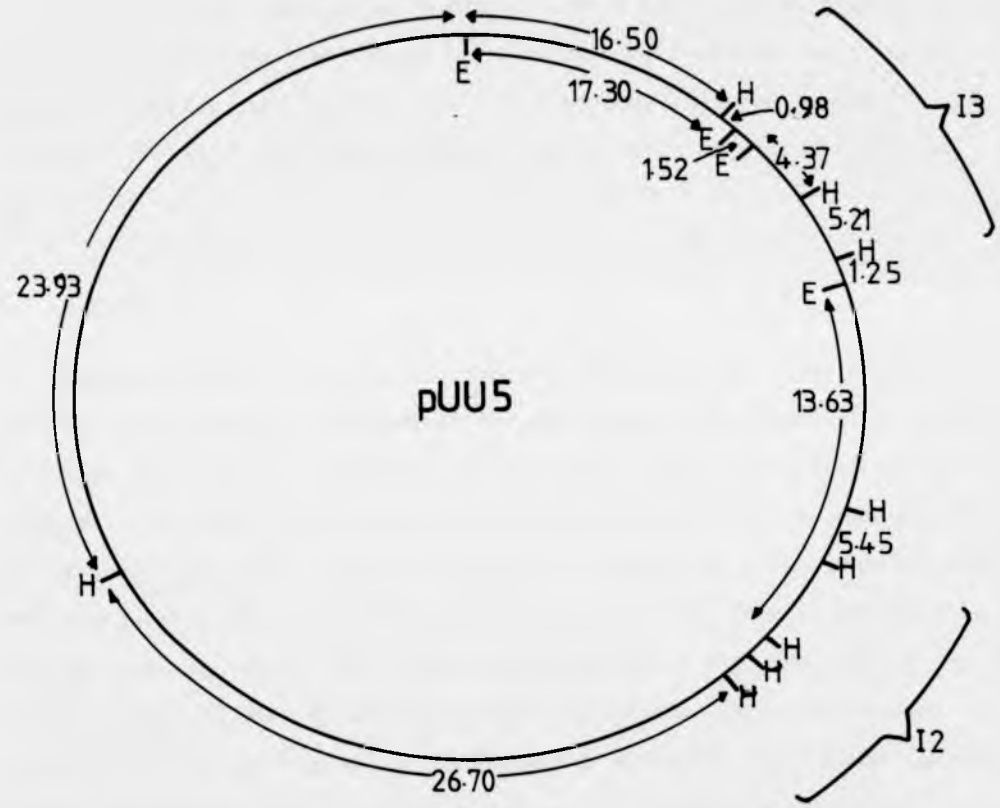
modification to I2 and the close proximity of I5 to I2 it was not always possible to conclusively identify all the new bands as being associated with one insert or the other. However because of the similarity in digest patterns between the I3 associated bands in pUU3 and some of the new bands in pUU5 it was possible to ascribe a core set of bands to I5, which was presumably very similar to I3.

In the case of the EcoRI or HindIII digests it was possible to map these core restriction fragments. This gave the same order of restriction sites as were deduced for I3. The detailed reasoning is not given as it follows closely that for I3 in Section 6.6.1.6. The EcoRI digest gave the simplest pattern, this enabled the position of the anticlockwise most EcoRI of I5 to be located 17.30kb in a clockwise direction from the original EcoRI site of R68. The band 5XE2 (17.30kb) was not present in the EcoRI/HindIII double digest, suggesting that it contained one or more HindIII sites. A probable combination of EcoRI/HindIII fragments to make up 5XE2 was 5XE/H2 (16.50kb) and 5XE/H10 (0.98kb). This enabled the core area to be orientated in the plasmid (Figure 6.20). Similarly 5XE/H3 (13.63kb) and 5XE/H10 (1.25kb) were a probable combination of EcoRI/HindIII produced bands to make up the HindIII produced band 5XH3 (14.56kb). 5XH3 would then project clockwise from the core area (Figure 6.20). 5XE/H4 (5.45kb) probably linked this region to the I2 area. The resulting map, Figure 6.20, can by no means be considered definitive, however it does give the most likely arrangement of I2 and I5 and a possible description of the intervening restriction sites.

It was more difficult to suggest positions for even a few of the new restriction sites in pUU5 for other enzymes. It is probably best not to attempt to do so. However, as mentioned above, these digests do confirm the presence in pUU5 of I5, an extra piece of DNA very similar to I3 from pUU3. This extra piece of DNA was inserted in the plasmid within the region represented by the overlapping SmaI SC and KpnI KB fragment of R68.

FIGURE 6.20

Map of pUU5 with probable EcoRI and HindIII sites



Not to scale.

E: EcoRI

H: Hind III

The region into which I5 had inserted contained some mapped genes including the genes for tetracycline resistance (Thomas, 1981a). P. putida PPW11, the organism containing pUU5, proved to be tetracycline sensitive. This suggested that the modifications associated with I5 had inactivated this gene. pUU5 was incapable of conjugal transfer. It is probable that the tra3 gene was inactivated either by I5 or by changes connected with I2.

It was not possible to prove whether or not pUU5 carried dehI as it was not possible to successfully mate or transform the plasmid. Restriction digests indicated that the bulk of I2 in pUU5 was unaltered compared to I2 in pUU2. Therefore it seemed probable that I2 still carried a functional dehI.

6.7 Discussion

P. putida PPW2 harbouring the R-prime pUU2 was grown under phosphate limitations on 2MCPA in a chemostat for 2600 hours. Five derivative strains were isolated from the chemostat culture based on PstI digestions of their plasmids. A strain indistinguishable by PstI digestion of its plasmid from the parental strain persisted throughout the experiment. Two other strains PPW9, containing pUU3, and PPW11 containing pUU5, contained in addition to the dehI bearing insert, I2, a second extra piece of DNA, known as I3 and I5 respectively. I3 and I5 contained common restriction patterns but were located in different regions of the plasmid. A fourth strain PPW10 contained a plasmid which had lost the bulk of I2, but it produced dehalogenase I and was still capable of growth on 2MCPA. The fifth strain, PPW12, contained no plasmid; it had lost the antibiotic resistances associated with the plasmid, but it produced dehalogenase I enabling it to grow on 2MCPA.

No one derivative strain came to dominate the chemostat population. Certainly the parental strain PPW2 was not out competed and persisted throughout the experiment. It is worthy of note that though the chemostat isolated

strains including the parental-like PPW2* had different specific growth rates from the original strain PPW2, these growth rates were higher than that for PPW2 and virtually identical for all the derivative strains. Therefore these batch growth experiments did not show any significant growth advantage to any particular derivative strain. It is worth pointing out that PPW11 was only isolated on one occasion, and that the plasmid minus strain, PPW12, though isolated from the chemostat over a considerable period of time, was not detected in the chemostat population during the latter part of the experiment.

The presence of a non-essential plasmid usually confers a competitive disadvantage to its host compared to plasmid minus strains (Melling et al., 1977; Wonters et al., 1978; Adams et al., 1979; Godwin and Slater, 1979; Jones and Primrose, 1979). Growth of a plasmid containing strain under limiting conditions in most cases led to the virtual elimination of the plasmid containing population. However Godwin and Slater (1979) observed that plasmid fragmentation occurred; certain plasmid markers were lost while others were retained. Competition experiments showed that E. coli containing the derivative plasmid TP120A outcompeted an isogenic strain containing the original plasmid TP120. The loss of plasmid markers in TP120A was shown to be associated with a 40% reduction of the plasmids' overall size compared with the parental TP120.

The chemostat culture examined here though similar was not identical to the experiments mentioned above. Though there was no selection for the plasmid's native antibiotic markers there was selection for the dehalogenase genes carried on the insert of foreign DNA, I2. Two of the derivative strains had lost plasmid DNA, PPW10 and PPW12. PPW10, the organism containing pUU4, had lost the bulk of I2 the dehI carrying insert. It was also tetracycline sensitive though no modification in this region was detected. It had regained a size comparable to the original plasmid R68. Certainly no fragmentation

of R68 plasmid features was observed. R68 may be particularly resistant to fragmentation. Attempts to reduce the size of R68 or its relatives by *in vitro* techniques have generally met with limited success (Hedges *et al.*, 1976; Meyer *et al.*, 1977b; Haque, 1979; Thomas *et al.*, 1982). This lack of success was probably due to the genes essential for maintenance of the plasmid not being highly clustered. However Ditta *et al.* (1980) did manage to obtain a derivative of RK2 of 20kb by a series of *in vitro* techniques. It is not possible to determine how closely *in vitro* techniques mimic the pressures on a plasmid in a chemostat. PPW10 still synthesised dehalogenase I, so presumably *dehI* had become translocated from the plasmid to the chromosome. It has been suggested that *dehI* is on a transposon (Slater, Weightman and Hall, unpublished observations) which could be the mechanism for this gene translocation. PPW10 was the most frequently isolated derivative strain (Figure 6.2), therefore though it was not capable of outcompeting the other strains it was successfully maintained in the population.

PPW12, the plasmid minus strain, could be seen as the next step from PPW10, i.e. translocation of *dehI* followed by elimination of the plasmid. However though PPW12 was isolated on frequent occasions it did not outcompete the other strains.

Analysis of PPW10 and PPW12 suggested that I2 was translocated to the bacterial chromosome. No derivatives of PPW2 were detected in the chemostat in which I2 had been translocated within the plasmid. A phenomenon which had been observed to occur during mating experiments with pUU2 described in Chapter 5.

PPW9 and PPW11 stood out from the others. Not only had their plasmids retained I2 they had increased in size over pUU2 by about 20kb. This extra piece of DNA had been inserted opposite I2 in pUU3 (from PPW9), and close to I2 in an anticlockwise direction in pUU5 (from PPW11). These two new inserts shared common restriction patterns which did not resemble restriction patterns

from pUU2. Therefore this novel DNA was probably not of plasmid origin but must have originated from the bacterial chromosome. It is tempting to postulate that this novel piece of DNA was an insertion sequence. In pUU5 its insertion may have led to the inactivation of the tetracycline resistance gene and tra3, and in pUU3 to the inactivation of tra1. It is interesting that this new insert was only observed in two sites in the plasmid. Perhaps the new insert requires a specific sequence in order to insert.

A strain indistinguishable from PPW2 by PstI plasmid digestion persisted at a significant frequency throughout the experiment, known as PPW2*. PPW2* did show an increased growth rate compared with PPW2, comparable with that of the other derivative strains. It is difficult to explain the persistence of a mixed derivative population, including the original strain, over such a long period of time, without one derivative outcompeting the rest. Especially in the light of the genetic rearrangements which were observed. It is necessary to postulate that in most cases these genetic rearrangements had no effect on the competitive fitness of the strains.

This long term chemostat experiment has demonstrated a few of the potential interactions between the bacterial chromosome and plasmids under conditions which approximate more to those pertaining in the natural environment than those usually studied in batch culture. The plasmid was shown to be capable of acquiring DNA, presumably chromosomally borne insertion sequences; inserted DNA was capable of translocation from the plasmid to the chromosome, and the plasmid could subsequently be lost.

CHAPTER 7

THE FORMATION OF R-PRIMES CARRYING *dehI* DERIVED FROM PPW12

Chapter 7

THE FORMATION OF R-PRIMES CARRYING dehI DERIVED FROM PPW12

7.1 Introduction

P. putida PPW12 a plasmid-minus derivative of PPW2 was isolated from a phosphate limited chemostat as described in Chapter 6. PPW12 produced dehalogenase I enabling it to utilize 2MCPA as sole carbon and energy source. dehI originally from P. putida PP3 had presumably integrated into the chromosomal DNA from the R-prime pUU2 and the plasmid had subsequently been lost in the formation of PPW12 from PPW2.

There were two purposes in producing R-primes from PPW12. Firstly a comparison of their restriction patterns with those of pUU2 would enable the origin of the dehalogenase gene in PPW12 to be established. Its derivation from dehI on pUU2 could be confirmed or denied. Secondly P. putida PP3 had not been an ideal donor for R-prime production as it modified R68.44 and R68.45 by the insertion of I1-like inserts and IS21 was always lost (Chapters 3 and 4). The use of a genuine R68.45 in a strain which did not modify plasmids would enable the comparison of the formation of R-primes carrying dehI with the results of others.

7.2 The introduction of R68.45 into PPW12

Pseudomonas aeruginosa PA025 (R.68.45) and P. aeruginosa PA02 were kindly provided by Dr. A.J. Weightman, University of Geneva (Table 7.1). Fresh colonies of PA025 (R.68.45) were checked for enhanced chromosome mobilizing ability (E.C.M.) by plate mating with PA02 (see Chapter 2, Methods for details). The plasmids from colonies which showed E.C.M. by this method were extracted by the rapid method and digested with PstI and SmaI to confirm the presence of the second copy of IS21 in R68.45. All the

TABLE 7.1

BACTERIAL STRAINS AND PLASMIDS USED OR DERIVED IN CHAPTER 7

Strain	Species[1]	Plasmid	Genotype/Phenotype	Derivation
PPW12	P.p.	-	Trp^-,2Mcpa$^+$,Sm^R	PPW2 in chemostat
PA025	P.a.	R.68.45	leu10,argF10,Ap^R, Km^R,Tc^R	A.J. Weightman
PA02	P.a.	-	Ser^-	A.J. Weightman
PPW4	P.p.	-	Leu^-,R^-,M^+,Nal^R	Mutant of PaWKT2440
PPW37	P.p.	R.68.45	Trp^-,2Mcpa$^+$,Sm^R,Ap^R, Km^R,Tc^R	PA025(R.68.45) X PPW12
PAU2	P.a.	-	leu38,rmoll1,Rif^R	Mutant of PA01162
PPW38	P.p.	pUU30	Leu^-,2Mcpa$^+$,R^-,M^+,Nal^R, Ap^R,Km^R,Tc^R	PPW37 X PPW4
PPW39	"	pUU31	"	"
PPW40	"	pUU32	"	"
PPW41	"	pUU33	"	"
PPW42	"	pUU34	"	"
PPW43	"	pUU35	"	"
PPW44	"	pUU36	"	"
PPW45	"	pUU37	"	"
PPW46	"	pUU38	"	"
PPW47	"	pUU39	"	"
PAU11	P.a.	pUU40	Leu^-,2Mcpa$^+$,R^-,M^+,Nal^R, Ap^R,Km^R,Tc^R	PPW37 X PAU2
PAU12	"	pUU41	"	"
PAU13	"	pUU42	"	"
PAU14	"	pUU43	"	"

Strain	Species[1]	Plasmid	Genotype/Phenotype	Derivation
PAU15	P.a.	pUU44	Leu^-,2Mcpa$^+$,R^-,M^+,Nal^R, Ap^R,Km^R,Tc^R	PPW37 X PAU2
PAU16	"	pUU45	"	"
PAU17	"	pUU46	"	"
PAU18	"	pUU47	"	"
PAU19	"	pUU48	"	"
PAU20	"	pUU49	"	"
PAU21	"	pUU50	"	"
PAU22	"	pUU51	"	"

1. Species: P.p.: Pseudomonas putida

P.a.: Pseudomonas aeruginosa

E.C.M. colonies screened contained genuine R68.45 plasmids.

One of these E.C.M. colonies of PA025 (R.68.45) was membrane mated with PPW12. Transconjugants were selected for on minimal medium supplemented with 2MCPA, tryptophan and Km ($100\mu gml^{-1}$). Transconjugants were obtained at a frequency of 9.2×10^{-4} per donor cell.

After purification a number of transconjugants were checked for the presence of E.C.M. plasmids by plate mating with PPW4, a leucine auxotrophic mutant of P. putida PaW KT2440. The plasmids from six transconjugants which gave positive results by this test were extracted by the rapid method and digested with PstI and SmaI. All six plasmids gave bands characteristic of the presence of the second copy of IS21 in R68.45. One of these transconjugants was designated PPW37 and was used in further experiments.

7.3 Formation of R-primes from P. putida PPW37

P. putida PPW37 (PPW12 containing R68.45) was membrane mated with P. putida PPW4 and separately with P. aeruginosa PAU2 (Table 7.1). Transconjugants were selected for on minimal media supplemented with 2MCPA, leucine and naladixic acid ($100\mu gml^{-1}$); and 2MCPA, leucine and Rif ($250\mu gml^{-1}$) respectively. In the mating of PPW37 with PPW4 transconjugants were obtained at a frequency of 2.8×10^{-4} per donor cell. In the mating of PPW37 and PAU2 transconjugants were obtained at a frequency of 6.7×10^{-6} per donor cell. These transconjugants were purified and checked for autotrophic markers and sensitivity to streptomycin. They all proved to have the correct recipient phenotype.

7.4 Analyses of Transconjugants

Ten transconjugants from the mating of PPW37 with PPW4 and twelve from the mating of PPW37 with PAU2 were chosen for further study (Table 7.1).

7.4.1 Antibiotic resistance profiles of transconjugants

The transconjugants were screened for the plasmid borne antibiotic resistance markers by pricking the colonies on to nutrient agar plates containing Ap ($300\mu gml^{-1}$), Km ($300\mu gml^{-1}$) or Tc ($300\mu gml^{-1}$) and scoring for growth over forty-eight hours (Table 7.2). Seven of the twenty-two transconjugants were sensitive to tetracycline. Two of these were also sensitive to kanamycin.

7.4.2 Restriction digests of the plasmids from the mating PPW37 X PPW4

The plasmids from PPW38 - PPW47, pUU30-39, were extracted by the rapid method and digested with PstI and SmaI. The digests were electrophoresed and the fragments sized and compared with those of R68.45 and pUU2 (Plate 7.1; Table 7.3; Figure 7.1). The rapid method did not reveal any restriction fragments smaller than 0.6kb, nor was it possible to identify unambiguously in many cases the smaller bands as doubles or singles.

The digest patterns of the new plasmids from PPW38-47, pUU30-39, showed similarities to those of R68.45 and the R-prime pUU2. Three of the new plasmids had retained the second copy of IS21, insofar as this was indicated by PstI and SmaI digests; these were pUU32, pUU33 and pUU35. The PstI digest of pUU39 appeared to indicate the presence of IS21, but the SmaI digest did not.

The restriction digests indicated an increase in size of all the new plasmids over R68.45 of about 10kb. By comparing the digest patterns with those of R68.45 it was possible to determine where in the published map of the plasmid the extra DNA had been inserted (Figure 7.2; Table 7.4). In most cases it was possible to identify the regions of insertion which were in various locations scattered around the plasmid. In other cases this was not possible, in some of these this could be due to the difficulty of identifying small size differences in the largest bands especially as only

TABLE 7.2

ANTIBIOTIC RESISTANCE PROFILES OF NEW TRANSCONJUGANTS

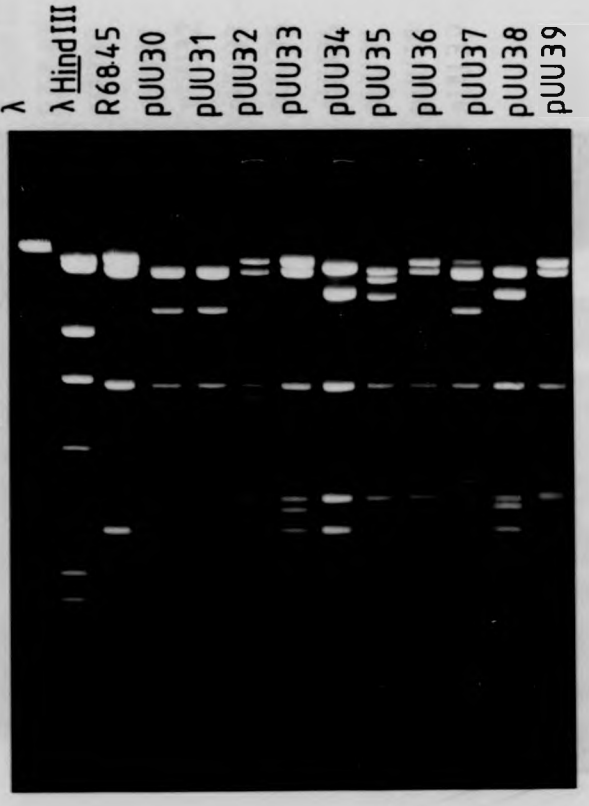
Strain	Plasmid	Ap[1]	Km	Tc
PPW38	pUU30	+	+	+
PPW39	pUU31	+	+	-
PPW40	pUU32	+	+	+
PPW41	pUU33	+	+	+
PPW42	pUU34	+	+	-
PPW43	pUU35	+	+	+
PPW44	pUU36	+	+	-
PPW45	pUU37	+	+	+
PPW46	pUU38	+	+	-
PPW47	pUU39	+	+	-
PAU12	pUU41	+	-	-
PAU13	pUU42	+	+	+
PAU14	pUU43	+	+	+
PAU15	pUU44	+	+	+
PAU16	pUU45	+	+	+
PAU17	pUU46	+	+	+
PAU18	pUU47	+	+	+
PAU19	pUU48	+	-	-
PAU20	pUU49	+	+	+
PAU21	pUU50	+	+	+
PAU22	pUU51	+	+	+

1. +: growth after 48h.
   -: no growth after 48h.

PLATE 7.1

Restriction digests of plasmids R68.45 and pUU30-39 with PstI and SmaI

a. PstI


λ
λ HindIII
R6845
pUU30
pUU31
pUU32
pUU33
pUU34
pUU35
pUU36
pUU37
pUU38
pUU39

a. PstI

λ
λ HindIII
R68.45
pUU30
pUU31
pUU32
pUU33
pUU34
pUU35
pUU36
pUU37
pUU38
pUU39

b. SmaI

λ
λ HindIII
R68.45
pUU30
pUU31
pUU32
pUU33
pUU35
pUU36
pUU37
pUU38
pUU39
R68.45
pUU34
λ HindIII
λ

b. Sma I

λ
λ Hind III
R68.45
pUU30
pUU31
pUU32
pUU33
pUU35
pUU36
pUU37
pUU38
pUU39
R68.45
pUU34
λ Hind III
λ

TABLE 7.3

FRAGMENT SIZES OF RESTRICTION DIGESTS OF PLASMIDS R68.45, pUU2, pUU30-39 WITH PstI and SmaI

a. PstI

Strain		PPW2	PPW38	PPW39
Plasmid	R68.45	pUU2	pUU30	pUU31
	32.67	32.67	24.84 *	24.84 *
	24.84	14.82	13.67	13.67
	6.52	12.38	6.52	6.52
	2.77	6.52	3.23	3.23
	1.34	3.23 *	2.77	2.77
	0.77 *	2.77	1.19	1.19
	0.66	1.19 *	0.87	0.87
		0.87 *	0.77	0.77
		0.77	0.66	0.66
		0.66	0.60	0.60
		0.60 *		
		0.29 *		
	70.34kb	82.95kb	79.96kb	79.96kb

(a) continued

Strain	PPW40	PPW41	PPW42	PPW43
Plasmid	pUU32	pUU33	pUU34	pUU35
	32.67	32.67	27.79	24.84
	24.84	24.84	17.54 *	21.65
	6.52	6.52	6.52	17.54
	5.94	3.23	3.23	6.52
	4.37	3.08	2.77	3.23
	3.23	2.77	1.19 *	2.77
	1.34	1.34	0.87 *	1.34
	1.19	1.19	0.77	1.19
	0.87	0.87	0.66	0.87
	0.77 *	0.77 *	0.60	0.77 *
	0.66	0.66		0.66
	0.60	0.60		0.60
	83.77kb	79.31kb	81.54kb	81.98kb

Strain	PPW44	PPW45	PPW46	PPW47
Plasmid	pUU36	pUU37	pUU38	pUU39
	32.67	24.84 *	24.84	32.67
	25.6	13.67	21.65 *	24.84
	6.52	6.52	6.52	6.52
	3.50	3.60	3.23	3.30
	3.23 *	3.23	3.08	3.23
	2.77	2.77	2.77	2.77
	1.19	1.19	1.19	1.34
	0.87	0.77	0.87	1.19
	0.77	0.66	0.77	0.87
	0.66	0.60	0.66	0.77 *
	0.60		0.60	0.66
				0.60
	81.61kb	82.69kb	87.83kb	78.76kb

b. SmaI

Strain		PPW2	PPW38	PPW39
Plasmid	R68.45	pUU2	pUU30	pUU31
	26.52	26.52	24.79	24.79
	17.55	16.60	17.55	17.55
	15.10	15.10	15.10	15.10
	7.15	8.45	9.97	9.97
	2.12	7.15	7.15	7.15
	0.76	3.80 *	2.51	2.51
		2.51 *	0.76	0.76
		0.76		
	69.20kb	87.20kb	77.83kb	77.83kb

Strain	PPW40	PPW41	PPW42	PPW43
Plasmid	pUU32	pUU33	pUU34	pUU35
	26.52	29.10	18.50	17.55 *
	17.55 *	17.55	17.55	15.10 *
	7.15	15.10	15.10	7.15
	4.71	7.15	9.97	3.76
	3.76	3.58	8.80	2.51 *
	2.51 *	2.51	7.15	2.12
	2.12	2.12	3.76	0.76
	0.76	0.76	2.51 *	
			0.76	
	85.14kb	77.87kb	86.61kb	84.11kb

b. continued

Strain	pPW44	PPW45	PPW46	PPW47
Plasmid	pUU36	pUU37	pUU38	pUU39
	26.52	24.79	19.74	26.52
	17.55	17.55	17.55	17.55
	16.73	15.10	13.29	15.10
	9.49	9.47	9.49	7.15
	7.15	7.15	7.15	5.34
	2.51	6.16	3.76	3.86
	0.76		2.51 *	2.51
	———	———	0.76	0.76
	80.71kb	80.72kb	76.76kb	78.80kb

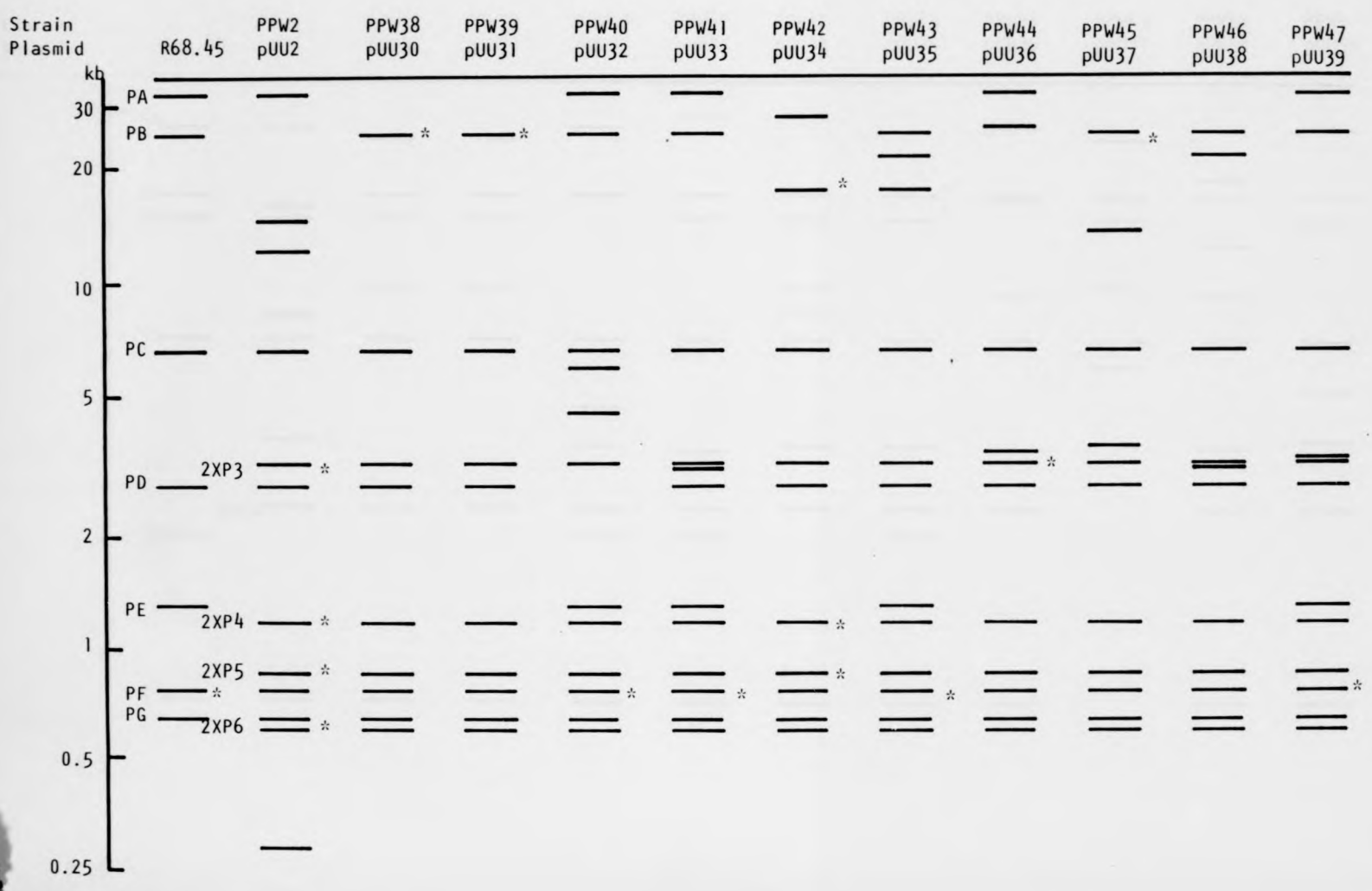
FIGURE 7.1

DIAGRAMS OF RESTRICTION DIGESTS OF R68.45, pUU2 AND DERIVATIVE PLASMIDS WITH *PstI* AND *SmaI*

a. *PstI*

b. *SmaI*

a. PstI


Strain
Plasmid
R68.45
PPW2 pUU2
PPW38 pUU30
PPW39 pUU31
PPW40 pUU32
PPW41 pUU33
PPW42 pUU34
PPW43 pUU35
PPW44 pUU36
PPW45 pUU37
PPW46 pUU38
PPW47 pUU39
kb
30
20
10
5
2
1
0.5
0.25
PA
PB
PC
PD
PE
PF
PG
2XP3
2XP4
2XP5
2XP6

b. SmaI

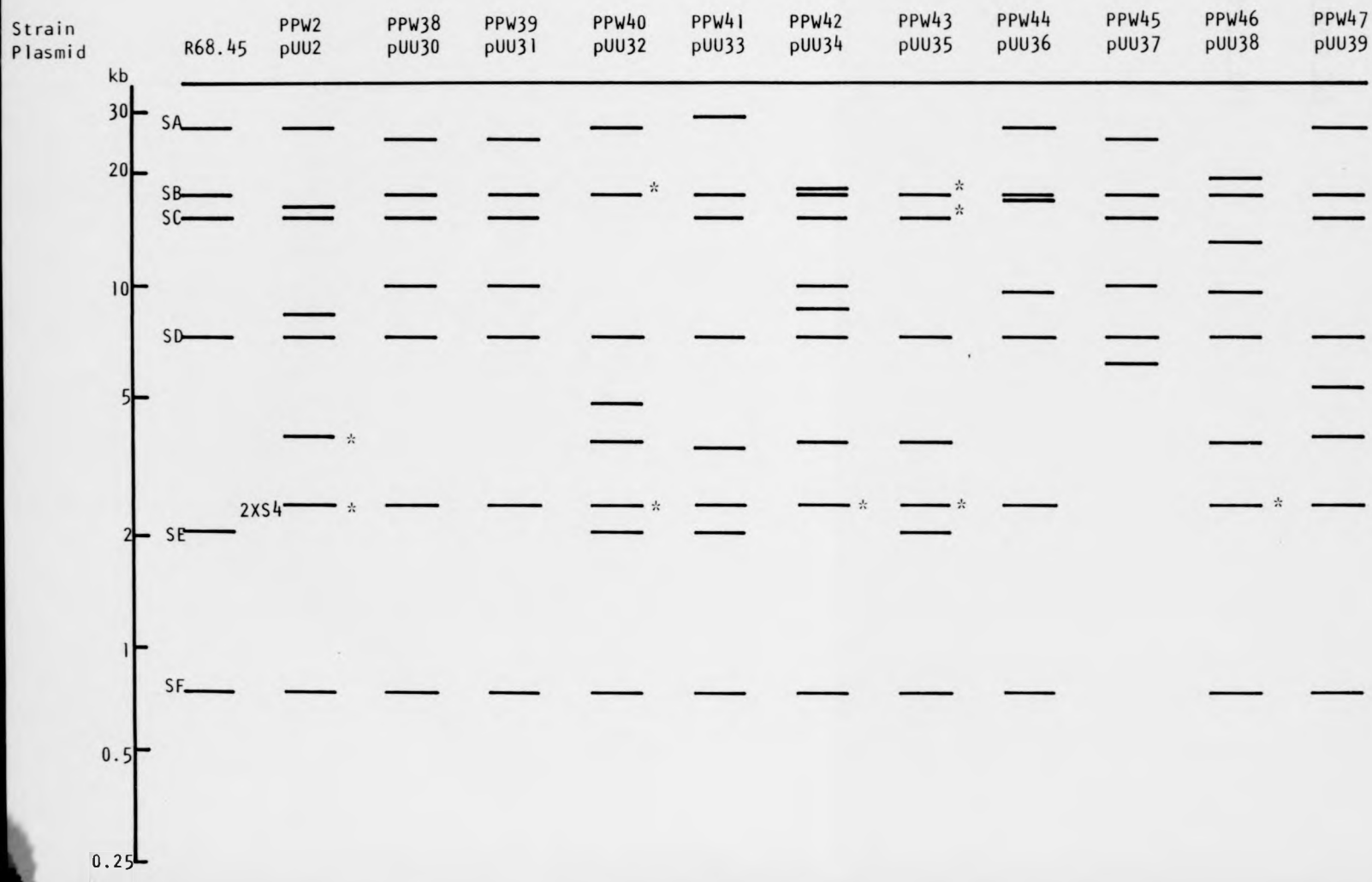

Strain
Plasmid
R68.45
PPW2 pUU2
PPW38 pUU30
PPW39 pUU31
PPW40 pUU32
PPW41 pUU33
PPW42 pUU34
PPW43 pUU35
PPW44 pUU36
PPW45 pUU37
PPW46 pUU38
PPW47 pUU39
kb
30
20
10
5
2
1
0.5
0.25
SA
SB
SC
SD
SE
SF
2XS4

FIGURE 7.2

MAP OF R68

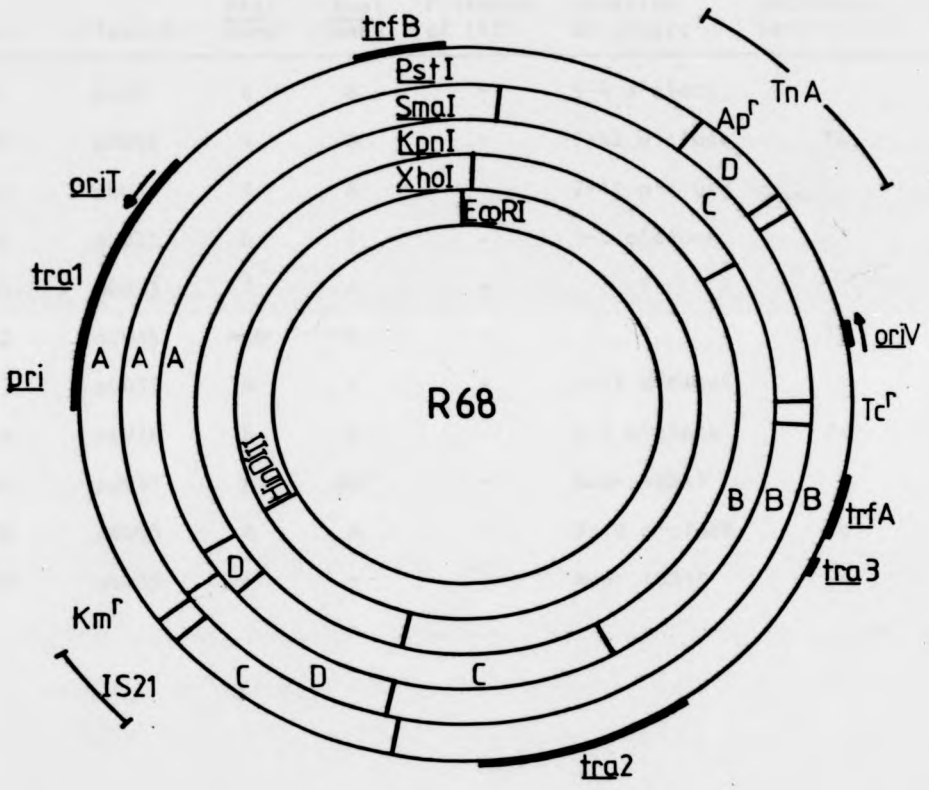

trfB
PstI
SmaI
KpnI
XhoI
EcoRI
TnA
Ap^r
D
C
oriV
Tc^r
trfA
tra3
B B B
tra2
C D C
Km^r
IS21
D
HindIII
A A A
pri
tra1
oriT
R68

TABLE 7.4

R68.45-DERIVED BANDS MISSING IN PLASMIDS pUU2, pUU30-39

Strain	Plasmid	PstI band	SmaI band	Presence of IS21	Location of insert	Antibiotic sensitivity
PPW2	pUU2	B	B	-	3-4 o'clock	
PPW38	pUU30	A	A	-	7-12 o'clock	Tc
PPW39	pUU31	A	A	-	7-12 o'clock	
PPW40	pUU32	D	C	+	1-2 o'clock	
PPW41	pUU33	?	A	+	?	
PPW42	pUU34	A&B	A	-	?	Tc
PPW43	pUU35	A	A	+	7-12 o'clock	
PPW44	pUU36	B	C	-	2-3 o'clock	Tc
PWW45	pUU37	A	A&F	-	Near IS21?	
PPW46	pUU38	A	A	-	7-12 o'clock	Tc
PPW47	pUU39	-	-	+/-	Near IS21?	Tc

one gel was run in such a manner as to reveal as many bands as possible. This may be the explanation for the results from pUU33 and pUU34. The PstI digest suggests that the latter had undergone two modifications in the PA and the PB bands. pUU37 showed modifications in the PA, SA and SF bands this might be due to an insertion into or near to IS21. The digests for pUU39 showed the presence of all R68.45 bands but ambivalent results on the presence of the second copy of IS21. This could indicate an insertion associated with IS21.

The new plasmids showed striking similarities to the original R-prime pUU2. In virtually all cases the PstI bands 2XP3, 2XP4, 2XP5 and 2XP6 and the SmaI band 2XS4 were present though not always as double bands. The plasmids contained other new bands but there was no consistancy about these. There appeared to be little correlation between the position and the insert and any antibiotic sensitivity of the host strain. Except in the case of PPW44 containing pUU36 which had acquired an insert in the 2-3 o'clock region and had become tetracycline sensitive.

7.4.3 Restriction digests of the plasmids from the mating PPW37 X PAU2

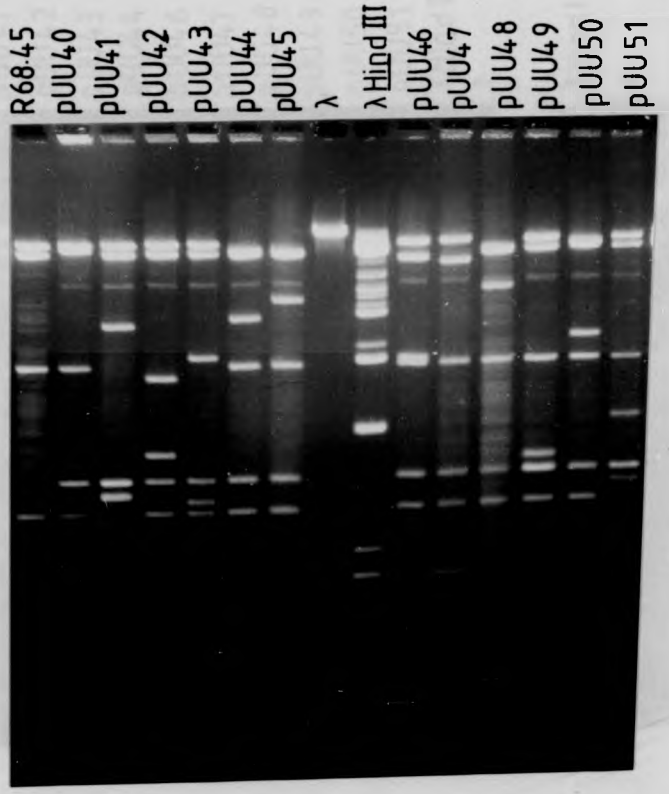
The plasmids from PAU11-22, pUU40-51, were extracted by the rapid method and digested with PstI and SmaI. The digests were electrophoresed and the fragments sized and compared with those of R68.45 and pUU2 (Plate 7.2, Table 7.5, Figure 7.3).

The digest patterns of the plasmids from PAU11-22, pUU40-51, showed similarities to those of R68.45 and pUU2. All but one (pUU41) of the new plasmids had retained the second copy of IS21, insofar as this is confirmed by PstI and SmaI digests. The new plasmids examined here were about 10kb larger than R68.45. By comparing the digests with those of R68.45 and the published map of R68.45 it was possible to determine roughly where, in most cases, the new DNA band inserted in the plasmid (Figure 7.2; Table 7.6)

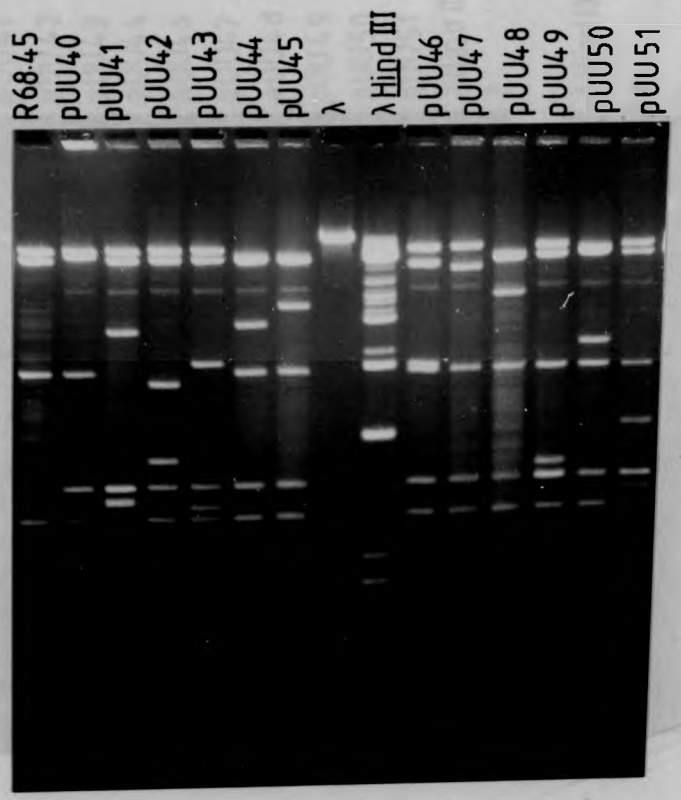
PLATE 7.2

RESTRICTION DIGESTS OF PLASMIDS R68.45 AND pUU40-51 WITH *PstI* AND *SmaI*

a. PstI


R68.45
pUU40
pUU41
pUU42
pUU43
pUU44
pUU45
λ
λ HindIII
pUU46
pUU47
pUU48
pUU49
pUU50
pUU51

a. PstI


R68.45
pUU40
pUU41
pUU42
pUU43
pUU44
pUU45
λ
λ HindIII
pUU46
pUU47
pUU48
pUU49
pUU50
pUU51

b. SmaI

R68.45
pUU40
pUU41
pUU42
pUU43
pUU44
pUU46
pUU47
pUU48
pUU49
pUU50
pUU51
λ HindIII
λ

λ HindIII
λ
R68.45
pUU2
pUU45

b. Sma I

R68.45
pUU40
pUU41
pUU42
pUU43
pUU44
pUU46
pUU47
pUU48
pUU49
pUU50
pUU51
λ Hind III
λ
λ Hind III
λ
R68.45
pUU2
pUU45

TABLE 7.5

FRAGMENT SIZES OF RESTRICTION DIGESTS OF PLASMID R68.45, pUU2, pUU40-51 WITH PstI AND SmaI

a. PstI

Strain		PPW2	PAU11	PAU12
Plasmid	R68.45	pUU2	pUU40	pUU41
	32.67	32.67	32.67	32.67
	24.84	14.82	26.50	24.84
	6.52	12.38	6.52	8.90
	2.77	6.52	3.23 *	3.23
	1.34	3.23 *	2.77	3.08
	0.77 *	2.77	1.34	1.19
	0.66	1.19 *	1.19	0.87
		0.87 *	0.87	0.66
		0.77	0.77 *	0.60
		0.66	0.66	
		0.60 *	0.60	
		0.29 *		
	70.34kb	82.95kb	81.12	76.04kb

a. continued

Strain	PAU13	PAU14	PAU15	PAU16
Plasmid	pUU42	pUU43	pUU44	pUU45
	32.67	32.67	24.84 *	24.84 *
	24.84	24.84	9.60	11.62
	5.90	7.01	6.52	6.52
	3.86	3.23	3.23	3.23
	3.23	3.00	2.77	2.77
	2.77	2.77	1.34	1.34
	1.34	1.34	1.19	1.19
	1.19	1.19	0.87	0.87
	0.87	0.87	0.77 *	0.77 *
	0.77 *	0.77 *	0.66	0.66
	0.66	0.66	0.60	0.60
	0.60	0.60		
	79.47kb	79.72kb	78.00kb	80.02kb

a. continued

Strain	PAU17	PAU18	PAU19	PAU20
Plasmid	pUU46	pUU47	pUU48	pUU49
	32.67	32.67	24.84 *	32.67
	21.63	20.73	13.49	24.84
	6.85	6.52	6.52	6.52
	6.52	3.23	3.23	3.76
	3.23	2.77	2.77	3.23 *
	2.77	2.01	1.34	2.77
	1.34	1.34	1.19	1.34
	1.19	1.19	0.87	1.19 *
	0.87	0.87	0.77 *	0.87
	0.77 *	0.77 *	0.66	0.77 *
	0.66	0.66	0.60	0.66
	0.60	0.60		0.60
	79.87kb	74.13kb	81.89kb	84.41kb

a. continued

Strain	PAU17	PAU18	PAU19	PAU20
Plasmid	pUU46	pUU47	pUU48	pUU49
	32.67	32.67	24.84 *	32.67
	21.63	20.73	13.49	24.84
	6.85	6.52	6.52	6.52
	6.52	3.23	3.23	3.76
	3.23	2.77	2.77	3.23 *
	2.77	2.01	1.34	2.77
	1.34	1.34	1.19	1.34
	1.19	1.19	0.87	1.19 *
	0.87	0.87	0.77 *	0.87
	0.77 *	0.77 *	0.66	0.77 *
	0.66	0.66	0.60	0.66
	0.60	0.60	____	0.60
	79.87kb	74.13kb	81.89kb	84.41kb

a. continued

Strain	PAU21	PAU22
Plasmid	pUU50	pUU51
	26.14	32.67
	24.84	24.84
	7.96	6.52
	6.52	4.60
	3.23	3.23 *
	2.77	2.95
	1.34	1.82
	1.19	1.34
	0.87	1.19 *
	0.77 *	0.87
	0.66	0.77 *
	0.60	0.66
	———	0.60
	77.66kb	87.25kb

b. SmaI

Strain		PPW2	PAU11	PAU12
Plasmid	R68.45	pUU2	pUU40	pUU41
	26.52	26.52	26.52	31.22
	17.55	16.60	17.55	17.55
	15.10	15.10	14.33	12.28
	7.15	8.45	8.67	9.81
	2.12	7.15	7.15	8.95
	0.76	3.80 *	3.67	2.51
		2.51 *	2.51	0.76
		0.76	2.12	
			0.76	
	69.20kb	87.20kb	83.28kb	83.08kb

Strain	PAU13	PAU14	PAU15	PAU16
Plasmid	pUU42	pUU43	pUU44	pUU45
	26.52	26.52	27.79	26.52
	17.55	17.55	17.55	17.55
	15.10	15.10	15.10	15.10
	9.97	8.23	7.15	9.03
	3.96	5.86	4.16	7.15
	2.51	2.51	2.51	2.51
	2.12	2.12	2.12	2.12
	0.76	0.76	0.76	0.76
	78.49kb	78.65kb	77.11kb	80.74kb

b. continued

Strain	PAU17	PAU18	PAU19	PAU20
Plasmid	pUU46	pUU47	pUU48	pUU49
	26.52	26.52	33.12	26.52
	17.55 *	15.10	17.55	17.55
	7.15	11.99	15.10	14.31
	4.95	7.15	9.97	8.67
	2.51	5.21	7.15	7.15
	2.12	2.51	3.67	3.67
	0.76	2.12	2.51	2.51
		0.76	2.12	2.12
			0.76	0.76
	79.11kb	71.36kb	91.95kb	83.26kb

Strain	PAU21	PAU22
Plasmid	pUU50	pUU51
	26.52	26.52
	17.55	17.55
	13.60	11.55
	9.03	10.67
	7.15	7.15
	2.86	6.01
	2.51	3.67
	2.12	2.51
	0.76	2.12
		0.76
	82.10kb	88.51kb

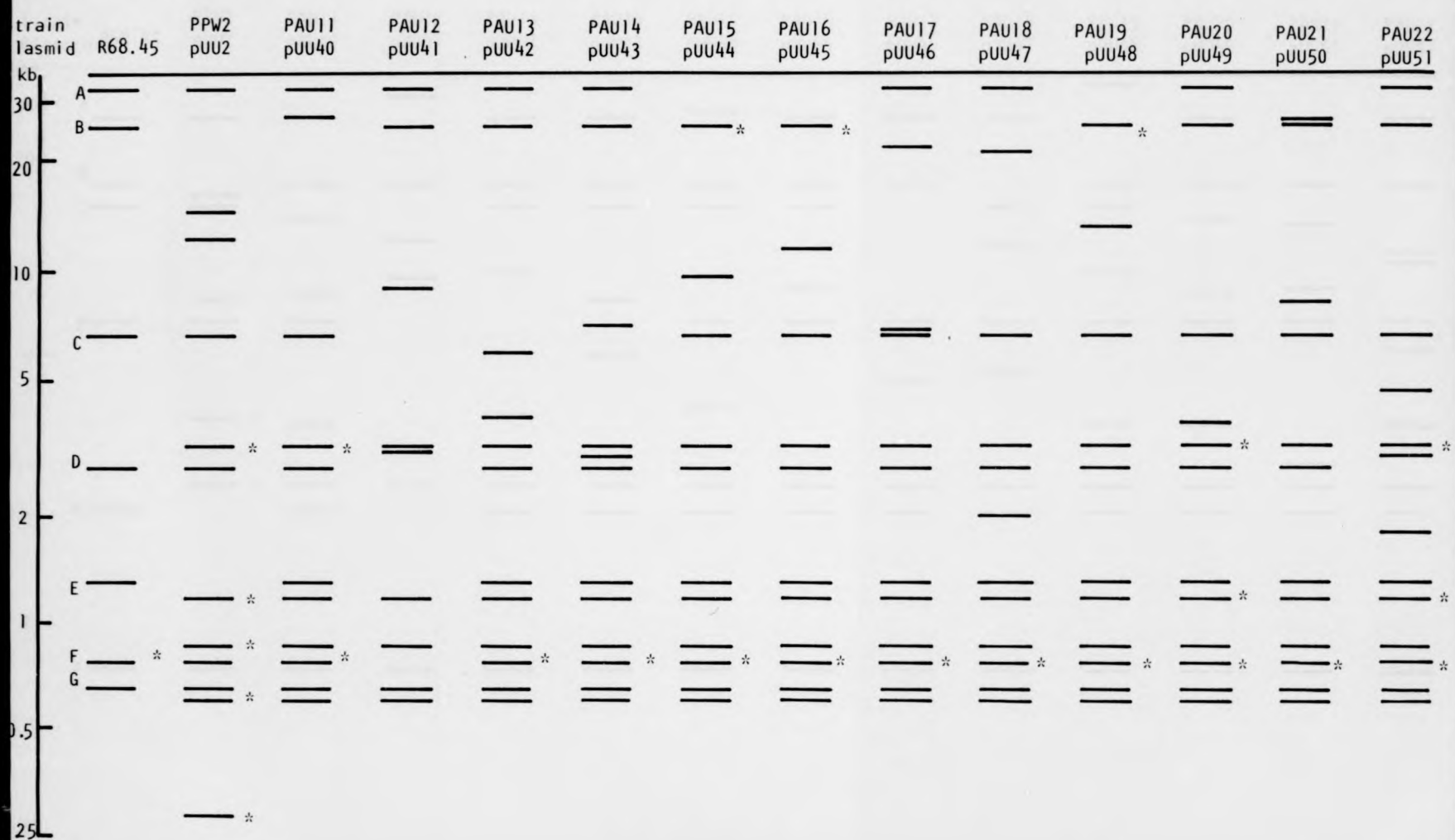
FIGURE 7.3

DIAGRAMS OF RESTRICTION DIGESTS OF R68.45, pUU2 AND DERIVATIVE PLASMIDS WITH *Pst*I AND *Sma*I

a. *Pst*I

b. *Sma*I

a. PstI


train
lasmid
R68.45
PPW2 pUU2
PAU11 pUU40
PAU12 pUU41
PAU13 pUU42
PAU14 pUU43
PAU15 pUU44
PAU16 pUU45
PAU17 pUU46
PAU18 pUU47
PAU19 pUU48
PAU20 pUU49
PAU21 pUU50
PAU22 pUU51
kb
30
20
10
5
2
1
0.5
25
A
B
C
D
E
F
G

b. SmaI

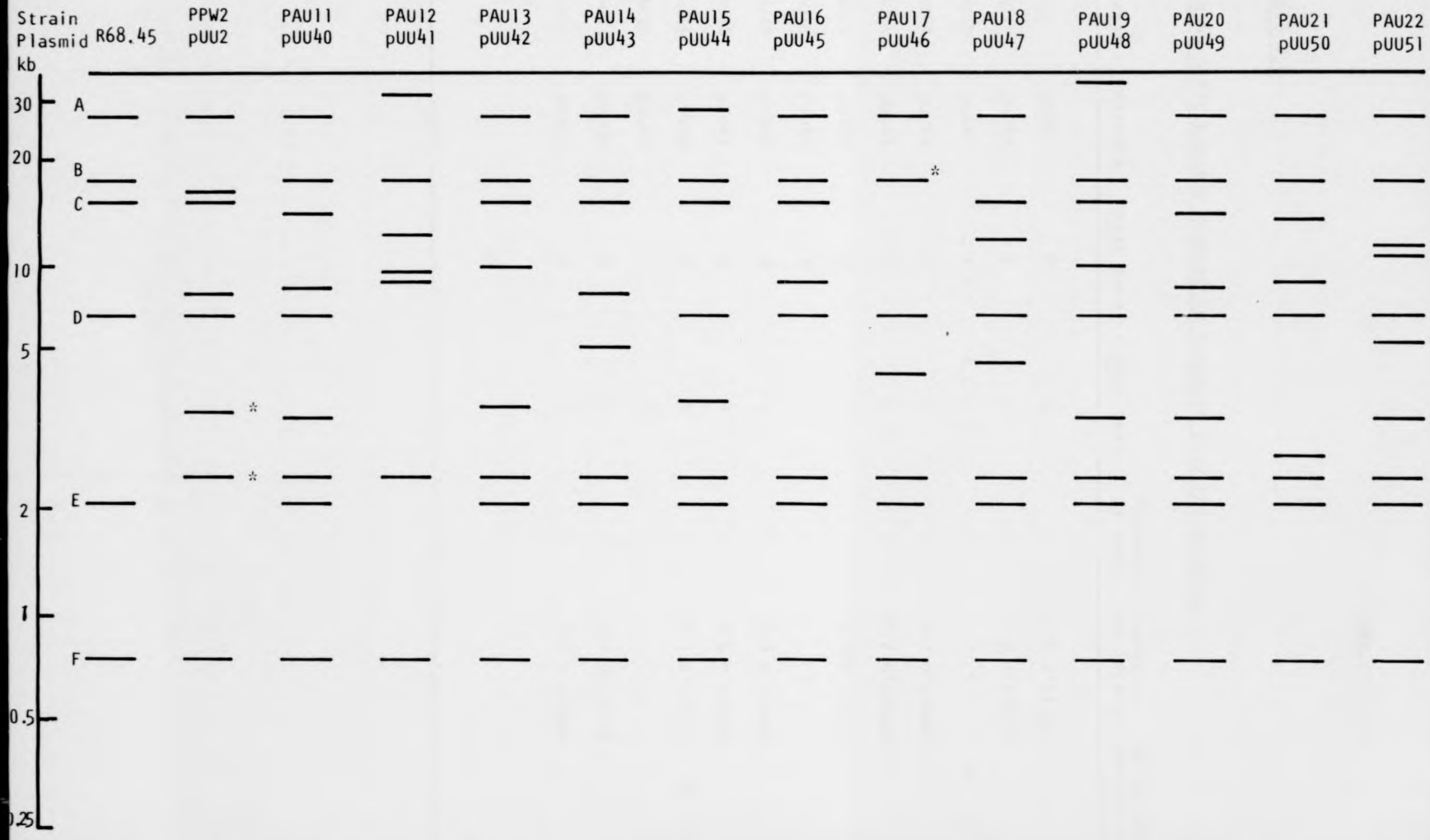

Strain
Plasmid
kb
R68.45
PPW2 pUU2
PAU11 pUU40
PAU12 pUU41
PAU13 pUU42
PAU14 pUU43
PAU15 pUU44
PAU16 pUU45
PAU17 pUU46
PAU18 pUU47
PAU19 pUU48
PAU20 pUU49
PAU21 pUU50
PAU22 pUU51
30
20
10
5
2
1
0.5
0.25
A
B
C
D
E
F

TABLE 7.6

R68.45-DERIVED BANDS MISSING IN PLASMIDS pUU2 AND pUU40-51

Strain	Plasmid	PstI band	SmaI band	Presence of IS21	Location of insert	Antibiotic sensitivity
PPW2	pUU2	B	B	-	3-4 o'clock	
PAU11	pUU40	B	C	+	2-3 o'clock	
PAU12	pUU41	C & F	A,C & D	-	?	Km Tc
PAU13	pUU42	C	D	+	6-7 o'clock	
PAU14	pUU43	C	D	+	6-7 o'clock	
PAU15	pUU44	A	A	+	8-12o'clock	
PAU16	pUU45	A	-	+	?	
PAU17	pUU46	B	C	+	2-3 o'clock	
PAU18	pUU47	B	B	+	3-6 o'clock	
PAU19	pUU48	A	A	+	8-12o'clock	Km Tc
PAU20	pUU49	-	C	+	?	
PAU21	pUU50	A	C	+	12-1o'clock	
PAU22	pUU51	D	C	+	1-2 o'clock	

For nine of the new plasmids it was possible to identify the regions of insertion of the acquired DNA into the plasmid. These regions were scattered round the plasmid. pUU41, the plasmid lacking the second copy of IS21, had lost several R68.45 bands, indicating modifications in several parts of the plasmid. pUU45 appeared to possess all the R68.45-derived SmaI bands, however it would be possible for a small change in size of the largest band to pass undetected. A similar argument applied to pUU49 which appeared to possess all its PstI bands.

These new plasmids, as with pUU30-39 discussed in the previous section, show similarities to the original R-prime, pUU2. Again bands of the same size as 2XP3, 2XP4, 2XP5, 2XP6 and 2XS4 were present in all cases, though generally as singles.

Only two of these transconjugants had lost plasmid coded antibiotic resistance. These were PAU12 (pUU41) and PAU19 (pUU48) which were both sensitive to kanamycin and tetracycline. pUU41 had undergone multiple modifications, some of which were in the vicinity of the kanamycin resistance gene and IS21. An insertion into this region could have led to kanamycin sensitivity. pUU8 had acquired an insert in the 8 - 12 o'clock region. This region included the kanamycin resistance gene.

7.5 Discussion

P. putida PPW37 containing a genuine R68.45 was mated with *P. putida* PPW4 and with *P. aeruginosa* PAU2 and produced 22 transconjugants capable of growth on 2MCPA as sole carbon and energy source. Their plasmids were extracted and digested with PstI and SmaI, the plasmids had all acquired an extra piece of DNA of about 10kb. This piece of DNA had inserted into a variety of regions of R68.45. The second copy of IS21 had been retained in 14 of the 22 new plasmids.

It was assumed that these new plasmids were all R-primes carrying a dehalogenase gene. No conclusive proof is offered, such as co-transfer of the 2MCPA$^+$ phenotype with the plasmid antibiotic resistance markers, and transformation of the plasmid. The transconjugants had acquired the ability to grow on 2Mcpa$^+$, and in all cases the plasmids had acquired extra DNA which was associated with new common restriction fragments. These results suggest that the new plasmids were R-primes carrying dehalogenase genes.

The new PstI and SmaI restriction fragments common to these plasmids were identical in size to the restriction fragments 2XP3, 2XP4, 2XP5, 2XP6 and 2XS4 from the original R-prime pUU2, though they were present usually as single as opposed to double bands. These restriction fragments had been associated with the dehI bearing insert I2 in pUU2. These results suggest: that the newly generated plasmids contained an insert of DNA which was basically identical to part of I2 from pUU2; that these bands represent the dehI containing part of I2; and that dehI from pUU2 had integrated into the PPW12 chromosome in order for it to be subsequently picked up by R68.45 in the formation of these new R-primes.

It is interesting to compare the frequencies of R68.45 transfer and R-prime formation in these cases with those associated with P. putida PP3. (Table 7.7) The frequency for mating R68.45 into P. putida PPW12 was 1000 times greater than for the similar mating into P. putida PP3. PPW12 was a derivative of P. putida PaW340 which was not restriction deficient. Therefore there must exist a barrier greater than the restriction system of PaW340 to the entry of R68.45 into PP3. The frequency of R-prime formation was 2×10^6 times greater when P. putida PPW4 was the recipient and 5×10^4 times greater when P. aeruginosa PAU2 was the recipient than when P. putida PPW1 was mated with P. putida PaW340. PPW4 was derived from P. putida PaW KT2440 and PAU2 from P. aeruginosa PAO1162, which were restriction deficient. The difference in frequency for the formation of

TABLE 7.7

FREQUENCIES OF PLASMID TRANSFER AND R-PRIME FORMATION

Donor	Plasmid	Recipient	Frequencies per donor cell	Comments
P. aeruginosa PA08	R68.45	*P. putida* PP3	1×10^{-6}	Acquired I1 or I1-like insertion. Lost second copy IS21.
P. putida PPW1	pUU1	*P. putida* PaW340	1.3×10^{-10}	R-prime formation. pUU2.
P. aeruginosa PA025	R68.45	*P. putida* PPW12	9.2×10^{-4}	Plasmid unchanged. Retained second copy of IS21.
P. putida PPW37	R68.45	*P. putida* PPW4	2.8×10^{-4}	R-prime formation 30% retain second copy of IS21.
P. putida PPW37	R68.45	*P. aeruginosa* PAU2	6.7×10^{-6}	R-prime formation 90% retain second copy of IS21.

the new R-primes in these strains was probably due to the former being an intraspecific and the latter an interspecific mating. In Chapters 4 and 5 it was suggested that the presence of I1 in pUU1 from PPW1 reduced the ability of this plasmid to transfer conjugally and reduced its R-prime forming ability. The conclusion was that if this reduction in R-prime forming ability was taken into consideration the R-prime carrying dehI, pUU2, had actually been found at a higher than expected frequency. Certainly this suggestion was borne out by the results presented here with frequencies of R-prime formation of 2.8×10^{-4} and 6.7×10^{-6} per donor cell. Frequencies for R-prime formation using R68.44 or R68.45 from the literature were considerably lower; 1×10^{-10} (Hedges et al., 1977); 1×10^{-11} (Hedges and Jacob, 1977a); 1×10^{-8} (Holloway, 1978); 1×10^{-9} - 10^{-10} (Johnston et al., 1978a); 1×10^{-8} (Morgan, 1982); and 1×10^{-8} (Moore et al., 1983). The higher than expected frequencies obtained here lend support to the suggestion that dehI was located on a transposable element (Slater, Weightman and Hall, unpublished observations).

In 17 of the 22 new R-primes the position of insertion of the dehI bearing DNA could be localised. These inserts were scattered around the plasmid and were not associated with IS21. It was not possible to locate the region of insertion in the remaining 5 plasmids; in two of the cases this could be in or near the IS21 region. Leemans et al. (1980) and Moore et al. (1983), found that the insert in R-primes of R68.44 and R68.45 was closely associated with IS21. Willetts et al. (1981), and Riess et al. (1983), suggest that a cointegrate intermediate was formed involving both copies of IS21 during R-prime formation; and that therefore the insert was always associated with IS21. They also found that one copy of IS21 was lost in the process. On the other hand inserts were found in other locations in R-primes derived from R68.44 and R68.45 (Johnston et al., 1978a; Kiss et al., 1980; Kowalczuk et al., 1981). In only one or two of the R-primes

examined here was the insert remotely associated with IS21, and certainly in over 60% of the R-primes the second copy of IS21 had been retained. Three possibilities arise: (a) IS21 has nothing to do with R-prime formation; (b) IS21 is involved in R-prime formation but this involvement does not necessarily entail the insert having to be intimately associated in the R-prime with IS21; or (c) the formation of R-primes carrying dehI is a special case due to some intrinsic peculiarity of dehI. Possibility (a) can be dismissed as the work discussed above all points to the involvement of IS21 in R-prime formation. Possibility (b) is supported by the work discussed above, inserts in the primes derived from R68.44 and R68.45 were not always associated with IS21. Possibility (c) is supported by the finding that the frequency for the formation of R-primes carrying dehI from R68.45 was higher than expected. If dehI were on a transposable element R-prime formation might not have to be mediated via IS21. Possibilities (b) and (c) are by no means mutually exclusive. The data presented here supports the view that IS21 may be involved in R-prime formation but that the insert does not have to be directly associated with IS21 in the R-prime and that there is probably something distinctive about R-prime formation involving dehI.

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CHAPTER 8

CLONING THE DEHALOGENASE I GENE

as in Table 8.1. 1/20 of the total volume of each digest was checked for complete cleavage on an agarose gel. The digests were heat-inactivated, phenol-extracted, and ethanol-precipitated. The dried pellets were re-dissolved in TE buffer pH 7.6. The DNAs were mixed, ligated and transformed into E. coli ED8654 according to the strategy in Table 8.1 and the methods in Chapter 2. E. coli ED8654 (SupE SupF hsd $N^-M^+S^+$ met$^-$ trpR$^-$) was kindly provided by Dr. A.J. Weightman, University of Geneva. ED8654 was shown to grow on minimal medium supplemented with lactate and methionine. It did not grow on 2MCPA. It therefore was a good candidate for growth on 2MCPA if it contained a functional dehalogenase gene.

Transformants were selected on L agar containing Tc (12.5μgml^{-1}) in the case of pAT153 and Sm (25μgml^{-1}) in the case of pKT231 (Table 8.1). Clones containing inserts were identified by insertional inactivation on L agar containing Tc (12.5μgml^{-1}) and Ap (10μgml^{-1}) for pAT153 or Sm (25μgml^{-1}) and Km (50μgml^{-1}). All the transformants were also checked for growth on minimal medium supplemented with 2MCPA and methionine. 8 clones from the 3 cloning experiments were identified which grew very slowly on 2MCPA (Tables 8.1 and 8.2). In the case of the pAT153 PstI clone CP24, it was observed that the clones pricked near the growing clone exhibited slight growth. This could be due to diffusion of the dehalogenase. Repeated sub-culturing of the clones on 2MCPA gave faster growing derivatives of the pKT231 - HindIII clones CH15 and CH16.

8.3 Restrictive analyses of plasmids from 2Mcpa$^+$ clones

The plasmids from the clones which grew on 2MCPA were extracted by the rapid method. They were digested with a range of restriction enzymes so as to identify and size the restriction fragments cloned.

CHAPTER 8

CLONING THE DEHALOGENASE I GENE

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TABLE 8.1

STRATEGY FOR CLONING *dehI*

DNA	Enzyme		Selection	N° transformants	N° insertional inactivants	N° 2MCPA$^+$
pAT153 1µg	PstI	Ligate transform	Tc^R	96	24	1
pUU2 2µg	PstI					
pAT153 0.5µg	-	Transform	Tc^R	500+	0	0
-	-	-	Tc^R	0	0	0
pKT231 1µg	HindIII	Ligate transform	Sm^R	86	21	6
pUU2 2µg	HindIII					
pKT231 1µg	XhoI	Ligate transform	Sm^R	56	7	1
pUU2 2µg	XhoI					
pKT231 0.5µg	-	Transform	Sm^R	500+	0	0
-	-	-	Sm^R	0	0	0

TABLE 8.2

BACTERIAL STRAINS AND PLASMIDS USED AND DERIVED IN CHAPTER 8

Strain	Species	Plasmid	Genotype/Phenotype	Derivation
		pUU2	2Mcpa$^+$,Ap^R,Km^R,Tc^R	PPW1 X PaW340
		pAT153	Ap^R,Tc^R	Twigg & Sherratt(1980)
		pKT231	Sm^R,Km^R	Bagdasarian *et al.*, (1981)
ED8654	*E. coli*	-	*Sup*E *Sup*F *hsd* R$^-$ M$^+$ Met$^-$ *trp*R	A.J. Weightman
CP24	*E. coli*	pUU52	As ED8654, 2Mcpa$^+$,Tc^R	*Pst*I digest of pUU2 into pAT153
CX1	*E. coli*	pUU53	As ED8654, 2Mcpa$^+$,Sm^R	*Xho*I digest of pUU2 into pKT231
CH6	*E. coli*	pUU54	"	*Hin*dIII digest of pUU2 into pKT231
CH7	*E. coli*	pUU55	"	"
CH12	*E. coli*	pUU56	"	"
CH14	*E. coli*	pUU57	"	"
CH15	*E. coli*	pUU58	"	"
CH16	*E. coli*	pUU59	"	"

8.3.1 Restriction analysis of pUU52 from CP24

CP24 was a derivative of *E. coli* ED8654 containing pUU52; pAT153 with a PstI insert from pUU2 cloned into the PstI site. It grew slowly on 2MCPA. pUU54 was extracted by the rapid method and digested with PstI the digest was compared with a PstI digest of pUU2 so as to enable the insert to be identified (Plate 8.1).

The PstI digest of pUU52 gave 3 restriction fragments, one corresponding to linearised pAT153 and two the same size as fragments in the PstI digest of pUU2. These cloned fragments corresponded in size to 2XP4 (1.19kb) and 2PF (0.77kb). 2XP4 was a band associated with I2 in pUU2, where it was present as a double. 2PF, however, was derived originally from R68 and was not associated with 2PF or I2 in pUU2. Therefore it was probable that these two bands were linked together in pUU52 through chance ligation, rather than as a result of partial digestion of pUU2. Certainly the gel used to monitor the digestion of pUU2 prior to ligation indicated total digestion of the plasmid.

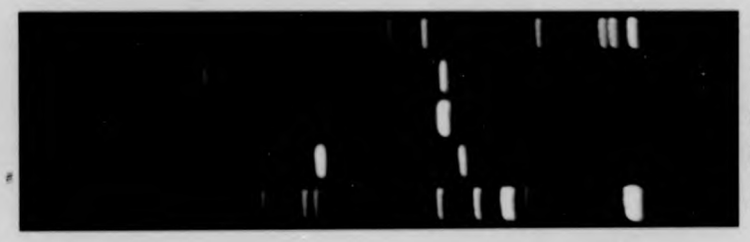
8.3.2 Restriction analysis of pUU53 from CX1

CX1 contained pUU53, pK231 with an XhoI insert from pUU2 cloned into the XhoI site. CX1 grew slowly on 2MCPA. pUU53 was extracted by the rapid method and digested with XhoI and PstI, both singly and together in order to identify the region of pUU2 cloned (Plate 8.2; Table 8.3; Figure 8.1).

The digest revealed that pUU53 contained an XhoI fragment identical in size to 2XX3 in pUU2 cloned into the XhoI site. This band was double in pUU2. Digestion of pUU53 with PstI showed that the cloned XhoI fragment, 53XX1, contained within it 3 PstI fragments which were present in PstI digest of pUU2; 53XP1 (3.23kb) = 2XP3; 53XP4 (0.87kb) = 2XP5; and 53XP5 (0.60kb) = 2XP6. These fragments were present as doubles in pUU2. By

PLATE 8.1

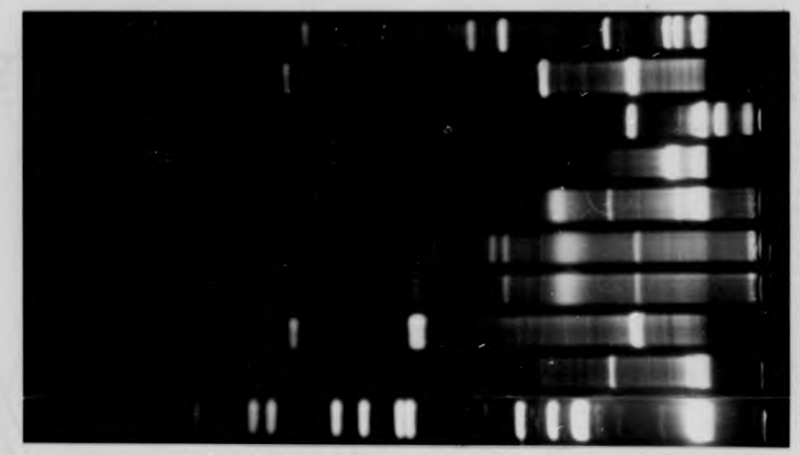
RESTRICTION DIGESTS OF pUU2 AND pUU52 WITH *PstI*


pUU2
pUU52
pAT153
pAT153
λEcoRI/HindIII
PstI


pUU2
pUU52
pAT153
PstI
pAT153
λEcoRI/HindIII

PLATE 8.2

RESTRICTION DIGESTS OF pUU2 AND pUU53 WITH *Xho*I AND *Pst*I


pUU2 PstI
pKT231 PstI
pKT231
pKT231 XhoI
pUU53 XhoI
pUU53 PstI
pUU53 XhoI/PstI
pKT231 XhoI/PstI
pUU2 XhoI
λ EcoRI/Hind III

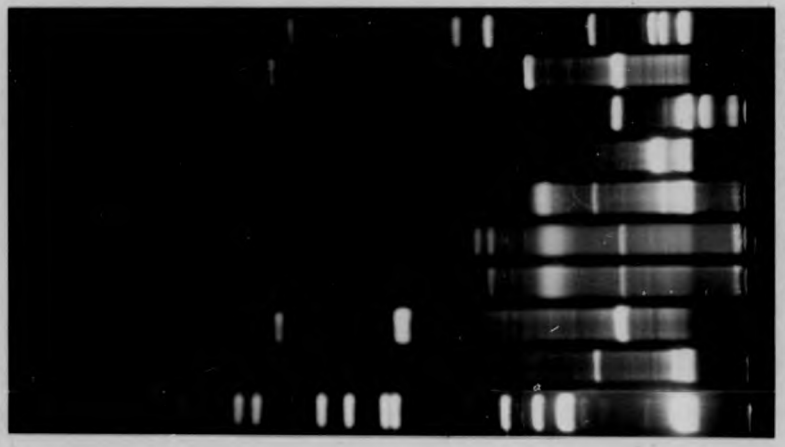

pUU2 PstI
pKT231 PstI
pKT231
pKT231 XhoI
pUU53 XhoI
pUU53 PstI
pUU53 XhoI/PstI
pKT231 XhoI/PstI
pUU2 XhoI
λ EcoRI/Hind III

TABLE 8.3

SIZES OF RESTRICTION FRAGMENTS OF pUU2, pKT231 AND pUU53 DIGESTED WITH XhoI AND PstI

a. pUU2

XhoI	PstI
33.98	32.67
28.46	14.82
18.20	12.38
6.55 *	6.52
	3.23 *
	2.77
	1.19 *
	0.87 *
	0.77
	0.66
	0.60 *
	0.29 *
87.19kb	82.95kb

b. pKT231

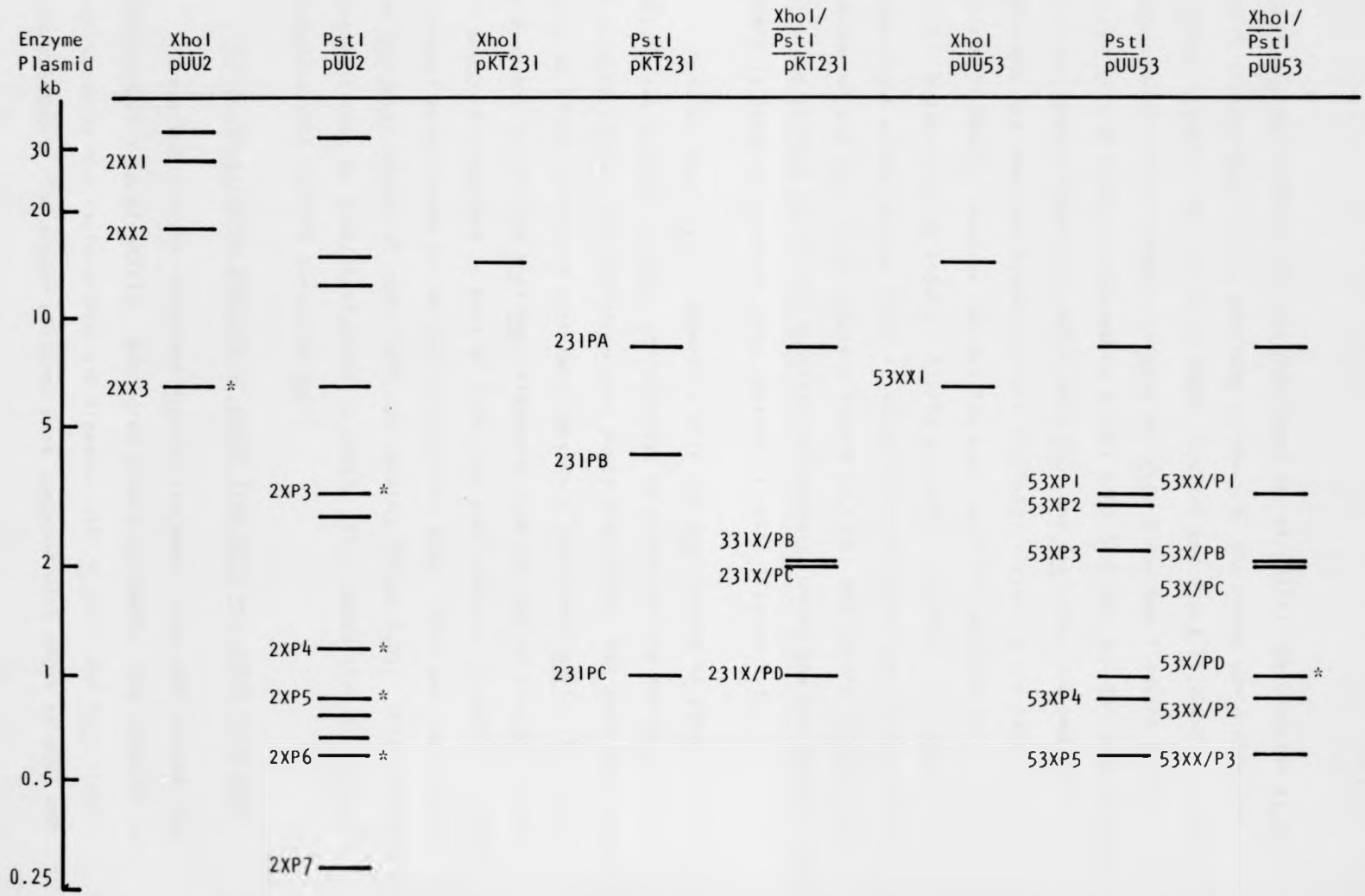
XhoI	PstI	XhoI/PstI
4.51	8.37	8.37
	4.18	2.07
	1.02	2.02
		1.02
14.51kb	13.57kb	13.48kb

c. pUU53

XhoI	PstI	XhoI/PstI
14.51	8.37	8.37
6.55	3.23	3.23
	3.02	2.07
	2.24	2.02
	1.02	1.02
	0.87	1.00
	0.60	0.87
		0.60
21.06kb	19.35kb	19.18kb

FIGURE 8.1

DIAGRAM OF RESTRICTION DIGESTS OF pUU2, pKT231 AND pUU53 WITH *XhoI* AND *PstI*


Enzyme
Plasmid
kb
XhoI
pUU2
PstI
pUU2
XhoI
pKT231
PstI
pKT231
XhoI/
PstI
pKT231
XhoI
pUU53
PstI
pUU53
XhoI/
PstI
pUU53
30
20
10
5
2
1
0.5
0.25
2XX1
2XX2
2XX3
*
2XP3
*
2XP4
*
2XP5
*
2XP6
*
2XP7
231PA
231PB
231PC
331X/PB
231X/PC
231X/PD
53XX1
53XP1
53XP2
53XP3
53XP4
53XP5
53XX/P1
53X/PB
53X/PC
53X/PD
53XX/P2
53XX/P3
*

comparing the digests with the published map of pKT231 (Bagdasarian et al., 1981) (Figure 8.2) it was possible to identify the bands containing the regions flanking the insert as 53XP2 (3.02kb) and 53XP3 (2.24kb). In the double digestion of pUU53 with XhoI and PstI these two flanking bands should give 2 bands corresponding to 231 X/PB (2.07kb) and 231 X/PC (2.02kb) from the double digest of pKT231 with XhoI and PstI plus two new bands. Only one new band was found, 53XX/P2 (1.00kb), virtually the same size as 53X/PD (1.02kb). However the missing band would be expected to be about 0.17 - 0.22kb in size (53XP3 - 53X/PB or 53XP3 - 53X/PC). A fragment of this size might either be too faint to detect or might have been lost off the bottom of the gel. Unfortunately 53X/PB (2.07kb) and 53X/PC (2.02kb) were so close in size it was not possible to determine which one was derived from 53XP2 (3.02kb) to yield it plus 53XX/P2 (1.00kb) (Figure 8.3).

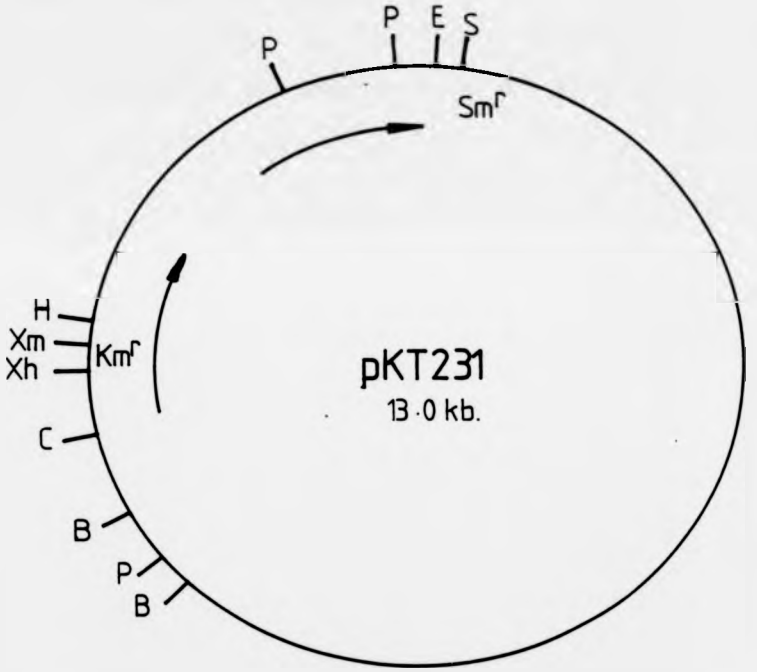
None of the 3 PstI fragments within the XhoI insert of pUU53 from CX1, which were present in pUU2, corresponded to either of the two PstI fragments from pUU2 cloned into pAT153 to give pUU52 from CP24. Yet both CP24 and CX1 grew on 2MCPA, therefore both must possess a functional dehI. Therefore, presumably one of the XhoI/PstI fragments from the end of the XhoI insert in pUU53 corresponded to part of 2XP4 from pUU2 present in pUU52 from CP24. In other words there was an XhoI site within 2XP4. This was confirmed by an XhoI/PstI digest of pUU2, 2XP4 was missing (Plate 8.2). This overlapping fragment must be 53XX/P2 (1.00kb) in pUU53. This comparison of results suggests that 53XX/P2 contained dehI.

8.3.3 Restriction analysis of pUU57 from CH14 and pUU58 from CH15

These two strains contained HindIII fragments from pUU2 cloned into the HindIII site of pKT231. Both grew slowly on 2MCPA. The plasmids were extracted by the rapid method and digested with HindIII and PstI singly and together. The digest patterns were compared with those of pUU2 and

FIGURE 8.2

MAP OF pKT231



S : SstI

E : EcoRI

P : PstI

H : HindIII

Xm: XmaI

Xh: XhoI

C : ClaI

B : BstEII

The arrows indicate directions of transcription.

FIGURE 8.3

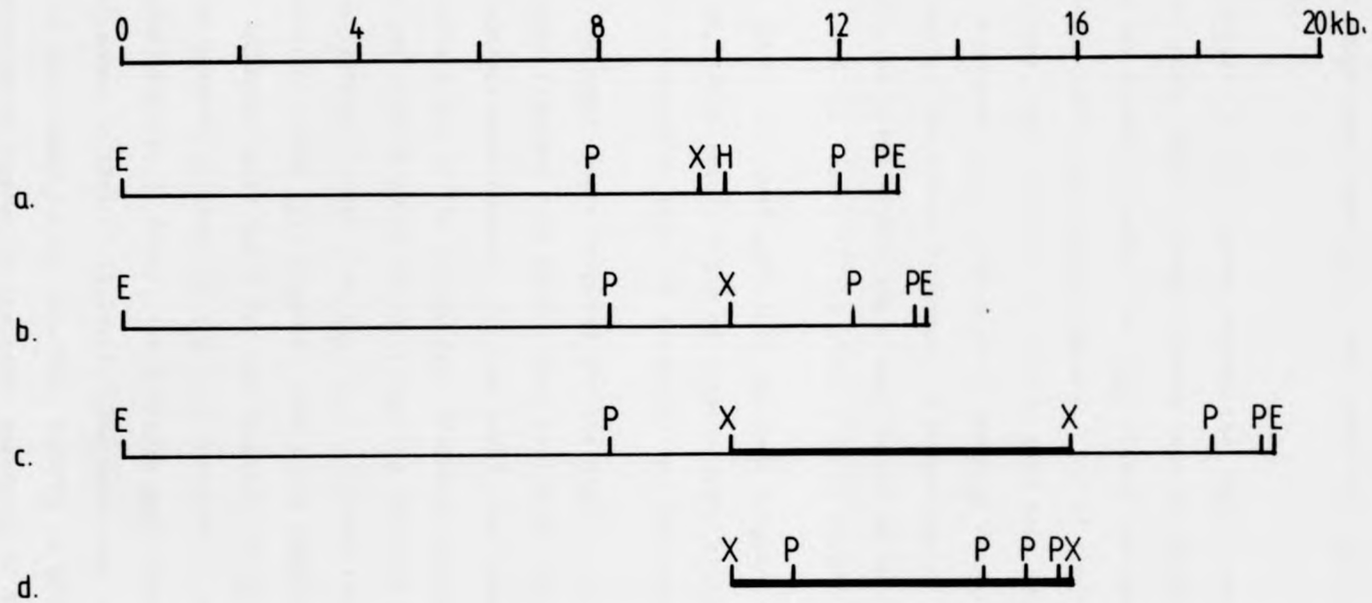
MAP OF XhoI INSERT IN pUU53

a. Linearised pKT231 from Bagdasarian et al. (1981).

b. Linearised pKT231 from PstI and XhoI digest data presented here.

c. Linearised pUU53 showing XhoI insert.

d. XhoI insert of pUU53. Internal PstI sites arbitrarily arranged. The orientation of the fragment could not be determined.


0
4
8
12
16
20kb.
a.
E
P
X
H
P
PE
b.
E
P
X
P
PE
c.
E
P
X
X
P
PE
d.
X
P
P
P
PX

pKT231 (Plate 8.3; Table 8.4; Figure 8.4). The restriction digests of pUU57 and pUU58 were identical. Only pUU57 will therefore be dealt with in detail.

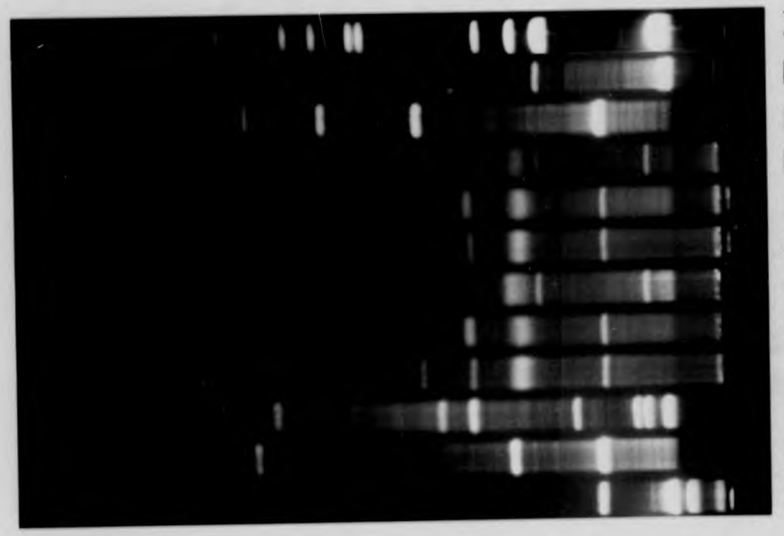
The HindIII single digests showed that pUU57 contained the HindIII fragment of pUU2, 2XH3 (5.04kb), cloned into the HindIII site of pKT231. This band was double in pUU2. The PstI digest indicated the presence of 2 PstI sites within the cloned fragment. One of the resultant fragments, 53XPI (3.23kb), was identical in size to 2XP3 from pUU2. 2XP3 was double in pUU2. A double digest with HindIII and PstI confirmed that this fragment was internal to the cloned fragment. A comparison of the digests enabled the restriction sites within the cloned insert to be mapped and the orientation of the insert in the plasmid to be determined (Figure 8.5).

Both pUU57 and pUU58 contained the same fragment cloned in the same orientation. This result might be coincidental, alternatively this particular orientation might be essential for the expression of dehI.

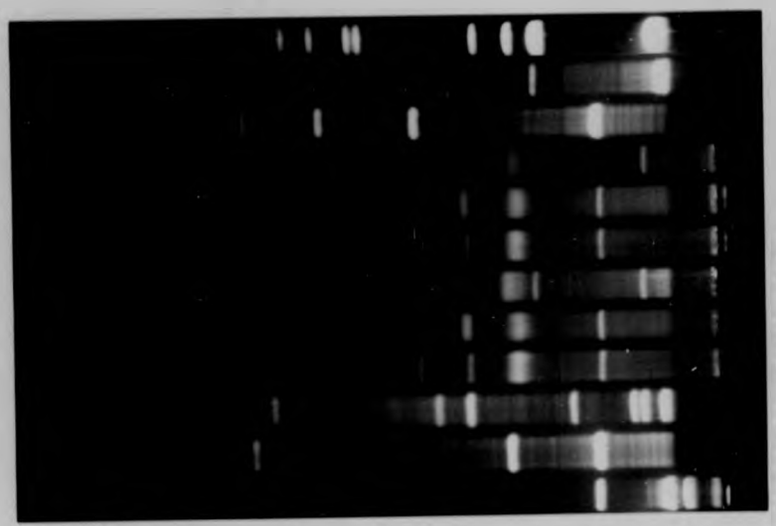
Both the XhoI insert in pUU53 and the HindIII insert in pUU57 contained the 2XP3 PstI fragment from pUU2. This indicated that the bulk of both cloned fragments were common. As with pUU53, the cloned insert of pUU57 did not contain any of the cloned PstI fragments present in pUU52, from CP24. In the case of pUU53 it was suggested (Section 8.3.2) that dehI was located in 53XX/P2 (1.00kb), an XhoI/PstI produced fragment, and that this overlapped with 1.19kb PstI fragment from pUU52 (equivalent to 2XP4 in pUU2). A similar argument would apply for the location of dehI in either 57XH/P2 (0.68kb) or 57XH/P3 (0.58kb) of the cloned insert in pUU57. This implies that in pUU53 53XX/P2 (1.00kb), the probable dehI containing fragment, was located adjacent to 53XX/PI (3.23kb). The remaining two PstI - PstI fragments internal to the cloned insert in pUU53, 53XX/P2 (0.87kb) and 53XXP3 (0.60kb), would therefore be located at the other extremity of 53XXPI (3.23kb). The

PLATE 8.3

RESTRICTION DIGESTS OF pUU2, pUU57 AND pUU58 WITH *HindIII* AND *PstI*



λ EcoRI/Hind III
pUU2 Hind III
pKT231 Hind III / Pst I
pUU57 Hind III
pUU57 Pst I
pUU57 Hind III/Pst I
pUU58 Hind III
pUU58 Pst I
pUU58 Hind III/Pst I
pUU2 Pst I
pKT231 Pst I
pKT231



λ EcoRI/Hind III
pUU2 Hind III
pKT231 Hind III / Pst I
pUU57 Hind III
pUU57 Pst I
pUU57 Hind III/Pst I
pUU58 Hind III
pUU58 Pst I
pUU58 Hind III/Pst I
pUU2 Pst I
pKT231 Pst I
pKT231

TABLE 8.4

SIZES OF RESTRICTION FRAGMENTS OF pUU2, pKT231 AND pUU57 WITH *HindIII* AND *PstI*

a.

pUU2 *HindIII*	pUU2 *PstI*
44.79	32.67
26.70	14.82
5.04 *	12.38
1.43 *	6.52
	3.23 *
	2.77
	1.19 *
	0.87 *
	0.77
	0.66
	0.60 *
	0.29 *
84.43kb	82.95kb

b. pKT231	HindIII	PstI	HindIII/PstI
	14.51	8.37	8.37
		4.18	2.62
		1.02	1.58
			1.02
	14.51kb	13.57kb	13.59kb

c. pUU57	HindIII	PstI	HindIII/PstI
	14.51	8.37	8.37
	5.04	3.23	3.23
		3.14	
		2.17	2.62
		1.02	1.58
			1.02
			0.69
			0.58
	19.55kb	17.93kb	18.09kb

FIGURE 8.4

DIAGRAM OF RESTRICTION DIGESTS OF pUU2, pKT231 AND pUU57 WITH *HindIII* AND *PstI*

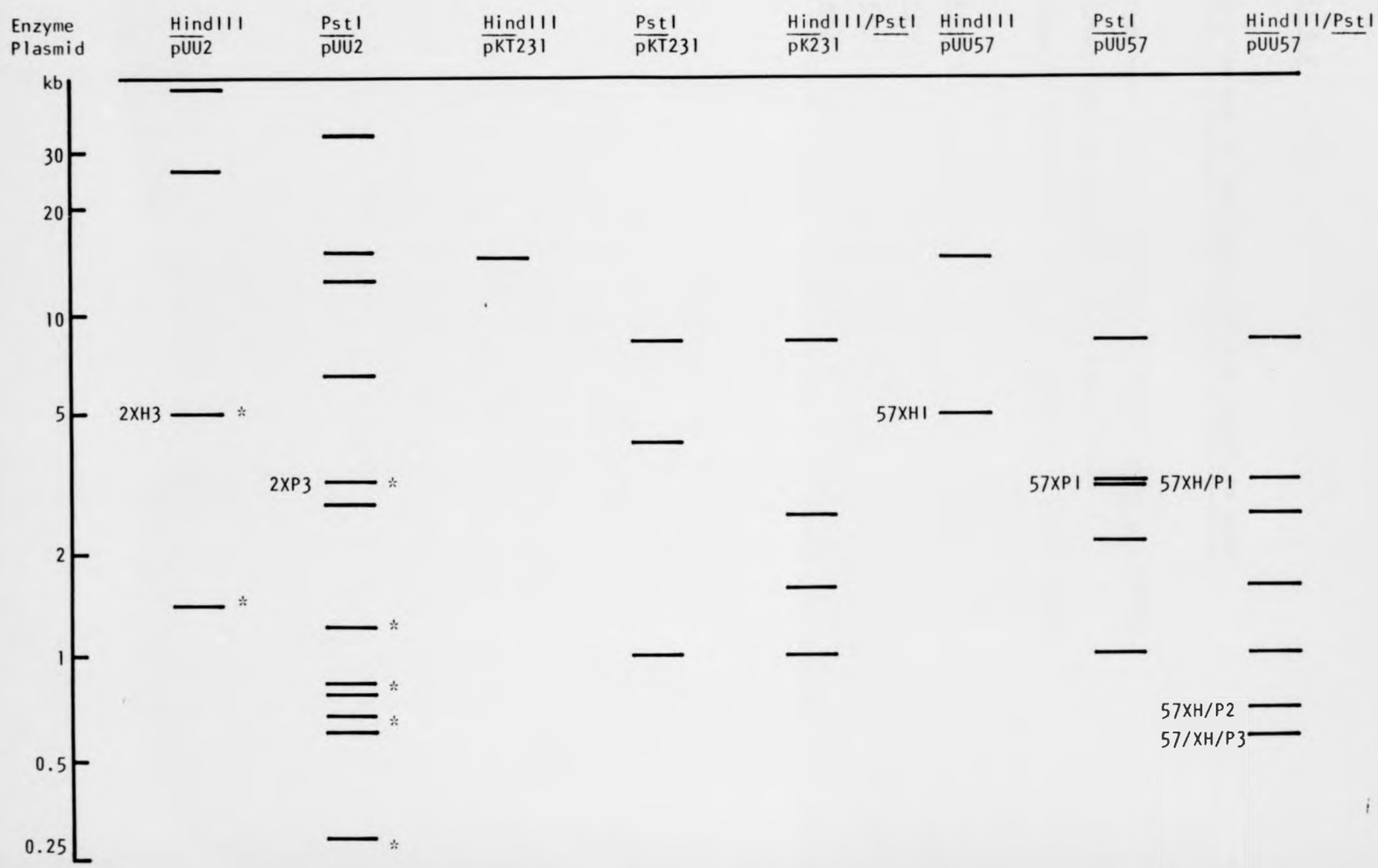

Enzyme
Plasmid
HindIII
pUU2
PstI
pUU2
HindIII
pKT231
PstI
pKT231
HindIII/PstI
pK231
HindIII
pUU57
PstI
pUU57
HindIII/PstI
pUU57
kb
30
20
10
5
2
1
0.5
0.25
2XH3
2XP3
57XH1
57XP1
57XH/P1
57XH/P2
57/XH/P3

FIGURE 8.5

RESTRICTION MAP OF THE *HindIII* CLONED INSERT IN pUU57

a. Linearised map of pKT231 from Bagdasarian *et al*. (1981).

b. Linearised map of pKT231 from digest data.

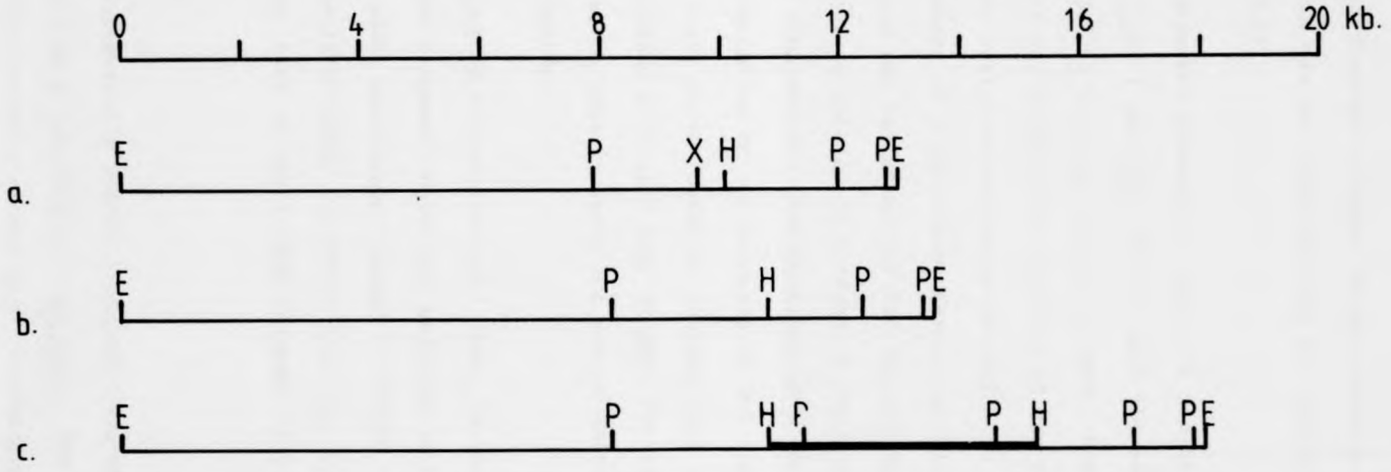
c. Location and orientation of the *HindIII* insert in pUU57.

E: *EcoRI*

H: *HindIII*

P: *PstI*

X: *XhoI*


0
4
8
12
16
20 kb.
a.
E
P
X
H
P
PE
b.
E
P
H
P
PE
c.
E
P
H
P
H
P
PE

small PstI - XhoI fragment of about 0.2kb, whose existence was predicted but whose presence was not detected must be located at the extremity of the insert (Figure 8.6).

The above argument presupposes that the 3.23kb PstI - PstI fragments present in the HindIII and XhoI inserts were the same. It must be borne in mind that this 3.23kb band was double in pUU2. However if the fragments were not the same this would imply the presence of two copies of dehI in I2 of pUU2. Certainly restriction digests of pUU2 with a variety of enzymes did suggest the presence of a duplicated feature within I2 (Chapter 3). This duplicated feature was not essential for the expression of dehI, as the same bands existed as singles in a range of new R-primes containing and expressing dehI (Chapter 7). The presence of a duplicated feature in I2 of pUU2 could be indicative of the presence of two copies of dehI. If that were the case there would be no reason to suppose that the restriction sites on the DNA associated with each copy of dehI should not map in the same positions. In which case it would not matter which copy had been cloned in these two plasmids.

It was suggested in Section 8.3.1 that, in the cloned PstI insert in pUU52, the 0.77kb fragment, which was believed to have originated from the R68 portion of pUU2, had become ligated by chance to the 1.19kb fragment originating from I2 of pUU2. In which case the 0.77kb fragment could be located on either side of the 1.19kb fragment (Figure 8.6).

8.4 Discussion

dehI was successfully cloned from pUU2 into pAT153 and pKT231 and expressed, albeit at a low level, in E. coli. The XhoI and HindIII inserts cloned into pKT231 shared a common PstI restriction band; but this band was not that cloned into pAT153. All three plasmids expressed a functional

FIGURE 8.6

CLONED FRAGMENTS FROM pUU52, pUU53 AND pUU57

a. HindIII insert from pUU57. dehI located in one of the PstI - HindIII fragments.

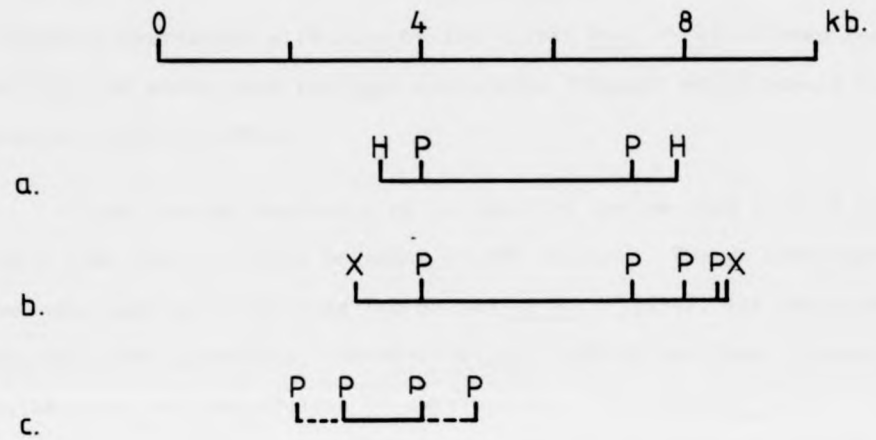
b. XhoI insert from pUU53. dehI located in the large XhoI - PstI fragment. The orientation of the insert was not determined. Neither was the order of the two smallest PstI - PstI inserts.

c. PstI insert from pUU2. Alternative locations of the 0.77kb PstI fragments.

H: HindIII site

P: PstI site

X: XhoI site


0
4
8
kb.
a.
H P
P H
b.
X P
P P PX
c.
P P P P

dehalogenase as shown by their host strains growth on 2MCPA. This led to the suggestion that *dehI* was located within one of the *PstI* - *HindIII* or *PstI* - *XhoI* fragments at the extremities of the cloned inserts in pUU53 and pUU57 adjacent to the common *PstI* - *PstI* fragment, and that these fragments overlapped with part of the 1.19kb *PstI* - *PstI* cloned fragment in pAT153. In which case the *dehI* containing fragment would have a maximum size of 0.58 or 0.69kb.

If the smaller maximum size is taken as the maximum size of *dehI*, *dehI* could code for a protein of about 21,000 daltons. Though dehalogenase I has been partially purified (Weightman *et al.*, 1979b), its molecular weight has not been determined. Kawasaki *et al.* (1981b) purified a plasmid-encoded haloacetate halidohydrolase (= dehalogenase) from *Moraxella* sp. This halidohydrolase dehalogenated MCA, but showed low activity towards DCA and 2MCPA and very low activity towards 2,2-DCPA. They obtained molecular weight determinations of 26,000 daltons by SDS-polyacrylamide gel electrophoresis and 43,000 daltons by Sephadex G-100 gel filtration. They were unable to account for the discrepancy between these two values, but suggest that the enzyme might exist as a dimer. The size of *dehI* postulated here could code for an enzyme near the lower of these two molecular weight determinations.

Experiments were not performed to determine whether the dehalogenase was inducible or constitutive in the three clones CP24, CX1 or CH14. These experiments would have indicated whether the control regions had been cloned together with *dehI*.

Though *E. coli* is very useful as a host for cloning and analysis of genes, it is not very suitable as a host when the functional expression of genes from soil bacteria is required. *E. coli* is thought of as not providing an appropriate "physiological background" for expression; perhaps because

its metabolism is "fermentative" and limited rather than "oxidative" and versatile as that of Pseudomonads is (Bagdasarian and Timmis, 1981). Certainly Pseudomonas genes for the catabolic enzymes of toluene/xylene degradation are poorly expressed (Jacoby et al., 1978; Ribbons et al., 1979; Franklin et al., 1981a). Yet in some circumstances Pseudomonas genes are expressed when cloned in E. coli (Franklin et al., 1981b). Franklin et al. (1981b) cloned XhoI and HindIII fragments of pWW0-161 into pK231 and the related plasmid pKT230. Resultant plasmids expressed the cloned degradative genes constitutively at high levels in E. coli. They concluded that expression was driven from the strong kanamycin resistance promoter located upstream from the XhoI - HindIII sites. They suggested that the poor expression of Pseudomonas catabolic genes often found in E. coli is due to inefficient transcription from natural Pseudomonas promoters, which may function less efficiently with RNA polymerase from E. coli than from Pseudomonas. Inadequacies of the E. coli translation and processing systems with the messages from Pseudomonas genes have probably an insignificant effect on expression. Unfortunately pUU53 and pUU57 were not transferred into Pseudomonas in order to compare their expression in that host with that in E. coli. It was therefore not possible to determine whether dehI was driven from its own promoter in these plasmids, as from the kanamycin resistance promoter.

In the strain CP24 dehI may or may not have been cloned with its promoter into the PstI site of pAT153. pAT153 has a copy number of 200-300 per cell. Therefore even very inefficient expression of dehI could lead to the production of sufficient enzyme to maintain growth. It was interesting that colonies near CP24 showed slight growth. This suggested that dehalogenase I was present extracellularly. This phenomenon was not observed in the other 2Mcpa$^+$ clones where dehI was inserted into pKT231. pAT153 is a high copy number derivative of pBR322 in which HaeII B and G

had been deleted (Twigg and Sherratt, 1980). The PstI site of pBR322 lies within the ampicillin resistance gene at a position corresponding to amino acids 183 and 184 of the β-lactamase (Bolivar et al., 1977; Sutcliffe, 1978, 1979). The N-terminal portion of the β-lactamase acts as a signal sequence to direct the polypeptide into the periplasmic space. This 23 amino acid leader sequence is cleaved from the protein as it traverses the membrane (Old and Primrose, 1979). In some cases genes cloned into the PstI site of pBR322 can lead to the expression of a fused gene product with part of the β-lactamase (Seeburg et al., 1978; Villa-Komaroff et al., 1978). Villa-Komaroff et al. (1978) cloned a GC-tailed cDNA of rat proinsulin into the PstI site of pBR322. This led to the expression of a fusion product in which the N-terminal portion of β-lactamase was linked through a run of glycines (resulting from the translation of the run of G residues transcribed from the homopolymer tails) to the fourth amino acid of proinsulin. This fusion product was exported from the cell and could be detected immunologically. For these events to occur the cloned gene must be in the correct orientation and in the correct reading frame, a one in six chance.

It was possible that CP24 may, in a similar fashion be producing and exporting a fusion product of β-lactamase and dehalogenase I. This would certainly account for the apparent extracellular dehalogenase activity observed with this strain. However while the synthesis of a fusion product was possible it is unlikely that such a protein would be biologically active. A comparison of the size of the dehalogenase from CP24 with dehalogenase I could shed some light on this question. It is most likely, therefore, that in CP24, dehI was driven by its own promoter. Additional data are required to shed light on the many questions raised by the results in this chapter.

CHAPTER 9

CONCLUSIONS

Chapter 9

CONCLUSIONS

A derivative of R68.45 was used to construct an R-prime, pUU2, carrying the dehalogenase I genes of Pseudomonas putida PP3. pUU1, the R68.44 derivative used to produce pUU2, had lost one copy of the insertion sequence IS21 and had acquired a small insertion of DNA, I1, presumably derived from the PP3 chromosome. I1 was shown to interfere with the conjugal transfer of the plasmid. I1 or I1-like inserts were capable of insertion into a variety of sites in R68. It could not be established whether or not I1 played a role in the formation of the R-prime pUU2.

In the R-prime, pUU2, dehI was located on a chromosomal insert, I2, in the plasmid of about 14-20kb, and was not associated with the remaining copy of IS21. I2 was capable of translocation within the plasmid.

Growth of PPW2, the strain harbouring pUU2, on 2MCPA under phosphate limitation led to the isolation of mutant strains. In two of these strains, PPW9 and PPW11, the plasmid had acquired an extra piece of DNA of about 18kb, presumably from the bacterial chromosome, and had retained I2. The plasmid of the third mutant strain, PPW10, had lost I2; presumably dehI had integrated into the chromosome. The fourth mutant strain, PPW12, had lost the plasmid altogether but had retained dehI after its translocation to the chromosome.

A new series of R-primes carrying dehI were generated from PPW12 using a genuine unmodified R68.45. These new R-primes shared common restriction fragments with I2 from pUU2. The dehI insert was located in a variety of sites in these new R-primes. These results indicated that though the two copies of IS21 may play a role in R-prime formation they need not be intimately associated with the insert as the cointegrate model of R-prime

formation would suggest (Riess et al., 1983). The frequencies at which these new R-primes were formed were substantially higher than expected. dehI was also shown to be capable of translocation within pUU2 and between the plasmid and the chromosome. These observations led to the suggestion that dehI might be on a transposable element. In which case the formation of R-primes carrying dehI might not be expected to conform to the general pattern of the R-prime formation in R68.45.

Overall these results amply demonstrate the ability of chromosomally located degradative genes to be transmitted between bacterial species by a broad host-range plasmid. These genes were shown to be capable of reintegration into the chromosome from the plasmid and subsequently to be picked up yet again.by a plasmid. In addition it was shown that other genetic material of unknown function but presumably of chromosomal origin, I1-like inserts and I3/5, could be translocated to the plasmid, thereby generating novel arrangements of DNA. This ability of plasmids to transfer chromosomal genes and to generate new permutations of genetic material was termed adventurous evolution in Chapter 1. Adventurous evolution involves the reassortment of large pieces of DNA via illegitimate recombination and was suggested to occur during environmental conditions outside the range normally encountered by the bacteria involved. Chromosomal genes, on the other hand, undergo conservative evolution via random mutation and homologous recombination. It was suggested in Chapter 1 that only after continued exposure to the novel environmental conditions for a considerable period of time would the gene or the assemblage of genes constructed via plasmid borne adventurous evolution to cope with these environmental conditions become stably integrated into the bacterial chromosome. This event had occurred in PPW12, the plasmid-minus derivative strain of PPW2, where dehI had become chromosomally integrated.

The novel recombinations of genetic material studied here were generated by *in vivo* events. It is impossible to say whether they were laboratory engendered flukes or whether they were representative of genetic events which occur in the environment. However science is based on such Cartesian reductionism, therefore the extrapolation from these laboratory findings to general microbial evolution, though qualified, is valid. Certainly other evidence supports the view presented here. For example the similarity between TEM-type β lactamases, the spread of antibiotic resistance, the close relationships between aromatic catabolic plasmids, and the ability of degradative and broad host-range plasmids to form cointegrates; discussed in Chapter 1. Overall the data presented and discussed here are powerful evidence for the important role of plasmids in aspects of microbial evolution.

APPENDIX

The formation of an R-prime carrying the fraction I dehalogenase gene from Pseudomonas putida PP3 using the IncP plasmid R68.44. (Beeching, J.R., Weightman, A.J. and Slater, J.H. (1983). Journal of General Microbiology, 129, 2071-2078.)

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The Formation of an R-prime Carrying the Fraction I Dehalogenase Gene from *Pseudomonas putida* PP3 using the IncP Plasmid R64.44

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Plasmid R68.44 was transferred to *Pseudomonas putida* PP3 at a frequency of approximately 10^{-6}. In the new strain, *P. putida* PPW1, the plasmid, designated pUU1, lost a 2·0 kb fragment corresponding to IS21 which had previously been implicated in enhanced chromosome mobilization, but gained between 3·4 and 5·7 kb of DNA. Plasmid pUU1 was used to mobilize the fraction I dehalogenase gene at a low frequency of approximately 10^{-10} into another strain of *P. putida*. The R-prime plasmid, designated pUU2, contained an additional 9·2 to 11·0 kb fragment which carried the fraction I dehalogenase.

INTRODUCTION

Pseudomonas putida strain PP3, isolated by Senior *et al.* (1976) as a result of its acquired capacity to grow on 2,2-dichloropropionic acid (22DCPA; the herbicide Dalapon) and 2-monochloropropionic acid (2MCPA), has been shown to contain two dehalogenases (Weightman *et al.*, 1979; Weightman & Slater, 1980). Dehalogenases, or alternatively halidohydrolases, catalyse the hydrolytic removal of halides from halogenated alkanoic acids, particularly chlorinated acetic and propionic acids (Goldman *et al.*, 1968; Little & Williams, 1971; Slater *et al.*, 1979). So far, a limited range of microbial genera have been grown on halogenated alkanoic acids (Slater *et al.*, 1979; Berry *et al.*, 1979), although some evidence suggests that a greater number of different bacterial species with dehalogenating capabilities can be isolated from environments which have been challenged by the presence of chlorinated alkanoic acids (Burge, 1969; Hardman & Slater, 1981) compared to those from unchallenged environments.

We are interested in the mechanisms of transfer of genes coding for catabolic functions in natural populations of loosely or closely associated communities (Slater 1978; Slater & Godwin, 1980; Slater & Bull, 1982). It would be of particular interest to know how the dehalogenase gene (or genes) of *P. putida* PP3 may be transferred initially to other pseudomonads and ultimately to other genera by mechanisms involving plasmid transfer. We have argued (Hardman & Slater, 1981) that the plasmid-mediated transfer of dehalogenase genes might explain the increased occurrence of different bacteria possessing dehalogenases after challenging the mixed microbial flora with halogenated alkanoic acids. Furthermore, plasmid-mediated mobilization of dehalogenase genes provides a possible mechanism for the evolution of strains carrying multiple dehalogenase genes (Hardman & Slater, 1981), and recent evidence suggests that the dehalogenase genes in a number of soil bacteria are plasmid-borne (Kawasaki *et al.*, 1981, 1982; D. J. Hardman, P. C. Gowland and J. H. Slater, unpublished observations).

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Abbreviations: MCA, monochloroacetic acid; DCA, dichloroacetic acid; 2MCPA, 2-monochloropropionic acid; 22DCPA, 2,2-dichloropropionic acid.

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Plasmid R68.44 is a derivative of R68 which exhibits enhanced chromosome-mobilizing ability (Haas & Holloway, 1976) and has been shown to be physically identical to the more stable R68.45 (Leemans *et al.*, 1980). R68.44 differs from R68 by a 2·12 kb duplicated region which is correlated with the possession of enhanced chromosome-mobilizing ability (Riess *et al.*, 1980; Leemans *et al.*, 1980; Willetts *et al.*, 1981).

In this paper, we report the introduction of plasmid R68.44 (Haas & Holloway, 1976) into *P. putida* PP3, its structural modification in this strain and the mobilization of chromosomal DNA carrying a dehalogenase gene into another strain of *P. putida*.

METHODS

Bacterial strains and maintenance. Pseudomonas putida PP3 (2Mcpa$^+$ 22Dcpa$^+$ Ap^S Km^S Sm^S Tc^S) was obtained as previously described (Senior *et al.*, 1976). *Pseudomonas putida* PaW340 (2Mcpa$^-$ 22Dcpa$^-$ Trp$^-$ Ap^S Km^S Sm^R Tc^S) was kindly provided by Dr P. A. Williams (University College of North Wales, Bangor). *Pseudomonas aeruginosa* PAC174 (2Mcpa$^-$ 22Dcpa$^-$ Lys$^-$) containing plasmid R68.44 (Ap^R Km^R Tc^R) was kindly provided by Professor P. H. Clarke (University College, London). *Pseudomonas aeruginosa* PAO1162 (*leu-38 rmo-11*) (Dunn & Holloway, 1971) was kindly provided by Professor K. N. Timmis (University of Geneva, Switzerland). A spontaneous rifampicin-resistant derivative of PAO1162, designated *P. aeruginosa* PAU2, was selected on nutrient agar containing rifampicin at 250 μg ml^{-1}. The parent and derivative strains capable of growing on 2MCPA were grown on defined medium containing 2MCPA (Slater *et al.*, 1979) supplemented, where appropriate, with amino acids (25 μg ml^{-1}), ampicillin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), tetracycline (150 μg ml^{-1}), rifampicin (250 μg ml^{-1}) and streptomycin (750 μg ml^{-1}). The other strains were maintained on nutrient agar (Oxoid) supplemented as necessary with drugs at the above concentrations. King's A and B media were made as described by King & Phillips (1978).

Measurement of dehalogenase activity. The dehalogenase activity was determined in organisms grown in medium containing 2MCPA at 0·5 g C l^{-1}. The cultures were incubated overnight at 30 °C on an orbital shaker rotating at 200 r.p.m. Late-exponential phase cultures were harvested by centrifugation at 5000 *g* for 15 min, and washed and resuspended in 0·02 M-Tris/sulphate buffer, pH 7·9. Crude cell-free extracts were prepared by two passages through a French pressure cell (Aminco International) at 83 MPa. The remaining whole organisms and cell debris were removed by centrifugation at 30000 *g* for 45 min.

Dehalogenase activity in the cell-free extract against MCA, DCA, 2MCPA and 22DCPA was determined as previously described (Weightman & Slater, 1980). The assay depended on measuring free chloride ion release in a Marius Chlor-O-Counter (F. T. Scientific, Tewkesbury) as previously described (Slater *et al.*, 1979). Protein concentration of the extracts was estimated by the Biuret method using BSA as the reference protein. Enzyme specific activities were expressed as μmol substrate converted min^{-1} (mg protein)$^{-1}$.

PAGE. The two dehalogenases of *P. putida* PP3 were separated and identified in derivative strains by an analytical discontinuous PAGE technique as described by Weightman & Slater (1980) and Hardman & Slater (1981).

Plasmid transfer and chromosome mobilization. A modified membrane mating technique due to Hedges & Jacob (1977) was used. The parent strains were grown in either LB broth (Miller, 1972) or defined medium containing 2MCPA and supplemented as appropriate. Samples of mid-exponential phase donor culture (5 ml) or maximum population phase recipient culture (5 ml) were centrifuged and each resuspended in 5 ml of 0·1 M-phosphate buffer pH 7·0. The suspensions were mixed and vacuum-filtered through a sterile 0·45 μm pore size membrane filter (Millipore). The filter was transferred to the surface of an LB agar Petri dish and incubated at 30 °C for 6 h. The membrane was transferred to a sterile test tube and the organisms resuspended in 2·5 ml 0·1 M-phosphate buffer pH 7·0. The suspension was serially diluted in 0·1 M-phosphate buffer and samples (0·1 ml) of appropriate dilutions spread on suitable selective media to screen for transconjugants.

Screening for the presence of plasmid DNA. The method developed by Wheatcroft & Williams (1981) was used.

Plasmid DNA isolation for restriction endonuclease analysis. The method of Birnboim & Doly (1979) was used.

Plasmid DNA digestion by restriction endonuclease. The restriction endonucleases *Eco*RI, *Hin*dIII, *Pst*I and *Sma*I were used in accordance with the manufacturer's instructions (Boehringer, Mannheim). After 2 h the digestion was terminated by heat inactivation at 65 °C for 10 min. The resulting DNA fragments were separated by electrophoresis in horizontal 0·7% (w/v) agarose gels in TB buffer containing 89 mM-Tris, 2·5 mM-EDTA and 89 mM-boric acid. The gels were run at 30 to 60 mA for 16 h, stained with ethidium bromide and photographed. The plasmid DNA fragments were sized by comparison with the DNA fragments produced by a double *Eco*RI and *Hin*dIII restriction endonuclease digest of λ DNA (Boehringer Mannheim). The standard sizes for the λ fragments were taken from Southern (1979).

Transformation. This was performed as described by Bagdasarian & Timmis (1981).

Materials. All materials for the growth and enzyme assays were of the highest commercially available purity as described previously (Slater *et al.*, 1979; Weightman & Slater, 1980), as were the materials used for the plasmid analyses.

RESULTS

The introduction of plasmid R68.44 into P. putida PP3

Plasmid R68.44 was transferred by conjugation to *P. putida* PP3 in a membrane mating with *P. aeruginosa* PAC174, selecting for transconjugants on defined medium containing 2MCPA, ampicillin, kanamycin and tetracycline. Plasmid transfer occurred at low frequencies of about 10^{-6}. The putative *P. putida* strains carrying R68.44 were checked for characteristic growth on King's A and B media. The transconjugants produced cream/white colonies with no pigment on King's A medium, characteristic of *P. putida* strains, whereas *P. aeruginosa* produced a green diffusible pigment. On King's B medium the transconjugants produced lime-green, yellow-fluorescing colonies, again characteristic of *P. putida* and in contrast to *P. aeruginosa* which produced darker green, white/blue-fluorescing colonies. A number of transconjugants were screened for the presence of plasmid DNA, including the strain *P. putida* PPW1 selected for the mobilization studies, and were shown to contain a plasmid of the same size as that in the donor strain.

A comparison of the digests using restriction endonucleases *Pst*I and *Sma*I showed unexpected, significant differences between the plasmids in *P. aeruginosa* PAC174 and *P. putida* PPW1 (Fig. 1). This was a consistent modification since in two other independent matings using *P. putida* PP3 as recipient, the same changes in the digests were obtained. In view of the differences, the plasmid found in *P. putida* PPW1 was designated pUU1. The second largest fragment (21·0 kb) in the *Pst*I digest of plasmid R68.44 was missing in the equivalent digest of plasmid pUU1 (Fig. 1*a*). It was replaced by two additional fragments of sizes 15·0 and 11·7 kb in pUU1. Thus the combined size of the additional DNA was 5·7 kb greater than the replaced 21·0 kb fragment of R68.44. However, the complete size of pUU1 calculated from the *Pst*I fragments was 61·7 kb compared with the size of R68.44 which was calculated to be 58·0 kb [this size for R68.44 includes the second copy of the 0·74 kb fragment identified by Riess *et al.* (1980)]. The difference of 3·7 kb was significantly smaller than the insert of DNA into the fragment of size 21·0 kb and was due to the concomitant loss of two *Pst*I fragments at some time during the formation of pUU1 from R68.44. The loss of these two fragments (1·3 kb and 0·74 kb) with a total of 2·0 kb and the addition of the 5·7 kb insert accounted for the net increase in size of pUU1 compared with plasmid R68.44.

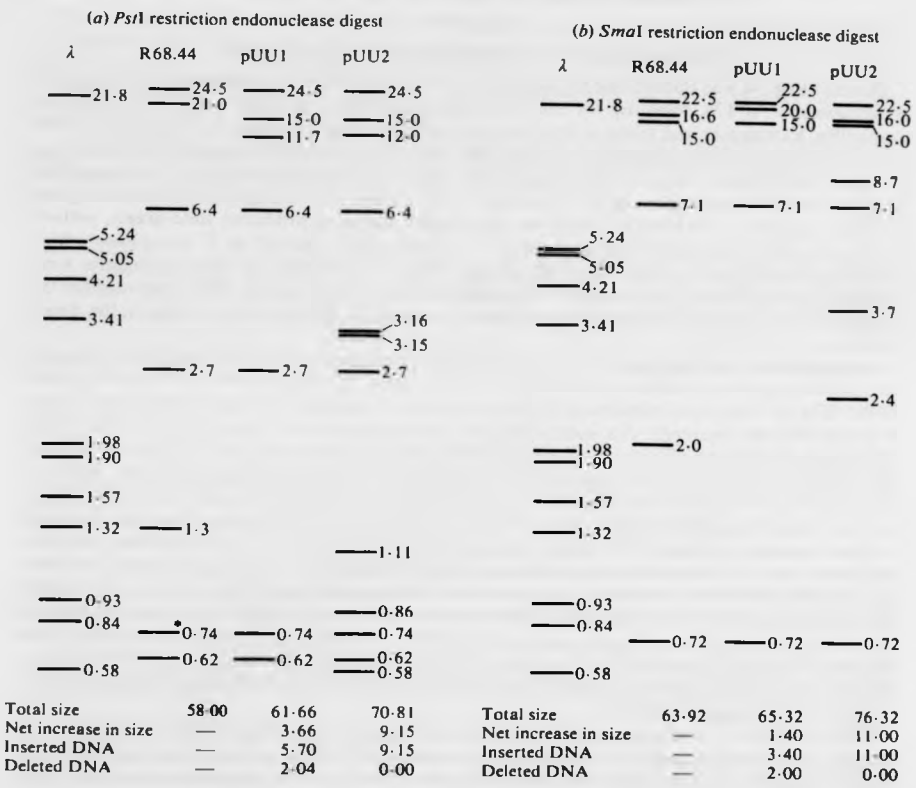
With restriction endonuclease *Sma*I, the second largest fragment (16·6 kb) was also lost in plasmid pUU1 and replaced by a single, larger fragment of 20·0 kb, giving an increase in size of 3·4 kb (Fig. 1*b*). This is comparable with the extra DNA (5·7 kb) determined from the *Pst*I digestion. Furthermore, in agreement with the *Pst*I digestion data, one *Sma*I fragment of 2·0 kb was lost, resulting in a net increase in the size of plasmid pUU1 of 1·4 kb.

The mobilization of the fraction I dehalogenase gene into P. putida PaW340

Pseudomonas putida PPW1 was mated on a membrane with *P. putida* PaW340 with selection for transconjugants on medium containing 2MCPA, tryptophan, ampicillin, kanamycin, streptomycin and tetracycline. Transconjugants were selected at a frequency of $1{\cdot}3 \times 10^{-10}$ per donor cell. This compared with a frequency of $0{\cdot}1 \times 10^{-6}$ to $1{\cdot}3 \times 10^{-6}$ per donor cell for the transfer of plasmid pUU1 into *P. putida* PaW340 in the absence of selection for growth on 2MCPA. The transconjugants proved to be tryptophan auxotrophs and contained a plasmid; one was designated *P. putida* PPW2.

Dehalogenase activity was demonstrated in *P. putida* PPW2 but the activity towards DCA was substantially lower than the activities in *P. putida* strains PP3 and PPW1 (Table 1). Indeed, the substrate activity ratios were indicative of an organism synthesizing only the fraction I dehalogenase, such as *P. putida* PP411-006, and compared favourably with the activity ratio for partially purified fraction I dehalogenase (Table 1). This was confirmed by discontinuous PAGE of *P. putida* strains PP3, PPW1 and PPW2 (Fig. 2). These results suggest the formation of a plasmid which carries *P. putida* chromosomal DNA encoding the fraction I dehalogenase gene.

Further evidence for the formation of such a plasmid was obtained by mating *P. putida* PPW2 with *P. aeruginosa* PAU2 as recipient and selecting for transfer of drug resistance markers.



Total size	58·00	61·66	70·81	Total size	63·92	65·32	76·32
Net increase in size	—	3·66	9·15	Net increase in size	—	1·40	11·00
Inserted DNA	—	5·70	9·15	Inserted DNA	—	3·40	11·00
Deleted DNA	—	2·04	0·00	Deleted DNA	—	2·00	0·00

Fig. 1. Restriction endonuclease digest fragment patterns for (*a*) enzyme *Pst*I and (*b*) enzyme *Sma*I for plasmids R68.44, pUU1 and pUU2 in *P. putida* PPW2. The fragment sizes are given in kb by calculation from a double digest of λ DNA with restriction endonucleases *Eco*RI and *Hin*dIII and using standard DNA fragment sizes given by Southern (1979). The asterisk indicates the position of two fragments of the same size.

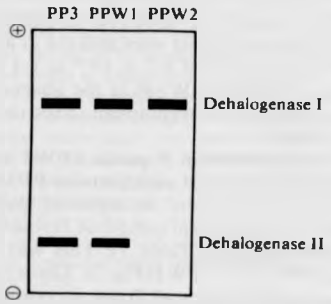


Fig. 2. Distribution of dehalogenases from *P. putida* strains PP3, PPW1 and PPW2 after separation by PAGE.

Table 1. *Dehalogenase specific activities and substrate activity ratios for various strains of P. putida and P. aeruginosa*

Strain	Dehalogenase specific activities [μmol substrate min⁻¹ (mg protein)⁻¹]* MCA	DCA	2MCPA	22DCPA
P. putida PP3	0·45	0·63	0·15	0·08
	(1·00)	(1·40)	(0·33)	(0·18)
P. putida PPW1	0·32	0·40	0·14	0·08
	(1·00)	(1·28)	(0·44)	(0·26)
P. putida PPW2	0·66	0·08	0·28	0·14
	(1·00)	(0·12)	(0·42)	(0·21)
P. putida PP411-006†	ND	ND	ND	ND
	(1·00)	(0·17)	(0·49)	ND
Partially purified fraction I dehalogenase‡	0·62	0·07	0·40	0·19
	(1·00)	(0·11)	(0·65)	(0·31)

ND, Not determined.
* Numbers in parentheses are dehalogenase substrate activity ratios with respect to MCA.
† Data from Weightman *et al.* (1982).
‡ Data from Weightman *et al.* (1979).

Transconjugants were selected at a frequency of $3{\cdot}8 \times 10^{-4}$ per donor cell. Of these transconjugants 95% co-inherited the ability to grow on 2MCPA as the sole carbon and energy source. As additional evidence, plasmid pUU2 was obtained from *P. putida* PPW2 and transformed into *P. aeruginosa* PAO1162 and transformants selected on the basis of drug resistance. Fifty of the transformants were tested and all grew on defined medium containing 2MCPA. No colonies grew on control plates on which recipient cells alone had been spread.

Restriction endonuclease digestion of plasmid pUU2 from P. putida PPW2

Digestion of plasmid pUU2 with restriction endonucleases *Pst*I and *Sma*I revealed that fragments were generated in the same positions as the equivalent digestions for the parent plasmid, with the exception of one fragment in both cases (Fig. 1). For the *Pst*I digestion the 11·7 kb fragment was missing whilst in the *Sma*I digestion the 20·0 kb fragment was not present in pUU2 (Fig. 1).

There were, however, a number of new DNA fragments in plasmid pUU2 which were not present in plasmid pUU1. Using the restriction endonuclease *Pst*I, six extra bands were formed with sizes of 12·0, 3·16, 3·15, 1·1, 0·86 and 0·58 kb; and for the *Sma*I digestion four new fragments were detected with sizes of 16·0, 8·7, 3·7 and 2·4 kb (Fig. 1). Thus, with these additional DNA fragments, plasmid pUU2 had a size of 70·8 to 76·3 kb, depending on which restriction endonuclease was used for the size determination. This represented an increase of 9·2 to 11·0 kb as a result of the formation of the R-prime. The inserted DNA carrying the fraction I dehalogenase gene therefore had an additional four *Pst*I sites and two *Sma*I sites.

DISCUSSION

The restriction endonuclease digest data for enzymes *Pst*I and *Sma*I were consistent with the previously published information (Riess *et al.*, 1980; Leemans *et al.*, 1980; Nayudu & Holloway, 1981; Willetts *et al.*, 1981) enabling the construction of a plasmid map showing the positions of the various restriction cleavage sites (Fig. 3). Plasmid R68.44 contained region I (Fig. 3) with two *Pst*I and one *Sma*I sites whereas plasmid pUU1 had lost this region; this was demonstrated by loss of two *Pst*I fragments totalling 2·0 kb and the equivalent loss of a single *Sma*I fragment of 2·0 kb size. The region lost has previously been associated with the enhanced chromosome-mobilizing capability of plasmid R68.44 and is probably the only difference with plasmid R68 which has no enhanced chromosome-mobilizing ability (Jacob *et al.*, 1977; Riess *et al.*, 1980;

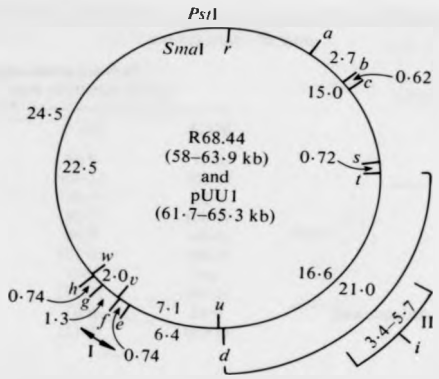


Fig. 3. Map of plasmids R68.44 and pUU1 showing the position of *Pst*I and *Sma*I restriction endonuclease digestion sites. Segment I constitutes the region of IS21 (Willetts *et al.*, 1981) which is present in R68.44 but not in pUU1. Segment II is present in pUU1 but not in R68.44; the position of the insertion is not precisely known but is located between restriction sites *d* and *t*. The map of R68.44 is derived by inserting the sizes of restriction fragments (kb) obtained in this study into the map of Riess *et al.* (1980).

Leemans *et al.*, 1980; Willetts *et al.*, 1981). Willetts *et al.* (1981) have designated this IS21 since its properties are similar to other transposable genetic elements. For example, it has been transposed to the pBR325-based vector pED815 at frequencies of approximately 2×10^{-3}. Furthermore, it can excise precisely at frequencies of approximately 2×10^{-4}, regenerating tetracycline resistance in plasmid pMO891 (Willetts *et al.*, 1981). This latter capability explains the high degree of instability of the enhanced chromosome-mobilizing ability of plasmids R68.44 and R68.5 (Haas & Holloway, 1976) and the ease with which plasmid pUU1 lost this region in the present study. In a number of independently isolated plasmids resulting from transfer of R68.44 from *P. aeruginosa* PAC174 to *P. putida* PP3, this region was repeatedly eliminated.

Another reproducible event, observed on several independent occasions when plasmid R68.44 was introduced into *P. putida* PP3, was the initial acquisition of 3·4 to 5·7 kb of new DNA, segment II. This was inserted in the overlapping regions of the 21·0 kb *Pst*I fragment (generated between *Pst*I sites *c* and *d*) and the 16·6 kb *Sma*I fragment (generated between the *Sma*I sites *t* and *u*) (Figs 1 and 3). Since the 21·0 kb *Pst*I fragment of plasmid R68.44 was replaced by two new fragments (15·0 and 11·7 kb) in pUU1, the newly inserted DNA must contain a single, additional *Pst*I site (site *i*, Fig. 3). The inserted DNA, however, does not contain an additional *Sma*I site since the parent 16·6 kb fragment was replaced by a single, larger *Sma*I fragment of size 20·0 kb in pUU1. The results do not yet enable segment II to be precisely located since neither the position of the *Pst*I site within the 5·7 kb fragment is known nor the orientation of the two new *Pst*I generated fragments. The origin of this extra DNA in pUU1 is at present unknown, but the possibility that it represented a duplicated IS21 region (since its size is almost exactly double that of IS21) can be excluded because the region does not contain appropriate *Pst*I and *Sma*I sites. We cannot exclude duplication of other regions of the parent plasmid but we consider it more likely that this addition represents 'foreign' DNA obtained from the *P. putida* chromosome.

The additional 9·2 to 11·0 kb of DNA inserted into plasmid pUU1 during the formation of the dehalogenase R-prime, plasmid pUU2, was inserted within the common *Pst*I and *Sma*I DNA contained between sites *d* (*Pst*I site) and *t* (*Sma*I site) (Fig. 3). This was determined on the basis of the loss of a single *Sma*I band (20·0 kb) which itself was a composite of the original R68.44 16·6 kb fragment (between sites *t* and *u*) and the added 3·4 kb to produce pUU1 which did not

have any further *Sma*I sites (Fig. 3). This was confirmed by the *Pst*I digest showing that the extra DNA of pUU2 was added into the fragment of 11·7 kb of pUU1 which had in turn been generated by the addition of segment II into the original *Pst*I fragment between sites *c* and *d* (Fig. 3). The exact location of the dehalogenase-encoding DNA cannot be determined with precision from the present information; however, the results do demonstrate that this must be between *Pst*I site *d* and *Sma*I site *t* (Fig. 3).

These results demonstrate the formation of an R-prime carrying the gene for a catabolic enzyme. Furthermore, it is clear that the mobilization of the fraction I dehalogenase gene was not linked topographically with the IS21 region implicated in the mobilization of other pseudomonad genes (Willetts *et al.*, 1981). Although we cannot pinpoint the time at which the 2·0 kb (IS21) segment was eliminated, its absence in pUU1 suggests that the R-prime formation proceeded without the involvement of the duplicate IS21 region. It is also clear that the site of integration of the dehalogenase gene is near the smaller segment of foreign DNA originally inserted during the formation of pUU1 (segment II, Fig. 3). It is possible that this region acts as a site for recombination with chromosomal DNA and hence a focal point for mobilizing chromosomal DNA. An analogous situation has been described for R68 and R68.45-like plasmids in *Escherichia coli* where the derivative plasmids contained 1·2 kb of *E. coli* K12 chromosomal DNA, possibly transposable elements, and were shown to have enhanced chromosome-mobilizing ability (Holloway, 1979). However, if this 3·4 to 5·7 kb region is associated with chromosome-mobilization in *P. putida* PPW1, it does not appear to be very efficient since the dehalogenase gene was transferred at very low frequencies (approximately 10^{-10}). In part this is due to the low rate of transfer of plasmids R68.44 and pUU1 between the various strains of *P. putida* and *P. aeruginosa* used in this study (approximately 10^{-6} to 10^{-7}). This contrasts with considerably higher rates of plasmid transfer demonstrated elsewhere. For example, Johnston *et al.* (1978) demonstrated frequencies of 10^{-2} for the transfer of R68.45 from *Rhizobium leguminosarum* to *Rhizobium meliloti* which was similar to the rates observed between different strains of *R. leguminosarum* (Beringer *et al.*, 1978). Thus, making the adjustment for poor plasmid transfer, the frequency of dehalogenase gene mobilization is about 10^{-6} which is comparable with the poorer mobilization frequencies recorded (Holloway, 1979).

Finally, we cannot exclude mechanisms of R-prime formation which have nothing to do with enhanced chromosome-mobilizing capabilities. It is possible, for example, that the plasmids act as suitable vectors for transposable elements which leave the chromosome independently of any plasmid-determined mechanism. Indeed, preliminary evidence suggests that the dehalogenase genes of *P. putida* PP3 are borne on transposable elements (J. H. Slater, A. J. Weightman and B. G. Hall, unpublished observations).

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