CHARACTERISATION OF TRIMETHOPRIM RESISTANCE TRANSPOSONS AND THEIR GENE PRODUCTS

By

STEPHANIE McKAY THOMSON

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ABSTRACT

A faecal enterobacterial strain, P-20, isolated from pigs, was shown to owe its trimethoprim resistance to two different plasmid/transposon mediated resistance genes. One gene, mobilised by RP4, was located on a 4 - 6 kb transposon designated Tn4135, and resulted in the mediation of transferable high level trimethoprim resistance of greater than 1000 ug/ml. Biochemical investigation of the transposon gene product revealed a close resemblence between the trimethoprim resistant dihydrofolate reductase (DHFR) of *Escherichia coli* J62(RP4::Tn4135) and that of the type I plasmid enzyme, encoded by RP4::Tn7. Despite the differences in transposon size, this marked similarity between the two DHFRs suggests a similar evolutionary origin for the two transposons and reiterates the potential of trimethoprim resistance transfer between animal and human resevoirs.

Detailed biochemical and genetic studies indicated that the second trimethoprim resistance gene of the pig isolate, P-20, mobilised by Sa-1 (Sa-1::Tn4135^{ORI}), bore very little similarity with any previously isolated DHFR. The specific activity of the enzyme was 10 fold lower than that of the type I-like enzyme encoded by RP4::Tn4135, and this, coupled with differences in enzymic properties and the failure to hybridize with type I or type II gene probes, suggests the presence of a new enzyme - a type VI - of distinct evolutionary origin.

The incompatibility group W plasmid, Sa, was investigated for its role as a stable recipient for amplifiication studies of Tn4135, but molecular weight determination, resistance testing and restriction enzyme analysis

revealed that this plasmid was not as stable as expected. This plasmid appeared in two forms, Sa-1 and Sa-2; the former being 15 kb smaller, which resulted from a deletion of DNA encoding the chloramphenicol resistance gene. This instability was further reflected in the size variations of Sa plasmid DNA harbouring trimethoprim resistance transposons. Examination of transconjugants from the transfer of Tn4135 from RP4 to Sa-1, in contrast with transfer to Sa-2, indicated that this transposon could transfer in an aberrant fashion, resulting in the formation of an enlarged plasmid species (Sa-1::Tn4135[a]). The mechanism(s) behind the generation of such a large species were obscure, but appear to involve a combination of multiple transposition, gene amplification, replicon fusion and transposon-mediated transfer of plasmid DNA. Examination of Tn7 transposition to Sa-2 indicated that this transposon was also capable of generating aberrant forms, and reiterated the similarity between the RP4::Tn7 and RP4::Tn4135 encoded genes.

'Your success is determined by your efforts and not by results, and you may come to realise that the most important journey is the journey inwards.'

The Adventure Alternative

Colin Mortlock 1984

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DECLARATION

The experiments and composition of this thesis are the work of the author unless otherwise stated.

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ABBREVIATIONS

Abbreviation	Meaning
Ap	ampicillin
bp	base pairs
BSA	Bovine serum albumen
ccc	covalently closed circular
Cb	carbenicillin
Cm	chloramphenicol
d	daltons
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DM	Davis-Mingioli
DNA	Deoxyribonucleic acid
DSTA	Diagnostic sensitivity agar
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium bromide
eop	efficiency of plating
His	histidine
ID_{50}	dose giving 50% inhibition
Inc	incompatibility
IR	inverted repeat
IS	Insertion sequence
ISO	Isosensitest broth
Ka	Kanamycin
Kb	kilobases
MIC	Minimum Inhibitory concentration
mls	millilitres
mtx	methotrexate
MW	molecular weight
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NB	nutrient broth
PABA	para-amino benzoic acid
PEG	polyethylene glycol
pI	isoelectric point
pro	proline
R-det	resistance determinant
Rif	Rifampicin
R-plasmid	resistance plasmid
s	seconds
SDS	sodium dodecyl sulphate
Sm	streptomycin
Sp	Spectinomycin
Su	sulphonamide
Tc	tetracycline
TE	Tris EDTA
THF	tetrahydrofolate
Thy	thymidine
Tn	transposon
Tp	Trimethoprim
Trp	tryptophan
u	units
ul	microlitres
v	volts

SOURCE OF BIOCHEMICALS AND CHEMOTHERAPEUTIC DRUGS

Ampicillin	Beechams Ltd., Brentford, Middlesex
Agarose	BDH Chemicals ltd., Poole, Dorset
Bacteriological agar	Oxoid Ltd., Basingstoke, Hants.
BSA	Sigma
Carbenicillin	Beechams Ltd., Brentford, Middlesex
Chloramphenicol	Parke Davis, Pontypool, Gwent
Chymotrypsinogen	Sigma
Caesium Chloride	Terochem Ltd., Edmonton, Canada
Cytochrome C	Sigma
DHF	Sigma
DNase	Sigma
DSTA	Oxoid Ltd., Basingstoke, hants.
EDTA	Sigma
Ethidium Bromide	Sigma
Ficoll	Sigma
Herring Sperm DNA	Sigma
L-Histidine	BDH
Ion agar	Oxoid ltd., Basingstoke, Hants.
Isosensitest broth	Oxoid Ltd., Basingstoke, Hants.
Kanamycin	Bristol laboratorees, Slough, Middlesex
Lambda DNA	Sigma
LB base	Gibco labs.
Lysozyme	Sigma

MacConkey agar	Oxoid Ltd., Basingstoke, Hants.
methionine	Sigma
mtx	Lederle lab., Gosport, Hants.
Mueller Hinton broth	Difco
NADPH	Sigma
Nalidixic acid	Sterling Research Labs., Surbiton, Surrey
NB	Oxoid Ltd., Basingstoke, Hants
Nitrocellulose filters	Schleicher and Schuell
Nucleotides (dCTP, dGTP, dTTP)	Sigma
Oxytetracycline hydrochloride	Glaxo Ltd., Greenford, Middlesex
PEG	Sigma
Polymerase I	BRL
polyvinyl pyrrolidone	Sigma
proline	BDH
^{32}P dATP	Sigma
Restriction enzymes	Boehringer Corporation(London) Ltd.
	NBL
Rifampicin	Le Petite, Milan, Italy
RNase	Sigma
SDS	BDH Chemicals Ltd., Poole
Sephadex	Pharmacia
Spectinomycin	Upjohn Ltd., Crawley. Sussex
Streptomycin	Glaxo Ltd., Greenford, Middlesex
Sulphamethoxazole	Wellcome Foundation Ltd., Beckenham, Kent
Tp lactate	Wellcome Foundation Ltd., Beckenham, Kent
L-tryptophan	BDH

TABLE i: STANDARD BACTERIAL STRAINS

STRAIN	PLASMID	TRANSPOSON	RESISTANCES	GENETICS	MOLECULAR WEIGHT (kb)	SOURCE/ REFERENCE
E coli J53	R40a		ApKaSu	IncC	130	Datta and Hedges, 1972a
E coli K12	R1		ApCmSuSm	IncF2	90	Meynell and Datta, 1966
E coli J53	RP4		ApKaTc	IncP	52	Datta et al, 1971
E coli J53	R1010-6		Ap	IncN	54	Nugent and Hedges, 1979
E coli J62	R6K		ApTc	IncX	38	Kontomichalou et al, 1970
E coli J62	Sa (Sa-2)*		CmSuSmKa	IncW	33	Watanabe et al, 1968
E coli J62	R388		Tp	IncW	29	Datta and Hedges, 1972
E coli J62	R751	Tn402	Tp	IncP	48	Jobanputra and Datta, 1974
E coli J62	R7K		Ap	IncW	32	Coetze et al, 1972
E coli J53						Bachman, 1972
E coli J62						Clowes and Rowley, 1954
E coli J62-2			Rif			Bachman, 1972
E coli C600						Bachman, 1972
E coli C600	pBR322		ApKaTc		4.3	Bolivar and Bachman, 1979
P aeruginosa Pa08						Isaac and Holloway, 1968
E coli J53	R483	Tn7	TpSm/Sp	IncIa	90	Barth et al, 1976
E coli J62+	RP4	Tn4135	ApKaTcTp (Sm/Sp)			This lab. - unpublished results
P-20F			TpTc			This lab. - unpublished results
P-20S			Tp			This lab. - unpublished results

Bacterial symbols are those used by Bachman et al (1976) and plasmid markers are used according to the proposals of Norvick et al (1976)

+Tn4135 was initially mobilised from the pig isolate stored on an agar slope (P-20S)

*See chapter 2

INTRODUCTION

INTRODUCTION

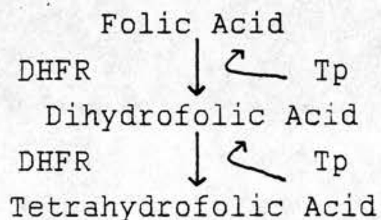
Trimethoprim (Tp) (2,4,-diamino-5-(3',4',5'-trimethoxybenzyl)-pyrimidine) is one of a series of compounds, first fully described by Roth et al (1962), possessing both antimalarial and antibacterial activity. It has been used in combination with sulphonamides (Co-trimoxazole) since 1968 (Burchall and Hitchings, 1968) for the treatment of a wide spectrum of infectious deseases in human and veterinary medicine (Wormser, 1978; Salter, 1982). It is active against a wide range of gram negative and gram positive aerobic bacteria, is readily absorbed by the oral route (Schwartz and Ziegler, 1969) and is widely distributed in body fluids and tissues with few side effects (Brogden et al, 1982). Among anaerobic bacteria, at least partial resistance to Tp is a widespread feature; the basis of which is not yet understood. Clostridia are resistant in most cases (Then and Angehrn, 1979), Pseudomonas aeruginosa are not susceptible at most concentrations attainable in the body tissues, blood or urine (Grey and Hamilton - Millar, 1977; Kasanen et al, 1978), and Bacteroides fragilis and lactobacillus are relatively insensitive (Then and Angehrn, 1979) to Tp requiring high concentrations for inhibition. As a single agent Tp has been used in the treatment and prophylaxis of acute uncomplicated urinary tract infections, caused mainly by Escherichia coli (review: Brogden et al, 1982), but has been found to be less effective in the treatment of complicated and recurrent urinary tract infections.

As a synthetic analog of the natural substrate, dihydrofolate (DHF), Tp acts as a potent competitive inhibitor of bacterial diydrofolate reductase (DHFR) by binding to the hydrophobic substrate binding pocket (Baker _et al_, 1981) of this enzyme. It binds 1000 times more strongly to this active site than does the natural substrate (Amyes and Smith, 1978) and is capable of descriminating between mammalian and bacterial DHFR's, since it has a far lower affinity for the former enzyme (Burchall, 1973). DHFR catalyses the NADPH dependent reduction of DHF to tetrahydrofolate (THF) (Figure i); a stage in the sequence leading to the synthesis of purines and ultimately of DNA, and its inhibition is thus toxic to cells. Although both mammalian and bacterial cells reduce DHF to THF by the enzyme DHFR (Figure i), the ability to synthesize THF from basic constituents is confined to bacteria. However, the mammalian DHFR can reduce dietary folic acid to DHF; a function not possessed by the bacterial enzyme, and this fortunate biochemical distinction forms the basis of the therapeutic potential of antifolates. Few bacteria can absorb preformed folic acid (Wood _et al_, 1961) and therefore have to synthesize their own.

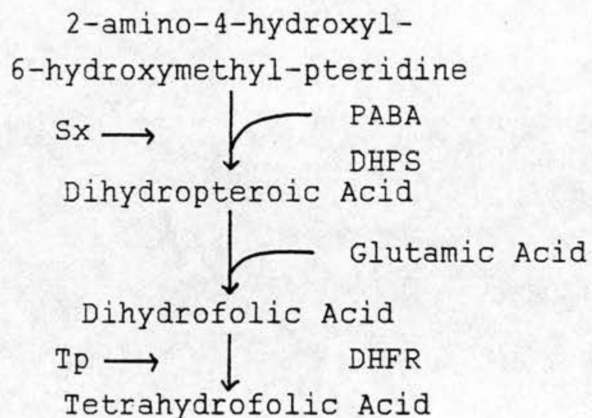
The stage inhibited by Tp immediately follows that blocked by the sulphonamides (Su) (Brown,1962), hence the strongly synergic effect of the combination of Su and Tp (Bushby and Hitchings, 1968). Whilst the action of Su is purely bacteriostatic that of the combination was found to exhibit bacteriocidal properties (Darrell _et al_, 1968) and it was therefore hoped that the use of this combination would reduce the incidence of bacteria able to resist both drugs (Bushby and Hitchings, 1968). However, when co-trimoxazole was first used widely, the bacterial population already contained a high percentage of sulphonamide resistant strains (Datta, 1969) and there have been reports of sulphonamide

FIGURE i: THE TETRAHYDROFOLIC ACID BIOSYNTHETIC PATHWAY IN MAMMALS AND BACTERIA

MAMMALIAN



BACTERIAL



DHPS: Dihydropteroate synthase; DHFR: Dihydrofolate reductase; Sx→: Sulphamethoxazole inhibits; Tp→: Trimethoprim inhibits; Tp↩: Trimethoprim does not inhibit.

apppearing to antagonise the bacteriocidal effects of Tp (Anderson et al, 1973). Furthermore no synergistic effect was found between Tp and Su against bacteria highly resistant to Su (MIC > 100 ug/ml) although there was synergism against bacteria with moderate resistance to Su (Grey et al, 1979). A number of surveys have therefore questioned the rational for combining Tp and Su (Grunberg, 1979; Amyes, 1982) as its use has not obviously prevented the emergence of Tp resistant strains in urinary tract infections (Amyes et al, 1981). In addition the choice of media is now known to be a crucial factor in determining the sensitivity of organisms (Amyes et al, 1981) and this will have undoubtably effected the accuracy of reports on the benefits of co-trimoxazole usage.

Although Tp resistant gram negative bacteria were first reported shortly after the introduction of co-trimoxazole, the emergence of acquired resistance to Tp was infrequent during the early years of its therapeutic use. However, with the introduction of Tp alone in October 1979 there was concern about the rapid development of such resistance in the absence of the Su moiety (Barry and Pattishall, 1983; Hamilton-Millar, 1984; Rich and Mee, 1985). Resistance to antibiotics in bacteria has shown an explosive increase in recent years, which in some instances has more than kept pace with the commercial production and clinical use of antibiotics and chemotherapeutic agents. However, despite laboratory surveys sugesting that resistance to Tp among enterobacteria was increasing, there has been no convincing evidence to suggest that the use of Tp alone in urinary tract infections has been associated with a 'rapid' increase in the incidence of bacteria resistant to the drug (Lacey, 1982; Brumfitt et al, 1983). In Finland, where Tp has been used alone since 1973 (Burman, 1980), the frequency of resistant E coli causing urinary tract infections remained fairly constant between 1972 and 1977 (Kasanen

et al, 1978) and the results of Towner et al (1986) for Enterobacteriacea isolated from patients with urinary tract infections, seem to suggest that the overall incidence of resistance has been only slightly affected in strains of E coli. However this may vary with environmental conditions (country) and may reflect the continuing use of co-trimoxazole, despite the availability for use of Tp alone. Although there appears to have been no overall increase in Tp resistance there is an increased proportion of Tp resistant bacteria with high level resistance to the drug, suggesting the continuing spread of plasmid or transposon mediated resistance (Towner, 1982; Brumfitt et al, 1983). Further complicating the issue is the possibility of resistance selection by other drugs. Amyes et al (1981) indicated that Ap was particularly effective at selecting bacteria that were not only Ap resistant but Tp resistant as well.

Although it is known that bacteria can mutate to become drug resistant, this process is generally not thought to be responsible for the resistance of clinical bacterial isolates to antibiotics. One reason for this is perhaps because such mutants often exhibit reduced pathogenicity (Knox and Smith, 1971). Most clinically significant antibiotic resistance is determined by genes located on extrachromosomal DNA elements called plasmids (Falkow, 1975; Broda, 1979; Hardy, 1981). Different species of bacteria harbour characteristic types of plasmids, some of which can mediate their own transfer, and thus the transfer of resistance genes, (Akiba et al, 1960), by conjugation. In addition, resistance genes are often incorporated into discrete genetic units called transposons (Kleckner, 1981), which have the capacity to transpose from one DNA molecule to another. This has undoubtably contributed to the rapid dissemination of antibiotic resistance by providing an efficient mechanisms for incorporating resistance determinants into new vectors

which can transfer to and stably replicate in diverse hosts. The distinction between plasmid-encoded and chromosomally encoded resistance can therefore become blurred because transposons can integrate into the chromosome and have been found in this location in clinical isolates. However, the two types of chromosomal resistance, transposon encoded and mutational, can be readily distinguished by biochemical and genetic tests.

There have been many mechanisms proposed by which bacteria may resist antibacterial drugs (Goldstein et al, 1968; Davies and Smith, 1978; for review see Foster, 1983). The mechanisms most favoured by bacteria are lowering of the intracellular drug concentration (impermeability), destruction or modification of the drug to an inactive form, alteration of the target site of a drug so that it no longer binds the inhibitor, increasing the synthesis of the target enzyme in excess of the inbibitor, or the production of an alternative biochemical pathway by by-passing the drug sensitive target site. Whilst resistance to B lactam antibiotics is due to a drug inactivation mechanism (Richmond and Sykes, 1973; Sykes and Mathew, 1976) resistance to Tp is largely due to the production of a plasmid encoded Tp resistant DHFR (Amyes and Smith, 1974,1984; Skold and Widh, 1974; Pattishall et al, 1977; Smith ,1980; Then ,1982; Acar and Goldstein, 1983; Hamilton Miller, 1984), thus affecting a bypass of the inhibited chromosomal enzyme. The discovery of such an enzyme on R388 was thought to be the first example of an R factor conferring an altered target site mechanism of resistance to a chemotherapeutic agent (Amyes and Smith, 1974). An extensive search had been caried out to find specific modifying enzymes or a plasmid determined mechanism that prevented drug uptake, that would explain the resistance, but none had been found (Amyes, 1974). Since the inhibitors of enzymes involved in THF synthesis are

synthetic, and not naturally occurring, it is unlikely that specific inactivation enzymes could have evolved, but there has subsequently been a report of plasmid mediated resistance due to reduced permeability (Amyes and Smith, 1976). However, this appears to additionally involve a chromosomal gene and Amyes and Smith have indicated that there may be other R plasmid mediated resistance mechansisms, that involve a combination of bacterial and chromosomal genes. Subsequent to the discovery of the DHFR of R388, another plasmid conferring Tp resistance, R483, was also shown to mediate the synthesis of a Tp resistant DHFR (Skold and Widh, 1974). These enzymes were found to possess the ability to differentiate between the substrate and its close structural analogue methotrexate (mtx) (Amyes and Smith, 1976), which may have some serious consequences in the search for new antibacterial inhibitors of DHFR. The plasmid mediated bypass mechanism exhibited by R388 and R483 generally results in high level resistance (MIC $>$ 1000 ug/ml) whilst resistance to low levels of Tp (4 - 512 ug/ml) results from mutations which either decrease the susceptibility of the chromosomal DHFR to Tp (Poe _et al_, 1979; Then and Herman, 1981) or impair the penetration of the drug in the cell (Glutman _et al_, 1985). (Plasmid mediated low level resistance has been described (Anderson, 1980; Towner and Pinn, 1981; Acar _et al_, 1973)) but the mechanism is still controversial). The only example of a chromosomally mediated bypass mechanism was in a mutant of _Streptococcus faecalis_ (SF/A) shown to produce two DHFRs. One resembled the wild type enzyme, possessing similar physical properties, whilst the other had a lower molecular weight and turnover number (Nixon and Blakely, 1968).

The type of resistance mechanism described above would be unsuitable for providing resistance to other antibiotics since the target sites of many of these drugs are complex structures involved in such processes as cell wall and protein synthesis. In the case of Tp, the target site is a single enzyme in a vitamin biosynthetic pathway, and the production of an additional resistant enzyme would be a negligible drain on the cell resources. There are however, other mechanisms of acquired resistance to Tp which are common to other antibiotics (Table ii), including overproduction of the sensitive enzyme (Breese et al , 1975; Sheldon and Brenner, 1976; Flensburg and Skold, 1984) and many diverse forms of chromosomal resistance (Goldstein, 1977). Occasionally Tp resistant strains causing urinary tract infection have a mutation in the gene encoding thymidylate synthase (Stacey and Simpson, 1965) which causes a thymine requiring phenotype and resistance to Tp because one of the major cellular requirements for reduced folic acid is eliminated (Stacey and Simpson, 1965).

With R plasmid mediated resistance to the B lactam antibiotics it was found that, when the enzymological properties of the B lactamases mediated by such R plasmids were analysed, an enzymic type termed TEM predominated. It was concluded that 'the ubiquity of the structural gene for the TEM like enzyme demonstrates its evolutionary success, which probably results from its ability to be translocated from one replicon to another (Hedges et al, 1974). Subsequently, this gene and other genes of R plasmids (such as Tc, Cm and Ka) have been classified as transposons (Shandler et al, 1979). The rapid spread of Tp resistance in France (Acar et al, 1977), Italy (Romero and Peduca, 1977), England (Amyes et al, 1978; Broad and Smith, 1982) and Scotland (Amyes et al, 1981) also suggests that the Tp genes were being broadcast on transposons. Barth et al (1976)

TABLE ii: MECHANISMS OF ACQUIRED RESISTANCE TO TRIMETHOPRIM

MECHANISM	ORGANISM(S) STUDIED
Thymine requirement	Enterobacteriaceae, Staphylococcus Streptococcus
Impaired penetration	Klebsiella, Enterobacteriaceae, Serratia
Altered DHFR	Enterobacteriaceae, Staphylococcus
Overproduction of DHFR	Enterobacteriaceae
Additional trimethoprim resistant DHFR (plasmid or transposon mediated)	Enterobacteriaceae, Vibrio cholerae, Acinetobacter, Pseudomonas aeruginosa

showed that one of the early Tp R plasmids,R 483, carried a Tp resistance gene on a 14 kb fragment of DNA known as Tn7, that is able to integrate into the chromosome as well as into other plasmids. Richards and Datta (1981) found that Tn7 was present in the majority of R plasmid-mediated Tp resistant bacteria isolated from human infections in hospitals and in general practice patients, and other Tp resistance transposons have since been found in clinical isolates (Amyes et al 1982; Tietze et al, 1982). Tp resistance in animal isolates is even more common than in human patients (Smith, 1979) and enzymological and resistance studies suggest that plasmids from these animal strains also possess Tn7 (Broad and Smith, 1982). It is hence possible that the source of Tp resistance in humans could have arisen in animals and that transposition is the mechamism of spread of this resistance, not only within bacterial populations, but also between them.

Tp has been introduced since the discovery of R-plasmids, and therefore provides a rare opportunity to monitor the initial development of an R plasmid borne resistance, and study its evolution. The spread of the Tp resistant determinant is uncharacteristic of conventional R plasmids in that resistance can spread rapidly amongst R plasmids of different incompatibility groups and thus through the bacterial population. With respect to the epidemiology of Tp resistance, greatest concern now centres around transposon encoded resistance, and there are now a number of different Tp resistance genes residing on transposons (and therefore R plasmids) which require further characterisation to ascertain their origins and relatedness. The purpose of this study was therefore to characterise one such transposon, a small Tp resistance transposon of animal origin, and its gene product(s), and to determine its relationship

to Tn7 by means of biochemical and molecular biological techniques. The examination of such a system will give a clearer understanding of the possible spread of resistance between resevoirs, and possibly the mechanisms that control transposition in and out of the chromosome and its stability. This information will be of value in preserving the efficacy of trimethoprim.

CHAPTER 1

COMPARISON OF RAPID SMALL SCALE TECHNIQUES FOR THE ISOLATION AND RESTRICTION OF PLASMID DNA

INTRODUCTION

Many experimental techniques and biological investigations require the ability to purify plasmid DNA. In the past, the spread of plasmids through the population was studied by examination of the characters that the plasmid carried (ie. antibiotic resistance) and its genetics (ie. incompatibility) (Datta and Hedges, 1971). More recently, molecular biological techniques have been used to determine the size of plasmids (Farrar, 1983). However, even a combination of these techniques may lack the sophistication to follow the progress of plasmids through a clinical population. This is especially true if it is considered that transposons may readily insert copies of themselves into the plasmid and affect not only the size of the plasmid but also its characteristics. Therefore, the plasmid DNA has to be examined more rigorously. This may be achieved by 'fingerprinting' the plasmid, by digesting it with restriction endonucleases, or by hybridising it with known radio-active probes. The latter technique, favoured by many molecular biological groups, is probably the best available but it is time-consuming and expensive. Fingerprinting, on the other hand, is simple to perform and will probably eventually win acceptance by many diagnostic laboratories.

The ability to restrict plasmid DNA successfully relies on the consistent isolation of pure DNA. Until fairly recently this involved centrifugation in caesium chloride - ethidium bromide density gradients (Bauer and Vinograd, 1968) to separate plasmid DNA from chromosomal DNA. Although efficient, this method is expensive, and time-consuming as gradients often have to be spun for about 40 hours, and the plasmid DNA

has to be extracted from the caesium chloride - ethidium bromide solution. In addition, epidemiological investigations were hampered by the inability of the currently available DNA preparation techniques to treat many isolates at the same time. In order to overcome these problems a number of rapid DNA isolation procedures were developed with a view that the extracted DNA might be used directly in restriction endonuclease analysis, in the absence of further purification procedures (Eckhardt, 1978; Birnboim and Doly, 1979; Klein _et al_, 1980; Kado and Liu, 1981; Sparks and Elder, 1983; Orberg and Sandine, 1984; Voquang _et al_, 1985). These methods exploit the physical characteristics of plasmid DNA to effect separation ie. the relatively small size of plasmid DNA (1 - 200 kilobases (Maniatis _et al_, 1982) compared with 4000 kb for the bacterial chromosome), its covalently closed structure and the fact that plasmids are not bound to other cellular components in a lysate.

A number of factors, such as the presence of large molecular weight RNA, failure to remove certain inhibitory chemicals (eg. phenol) and pH may be involved in determining whether a method successfully produces pure enough plasmid DNA for restriction. However, any method should satisfy the following conditions if it is to prove useful:- (i) the plasmid DNA must be pure enough for direct restriction, (ii) results should be reproducible, (iii) it should be possible to isolate sufficient DNA to give clear sharp bands, (iv) The preparation method should not affect the electrophoretic mobilities of the plasmid DNA, (v) it should be possible to isolate both large and small plasmid DNA's if the method is to have universal use, (vi) it should be applicable to as wide a range of bacteria as possible eg. _Enterobacteriaceae_, _Pseudomonas_ and _Haemophilus_, (vii) The examination of a large number of cultures at the same time should be possible and (viii) the presence of chromosomal DNA should be kept to a minimum.

There are significant problems in studying plasmids of clinical bacteria because many of the plasmid DNA isolation techniques were developed for work with small plasmids, such as pBR322 (4.3 kb)(Bolivar *et al*, 1977), which are easier to isolate than the larger clinical plasmids. Thus their application in the clinical situation is largely untested. The clinical bacteriologist therefore faces a dilemma; which of the many techniques available could be applicable to the larger plasmids (50 - 100 kb) found in clinical bacteria and what are the critical steps in a procedure? Recently there has been a move to try and draw some of the different techniques together (Trevors, 1985; Mazza, 1986), outlining the general principles of plasmid DNA isolation and separation by agarose gel electrophoresis. Although he alluded to the variety of methods available, Trevors (1985) made no comparison between them. Since clinical isolates and their transconjugants would be examined later, the purpose of this study was to compare a number of different methods for their ability to quickly and easily produce 'pure' DNA from strains containing plasmids, that would be involved in later resistance gene analysis (ie RP4 and Sa-1), and to identify the crucial steps where errors can arise.

MATERIALS AND METHODS

BACTERIAL PLASMIDS

The plasmids used in this study were Rl, RP4, R6K and Sa-1 of size range 30 - 90 Kb (See Table i). All plasmids were isolated from overnight Mueller Hinton (Difco, USA) broth cultures of *Escherichia coli*.

ISOLATION TECHNIQUES

Alkaline Denaturation

Plasmid DNA was prepared by the method of Birnboim and Doly (1979). An overnight broth culture (1.5 ml) was harvested in a microfuge tube by centrifuging for 1 minute (11500 g). The supernatant was discarded and the pellet resuspended in 100 ul of solution 1 (lysozyme 2mg/ml; 50 mM glucose; 10 mM EDTA; 25 mM Tris pH 8.0). After incubating at 0°C for 30 minutes, 200 ul of solution II (0.1 M NaOH; 1% weight/volume SDS) was added. This was followed by gentle shaking until the solution became clear and viscous. The tube was then maintained at 0°C for 5 minutes before 150 ul of solution III (3 M sodium acetate, pH 4.8) was added. The solution was gently mixed by inversion until a clot formed and then maintained at 0°C for 1 hour, followed by centrifugation for 10 minutes. The clear supernatant was transferred to a second microfuge tube and 1 ml of cold ethanol added. After mixing well DNA was precipitated by incubation at -70°C for 15 - 30 minutes. The precipitate was collected by centrifugation (5 minutes) and the supernatant discarded. The pellet was dissolved in 100 ul of 0.1 M sodium acetate/0.05 M Tris pH 8.0 and reprecipitated with two volumes of cold ethanol. After 10 minutes at -70°C the precipitate was again collected by centrifugation and the final pellet dissolved in 70 ul of 10 mM Tris; 1 mM EDTA pH 8.0.

Alkaline Lysis - Ish-Horowitz and Burke (1981)

Plasmid DNA was prepared by a modification of the above Birnboim and Doly (1979) method. The protocol was largely that of Ish-Horowitz and Burke (1981) with a few modifications. One ml of a 1.5 ml overnight culture, grown up in Mueller Hinton broth (Difco), was transferred to an eppendorf tube and the cells pelleted by centrifugation (11500 g; 15 seconds). The medium was removed with a Pasteur and the pellet resuspended in 100 ul of solution I (50 mM glucose; 10 mm EDTA, pH 7.5; 25 mM Tris/HCl, pH 8.0). After vortexing, 200 ul of solution II (0.2 M NaOH; 1% SDS, freshly prepared) was added and the tube rocked sharply to mix solutions. The tube was left on ice for 5 minutes before adding 150 ul of precooled solution III (5 M Sodium Acetate, pH 4.8, prepared as follows: to 60 ml of 5 M Sodium Acetate add 11.5 ml of glacial acetic acid and 28.5 ml of water). The tube was mixed by inversion and brief vortexing and left on ice for 5 minutes. After spinning for 1 minute, 360 ul of the supernatant was transferred to a fresh eppendorf tube and 420 ul of ethanol added with mixing. The tube was left at room temperature for 2 minutes before centrifuging for 2 minutes (microfuge, 11500 g). The ethanol was poured off and the tube left in an inverted position to allow the fluid to drain away. The pellet was washed with 500 ul of 70% ethanol, vortexed briefly and recentrifuged (11500 g, 2 minutes). The supernatant was removed and the pellet dried before resuspending in 50 ul of TE (pH 8.0) containing DNase-free RNase (20 ug/ml).

Kado and Liu

Cells were grown overnight in 5 mls of Mueller Hinton broth (Difco) and harvested by centrifugation (3980 g for 15 minutes). The pellet was resuspended in 1 ml of E buffer (40 mM Tris acetate; 2 mM sodium EDTA, pH 7.9). Lysing solution (3% SDS; 50 mM Tris pH 12.6) (2 ml) was added and the sample mixed by brief agitation. After incubation for 20 minutes at 60°C, two volumes of phenol/chloroform (1:1) were added. The solution was then shaken briefly and centrifuged for 30 minutes at 2544 g. Avoiding the precipitate at the interface, the upper aqueous phase was transferred to a fresh tube with a sawn off Pasteur pipette, ready for loading on an agarose gel.

A further purification step was included if preparations were to be restricted. Samples were extracted 2-4 times each with diethyl-ether to remove any phenol. One hundred microlitres of 3 M Sodium Acetate and 800 ul of cold ethanol were added and the solution left on ice for 5 minutes. Precipitated DNA was pelleted by centrifugation at 636 g for 5 mins. The pellet was resuspended in 40 ul of distilled water and 20 ul aliquots were used for restriction.

McMasters

DNA was prepared by a modification of the McMasters et al (1980) method. Bacteria were grown up overnight in 25 mls of Mueller Hinton broth (Difco) and harvested by centrifugation at 3980 g for 20 minutes. Two hundred microlitres of 25% sucrose in 50 mM Tris HCl (pH 8.0) was added and the solution vortexed. After transferring the sample to an eppendorf tube, 20 ul of lysozyme (5 mg/ml in 250 mM Tris HCl, pH 8.0) was added and the tube vortexed again. Forty microlitres of 250 mM EDTA; 10% SDS was added with further vortexing followed by 20 ul of fresh 5N NaOH and vortexing. After leaving at room temperature for 10 minutes, 20 ul of 1 M sodium acetate (pH 5.0) was added with vortexing. Phenol (400 ul) was added, again with brief vortexing, followed by centrifugation for 10 minutes in a microfuge (11500 g). The aqueous phase was pipetted into a new eppendorf tube and incubated at 37°C for 30 minutes with 5 ul of RNase (10 mg/ml). Twenty microlitres of 5 M NaCl and 150 ul of Polyethylene Glycol was added before incubating on ice for 2 hours. Precipitated material was then collected by centrifugation (microfuge, 30 minutes) and the pellet resuspended in 200 ul of 50 mM Tris-HCl, pH 8.0 containing 5 mM EDTA. After extracting with chloroform and precipitating for 30 minutes at -70°C with cold ethanol, the DNA pellet was resuspended in 70 ul of 10 mM Tris HCl (pH 8.0) containing 1 mM EDTA.

Rapid boiling method

Plasmid DNA was prepared by a modification of the Holmes and Quigley (1981) method. Five millilitres of Mueller Hinton broth (Difco) was inoculated with a single bacterial colony and incubated overnight with vigorous agitation on a Gallenkamp orbital incubator at 140 rpm. Approximately 1.5 mls of this was transferred to an eppendorf tube and centrifuged for 1 minute. The medium was removed with a Pasteur pipette leaving the pellet as dry as possible. The pellet was resuspended in 0.35 mls of lysing solution (8% sucrose; 0.5% Triton X-100; 50 mM EDTA; 10 mM Tris HCl, pH 8.0), and 25 ul of a freshly prepared lysozyme solution (10 mg/ml in 10 mM Tris HCl, pH 8.0) was added and the solution vortexed for 3 seconds. The tube was placed in a boiling water bath for 40 seconds before centrifuging for 10 minutes at room temperature in an eppendorf centrifuge (11500 g). After removing the pellet with a toothpick, 40 ul of 2.5 M sodium acetate and 420 ul of isopropanol was added to the supernatant and mixed by vortexing. The preparation was stored on ice for 15 minutes followed by 15 minutes centrifugation. The pellet was dried, resuspended in 70 ul of Tris-EDTA (pH 8.0) containing Dnase free Rnase (50 ug/ml) and the solution incubated at 37°C for 10 minutes, to remove any RNA.

TAKAHASHI TECHNIQUE

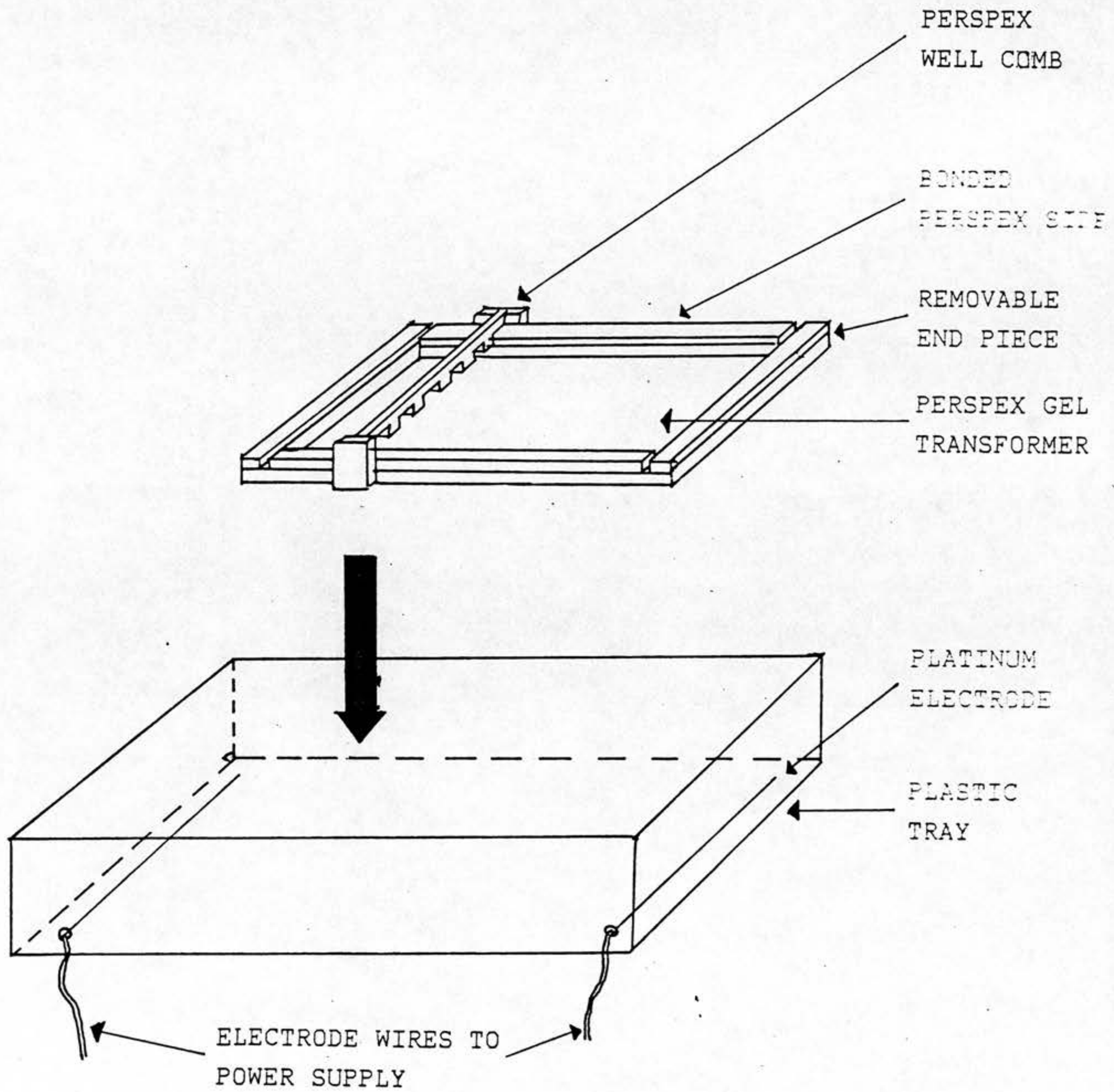
Cells were grown up overnight in 5 mls of Mueller Hinton broth (Difco) at 37°C and harvested by centrifugation (bench: 3980 g for 15 minutes). The cells were thoroughly resuspended in 200 ul of Buffer A (40 mM Tris acetic acid; 2mM disodium EDTA, pH 8.0) and then transferred to an eppendorf tube. Four hundred microlitres of lysing solution (4% SDS/100 mM Tris solution made up in double distilled water, to which was added an equal volume of freshly prepared 0.4 N NaOH solution) was added and the tube gently inverted 5-10 times. After allowing to stand at room temperature for 5 minutes the solution was neutralised with 300 ul of cold Buffer B (3 M sodium acetate, pH 5.5) and gently mixed by inversion 10-20 times. The solution was maintained at 0°C for 5 minutes before centrifuging at room temperature (Microfuge: 2576 g for 5 minutes) and the supernatant transferred to another tube by decantation. Chloroform (0.7 mls) was added and emulsified by inversion 5-10 times. After centrifuging at 0°C for 5 minutes (microfuge: 2576 g), 500 ul of the upper aqueous phase was carefully transferred to another tube with a micropipette tip which was cut so that it had a diameter of more than 5 mm in diameter. Cold ethanol (1 ml) was added to this and the solutions mixed by inversion 5-10 times. After maintaining at 0°C for 5 minutes the precipitate was collected by centrifugation at 0°C (Microfuge: 2576 g for 5 minutes). The pellet was resuspended in 100 ul of Buffer C (1 M sodium acetate; 10 mM Tris acetic acid; 2 mM disodium EDTA, pH 8.0) giving a final volume of approximately 130 ul.

The Takahashi method requires additional purification steps before the DNA can be digested with restriction endonucleases. Buffer C (350 ul of 10 mM Tris acetic acid; 2 mM disodium EDTA, pH 8.0) and Buffer D (50 ul of 1 M sodium acetate; 10 mM Tris acetic acid; 2 mM disodium EDTA, pH 8.0) was added to each sample. One millilitre of cold ethanol was then added and the tube inverted 5-10 times. DNA was then precipitated at 0°C for 5 minutes and collected by centrifugation at 0°C (5 minutes; 2576 g). The supernatant was completely removed and the pellet resuspended in 40 ul of sterile distilled water.

AGAROSE GEL ELECTROPHORESIS

Electrophoresis was performed in horizontal slab gels of 0.7% agarose (BDH Chemicals Ltd., Poole, Dorset) dissolved in Buffer A (40 mM Tris acetic acid; 2mM disodium EDTA, pH 8.0) following the method of Meyers *et al* (1976). Figure 1.1 illustrates the set up used. The apparatus comprises a plastic tray with platinum wire electrodes running along either end. The gel former is made of perspex as is the well comb. The sides of the gel former are made of bonded perspex whereas the ends are removable pieces, held together by clips whilst the gel is poured, and removed before it is run. Thirty microlitres of undigested DNA samples were mixed with 10 ul of bromophenol blue (0.025 mM; 50% glycerol) before loading with an eppendorf pipette. The gels were run overnight at 60 - 100 v in Buffer A and stained for 30 minutes with Ethidium Bromide (5 mg/ml). After destaining for 30 minutes in distilled water the gels were examined under UV light (330 nm) and the clarity of bands, presence of chromosomal DNA and relative mobilities of the plasmids observed. Electrophoretic patterns were photographed under UV light.

FIGURE 1.1: <u>EXPLODED VIEW OF HORIZONTAL GEL APPARATUS</u>



RESTRICTION ENZYME ANALYSIS

METHOD 1 :-

Samples prepared by each of the methods were restricted with a number of restriction enzymes [Hind III and Bgl II (Boehringer Corporation Ltd.) and Pst I (NBL Enzymes Ltd.)]. Twenty microlitres of each sample was mixed with 2.2 ul of the appropriate restriction buffer (10 x strength), prepared according to the manufacturers instructions, before restricting with the enzymes [Hind III - 32 u; Bgl II - 20 ul; Pst I - 24 u]. All samples were incubated for 1 hour at 37°C before stopping the reaction with 15 ul of stopping buffer (4 M urea; 50% sucrose; 50 mM EDTA, pH 7.0; 0.1% bromophenol blue weight/volume). Fragments were separated on 1% agarose gels run at 140 v for 4 hours in Buffer A (40 mM Tris acetic acid; 2 mM disodium EDTA, pH 8.0). The molecular weight of each fragment was compared with λ DNA fragments (Boehringer Corporation Ltd.) restricted with Hind III as above. DNA was also restricted with Bgl II and Pst I to check the enzyme activity.

Method II :-

Ten microlitre samples, prepared as for method 1, except for an additional drying step (all traces of alcohol were removed by drying under vacuum before resuspending the pellet) were dispensed into sterile eppendorf tubes. Solutions were then added in the following order: 2 ul 10x strength *Hind* III reaction buffer (NBL Enzymes Ltd), 7 ul distilled water and 1 ul (16 units) *Hind* III (NBL Enzymes Ltd). (λ DNA was also digested with *Hind* III to act as molecular size markers). Tubes were mixed thoroughly and centrifuged briefly (1 second). After incubating for 2 hours at 37°c, 5 ul of loading buffer (30% sucrose; 10 mM EDTA; 1% bromophenol blue) was added and samples loaded immediately on a 1% gel. Electrophoresis was carried out in Buffer A overnight at low voltage (40 - 60 V) or for 3 - 4 hours at high voltage (140 V).

RESULTS

COMPARISON OF THE DIFFERENT TECHNIQUES FOR THEIR ABILITY TO PRODUCE PLASMID DNA.

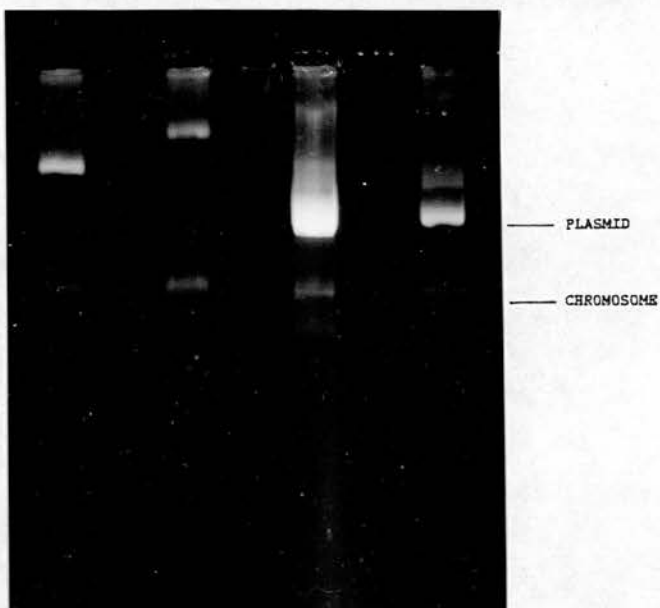
DNA was isolated from overnight broth cultures of Escherichia coli strains harbouring the plasmids Rl, RP4, R6K and Sa by the methods of Birnboim and Doly (1979), McMasters et al (1980), Holmes and Quigley (1981), Ish-Horowitz and Burke (1981), Kado and Liu (1981) and Takahashi and Nagano (1984). The DNA was separated by agarose gel electrophoresis on 0.7% gels for 16 hours at between 70 and 100 V, the results of which are shown in Figure 1.2.

Chromosomal DNA is clearly visable in both the Birnboim and Doly and the Ish-Horowitz and Burke preparations, but is greatly reduced in the other two (Figures 1.2b and 1.2c). The methods of Holmes and Quigley and McMasters both failed to produce any results, despite modifications to the volume of culture used, the length of the precipitation step and the amount of PEG added to the latter method. Of the methods which allowed DNA resolution, only the Kado and Liu technique failed to give adequate results: only R6K producing a significant band.

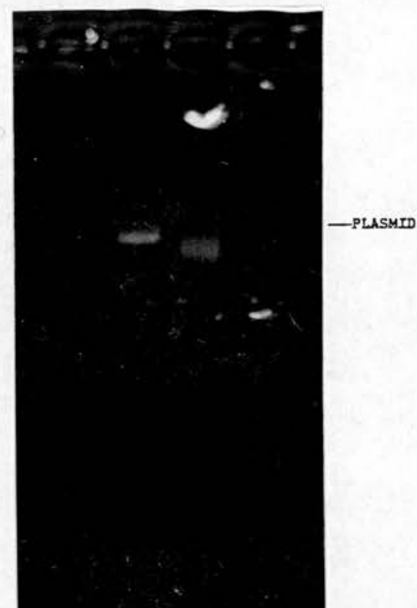
FIGURE 1.2: A COMPARISON OF THE DIFFERENT SMALL SCALE TECHNIQUES FOR THE PREPARATION OF PURE PLASMID DNA

TRACK 1. R1; TRACK 2. RP4; TRACK 3. R6K; TRACK 4. Sa

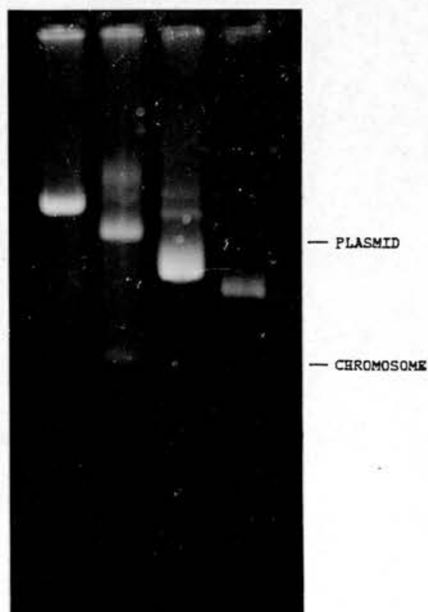
a. BIRNBOIM AND DOLY



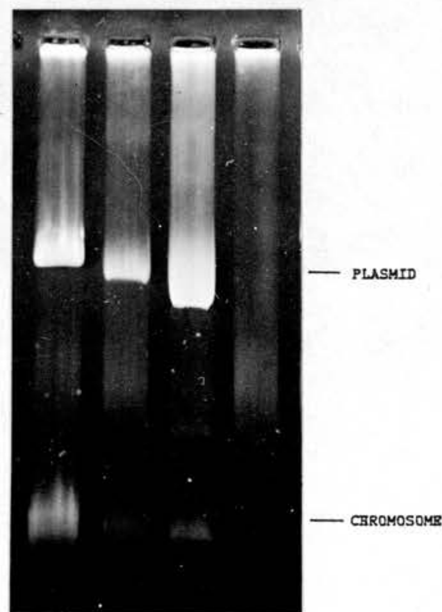
b. KADO AND LIU



c. TAKAHASHI AND NAGANO



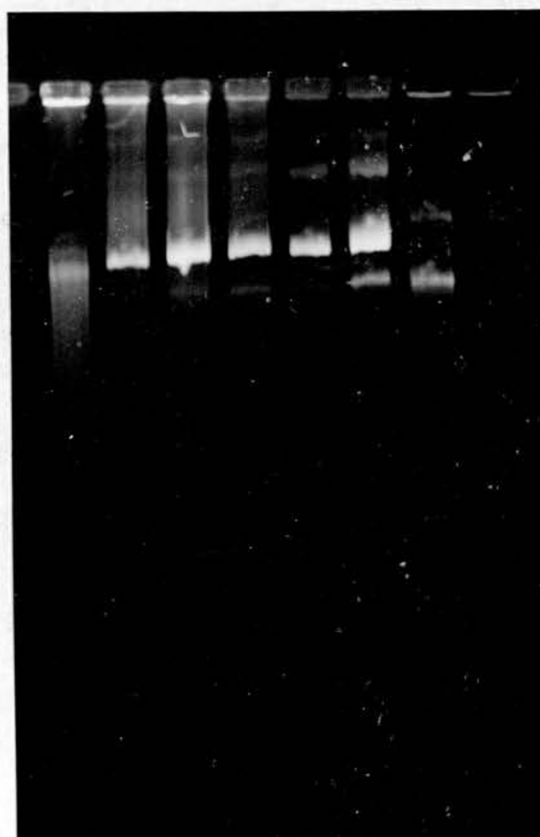
d. ISH-HOROWITZ AND BURKE



MODIFICATIONS TO THE TAKAHASHI AND NAGANO PROCEDURE TO IMPROVE CLARITY

The Takahashi and Nagano procedure was repeated with E coli cultures harbouring Sa. However, the concentration of NaOH in the lysing solution was varied to find the optimum for removing chromosomal DNA and still maintain clarity of plasmid bands. The results and concentrations of NaOH used are shown in Figure 1.3. As the molarity of NaOH is increased the intensity of the chromosonal band increases. The clarity of the plasmid band is only affected by concentrations of NaOH at the higher end of the scale: 0.56 - 0.64. These high concentrations also appear to affect the molecular size of Sa but the plasmid band is uniform compared with concentrations of NaOH in the middle of the range ie. there is only one molecular species (band).

FIGURE 1.3: EFFECT OF NaOH CONCENTRATION ON THE PREPARATION OF Sa PLASMID DNA BY THE TAKAHASHI AND NAGANO METHOD



REGRESSION ANALYSIS

The distance travelled by each of the plasmids R1, RP4, R6K and Sa was correlated with the corresponding molecular weight for each experiment performed. A comparison of the different preparation methods for their accuracy, as measured by the ability of the standard plasmids to lie on a straight line, is shown in Figure 1.4. (For raw data see Appendix 1.1). There is no significant difference in the relative sizes of plasmid bands obtained by the different preparation techniques. The coefficients of correlation for each set of data were determined and are shown in Table 1.1 along with their standard errors. They are generally high, indicating a high correlation between molecular weight and distance travelled, but the errors are large.

The Takahashi and Nagano and the Ish-Horowitz and Burke methods were repeated 9 and 4 times respectively, to determine the consistency of the method. Molecular weights were correlated with distance travelled, for each set of data, by plotting log 10 molecular weight against distance travelled (Figures 1.5 and 1.6) and by determining coefficients of correlation (Tables 1.2 and 1.3). (For raw data see Appendix 1.2)

The correlations between molecular weights and distance travelled were redetermined for the Takahashi and Nagano data, using only 3 of the 4 standards each time, to determine whether a particular plasmid is responsible for the inaccuracies. (Figure 1.5 would indicate that RP4 may be inaccurate). The results were plotted (Figure 1.7) and coefficients of correlation computed (Table 1.4) for data from experiment 1 (see Appendix 1.1). The coefficient of correlation is greatest in the absence of RP4. From the computerised correlation data in Table 1.4 the molecular weight of each plasmid, for each set of data, was estimated (Table 1.5).

FIGURE 1.4: COMPARISON OF SMALL SCALE TECHNIQUES FOR THE PREPARATION OF PLASMID DNA

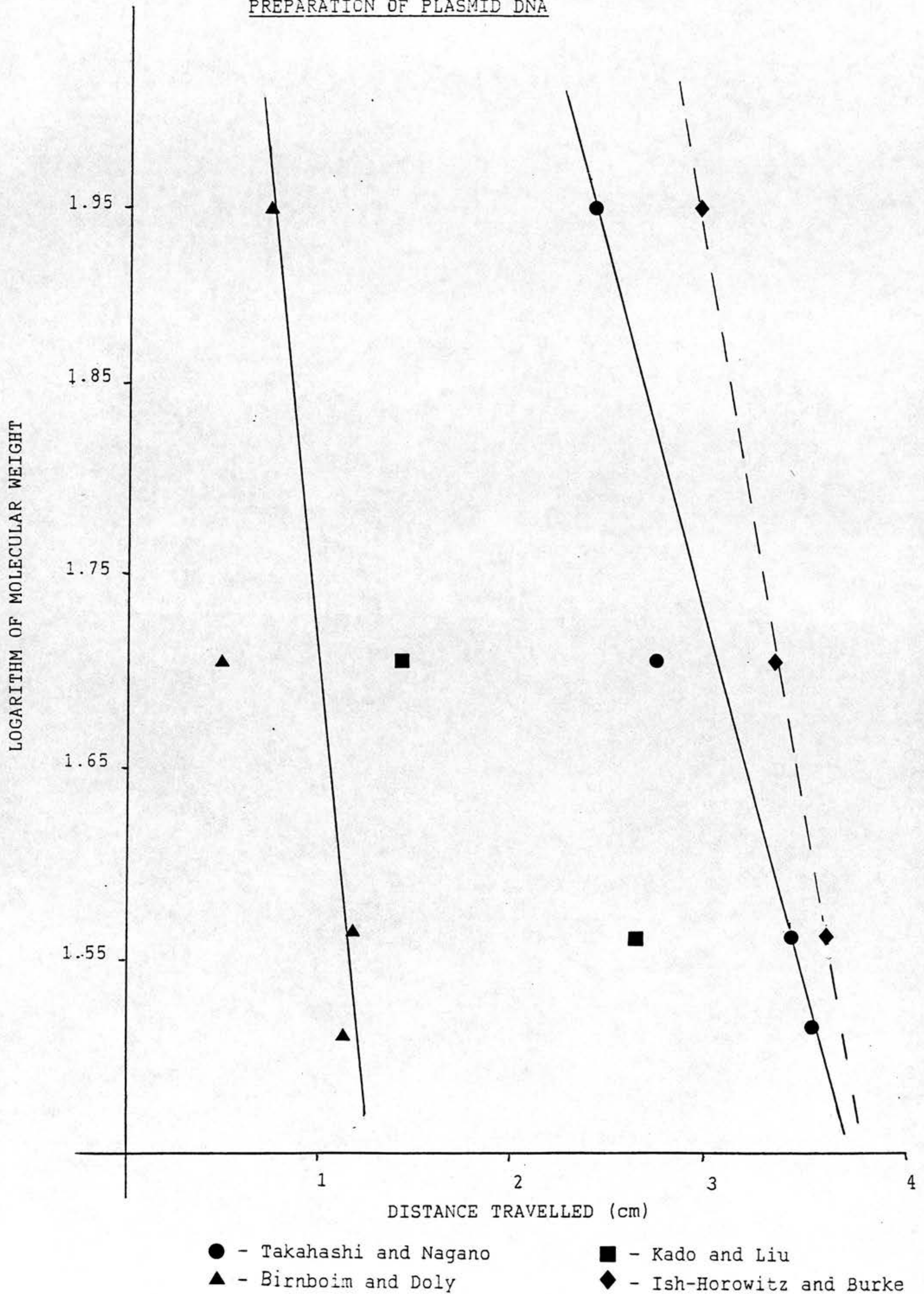


TABLE 1.1 THE COEFFICIENTS OF CORRELATION AND STANDARD ERRORS FOR EACH DNA PREPARATION METHOD

METHOD	CORRELATION COEFFICIENT	STANDARD ERROR
Birnboim and Doly	0.655	0.17779
Ish-Horowitz	0.944	0.08800
Takahashi and Nagano	0.955	0.6950

FIGURE 1.5: THE CONSISTENCY OF TAKAHASHI AND NAGANO METHOD FOR PREPARING PLASMID DNA

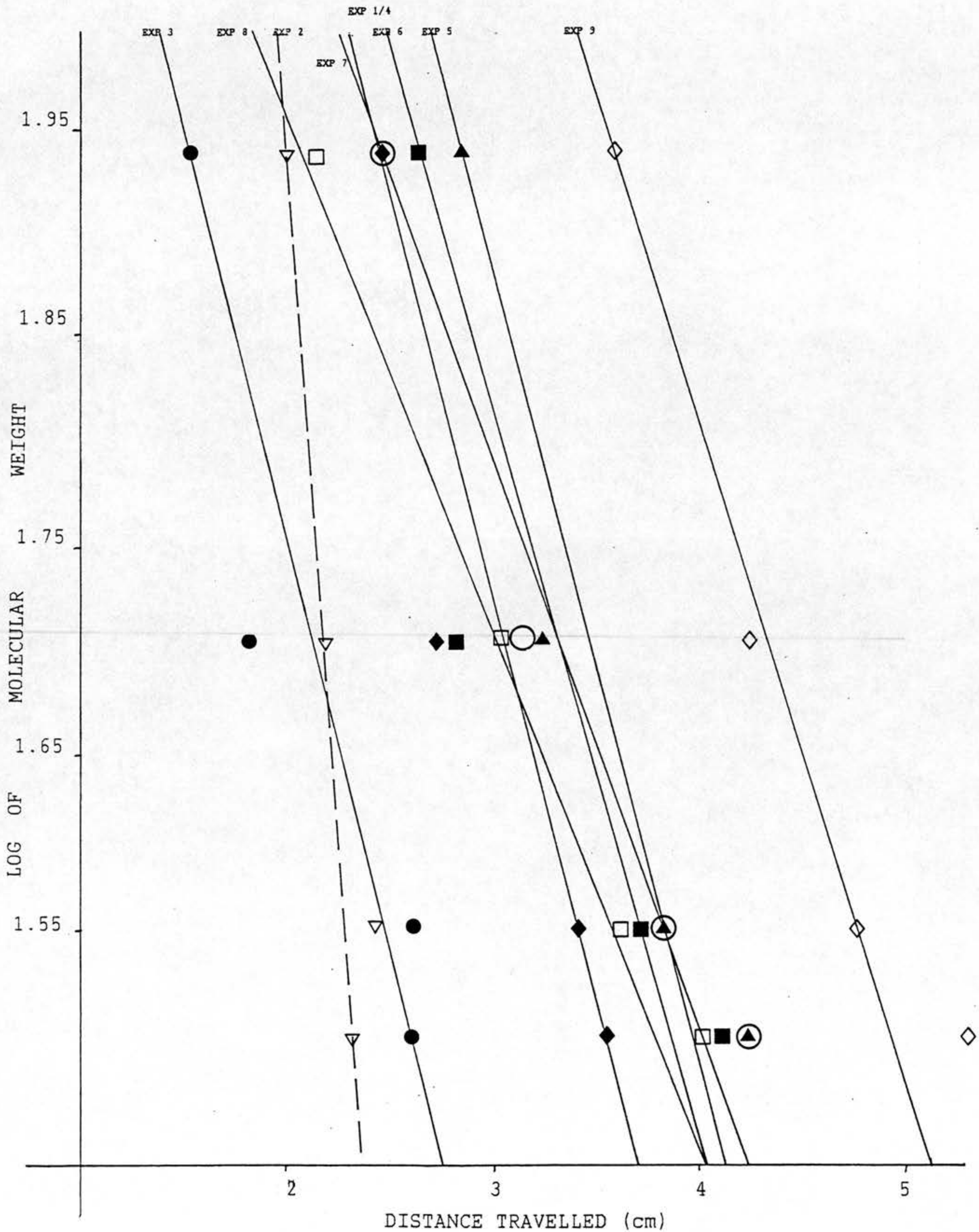


FIGURE 1.6: THE CONSISTENCY OF THE ISH-HOROWITZ AND BURKE METHOD FOR PREPARING PLASMID DNA

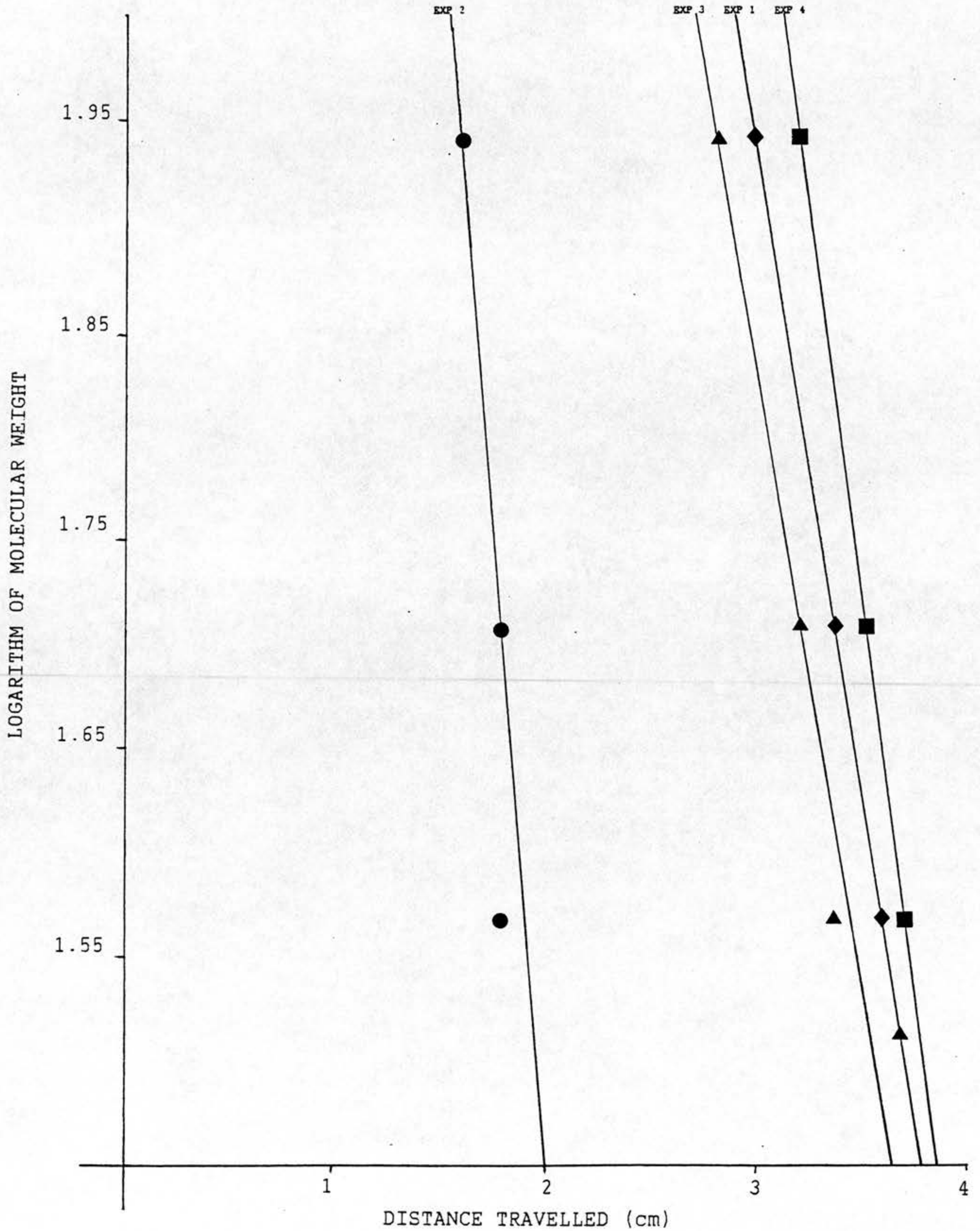


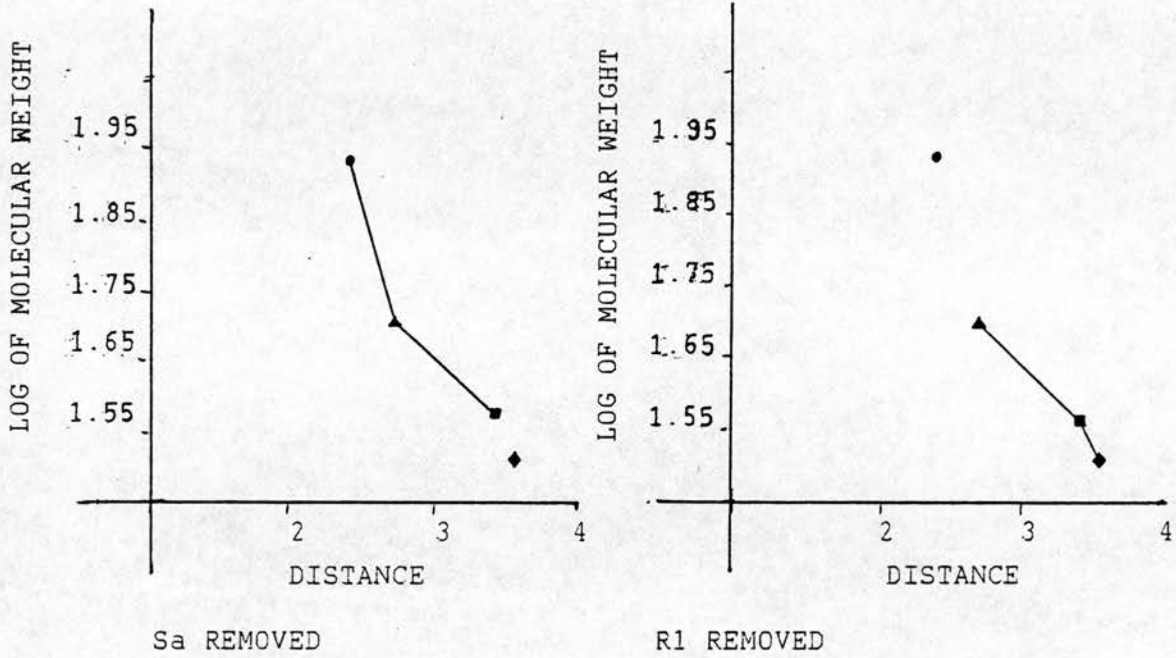
TABLE 1.2 CORRELATION COEFFICIENTS FOR REPEATED TAKAHASHI AND NAGANO PREPARATIONS

EXPERIMENT NUMBER	CORRELATION COEFFICIENT	STANDARD ERROR
1	0.955	0.0695
2	0.9378	0.08169
3	0.946501	0.0759
4	0.952610	0.07086
5	0.957741	0.06789
6	0.912336	0.09635
7	0.967417	0.05958
8	0.99465	0.0243
9	0.09701	0.057057

TABLE 1.3 CORRELATION COEFFICIENTS FOR REPEATED ISH-HOROWITZ AND BURKE PREPARATIONS

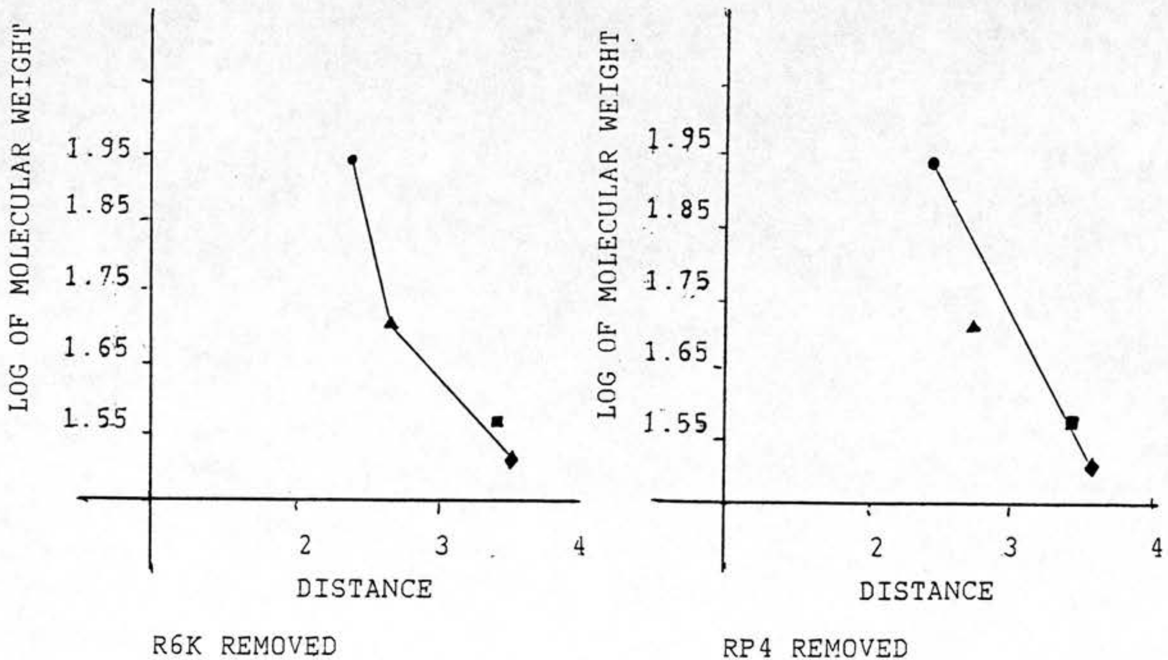
EXPERIMENT NUMBER	CORRELATION COEFFICIENT	STANDARD ERROR
1	0.944	0.088
2	0.760	0.120
3	0.913	0.086
4	0.977	0.06479

FIGURE 17: PLOT OF THE ELECTROPHORETIC MOBILITIES OF PLASMID DNA, PREPARED BY THE METHOD OF TAKAHASHI AND NAGANO, TO DETERMINE WHICH OF THE PLASMIDS IS RESPONSIBLE FOR THE INACCURACIES



Sa REMOVED

R1 REMOVED



R6K REMOVED

RP4 REMOVED

TABLE 1.4 CORRELATION COEFFICIENTS FROM TAKAHASHI AND NAGANO PREPARED DNA (EXPERIMENT 1), UTILISING DIFFERENT PLASMID SETS TO DETERMINE WHICH PLASMID(S) IS RESPONSIBLE FOR THE ERRORS.

DATA SET	PLASMIDS	CORRELATION COEFFICIENT	STANDARD ERROR
1	ALL	0.955	0.0695
2	R1, RP4, R6K	0.931	0.0978
3	RP4, R6K, Sa	0.993	0.0172
4	R1, RP4, Sa	0.949	0.0960
5	R1, R6K, Sa	0.999	0.0263

TABLE 1.5 ESTIMATED PLASMID MOLECULAR WEIGHTS FOR EACH DATA SET

DATA SET	R1	RP4	R6K	Sa
1	79.49	62.98	36.59	33.07
2	79.77	62.82	35.97	32.43
3	61.32	52.76	36.84	34.46
4	79.57	62.52	35.61	32.08
5	90.48	69.63	37.78	33.73
PUBLISHED MW	90	52	38	33

Molecular weights are most accurate ie are closest to published figures when RP4 is absent from the calculation (data set 5), confirming that RP4 is not running as predicted.

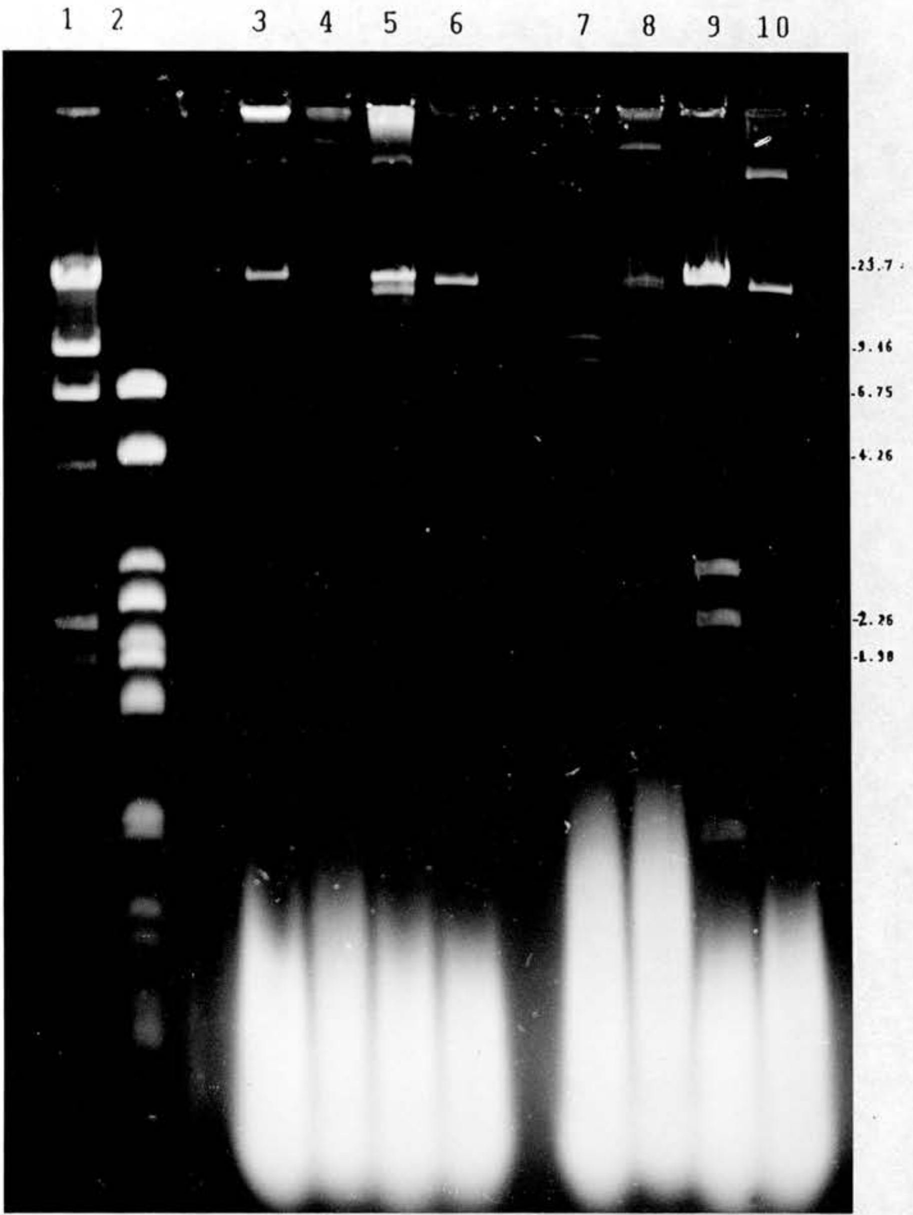
ASSESMENT OF THE DIFFERENT TECHNIQUES FOR THEIR ABILITY TO PRODUCE DNA SUITABLE FOR RESTRICTION

Twenty microlitre samples of R1, RP4, R6K and Sa DNA, prepared by the methods of Birnboim and Doly (1979), Ish-Horowitz and Burke (1981), Kado and Liu (1981) and Takahashi and Nagano (1984), were restricted with Hind 111 and Pst1 or Bgl 11, by method 1. Fragments were separated by agarose gel electrophoresis in 1% horizontal gels run for 4 hours at 140 v (Figure 1.8).

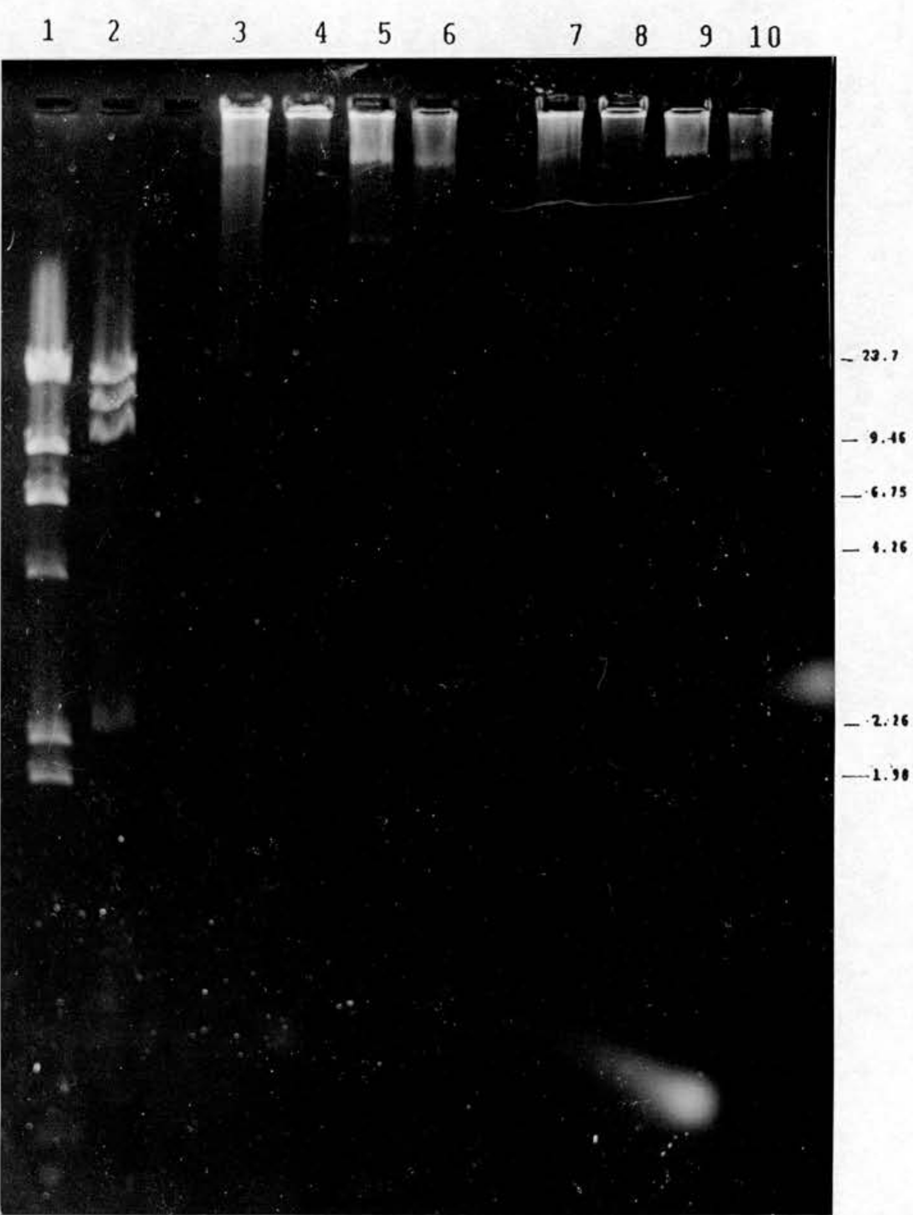
No results were obtained for the restriction of Ish-Horowitz prepared DNA, and only unrestricted DNA was present in samples prepared by the method of Kado and Liu: the single band in Figure 1.8b track 5 corresponds to unrestricted R6K DNA. A comparison of Figure 1.8a and 1.8c indicates that DNA has been restricted in both cases but undigested DNA is still found in the Birnboim and Doly preparations. An uncharacteristically large number of small DNA fragments are also apparent in both preparations, indicative of some non specific cleavage.

FIGURE 1.8: A COMPARISON OF THE RESTRICTION DIGESTS OF PLASMID DNA PREPARED BY DIFFERENT SMALL SCALE TECHNIQUES

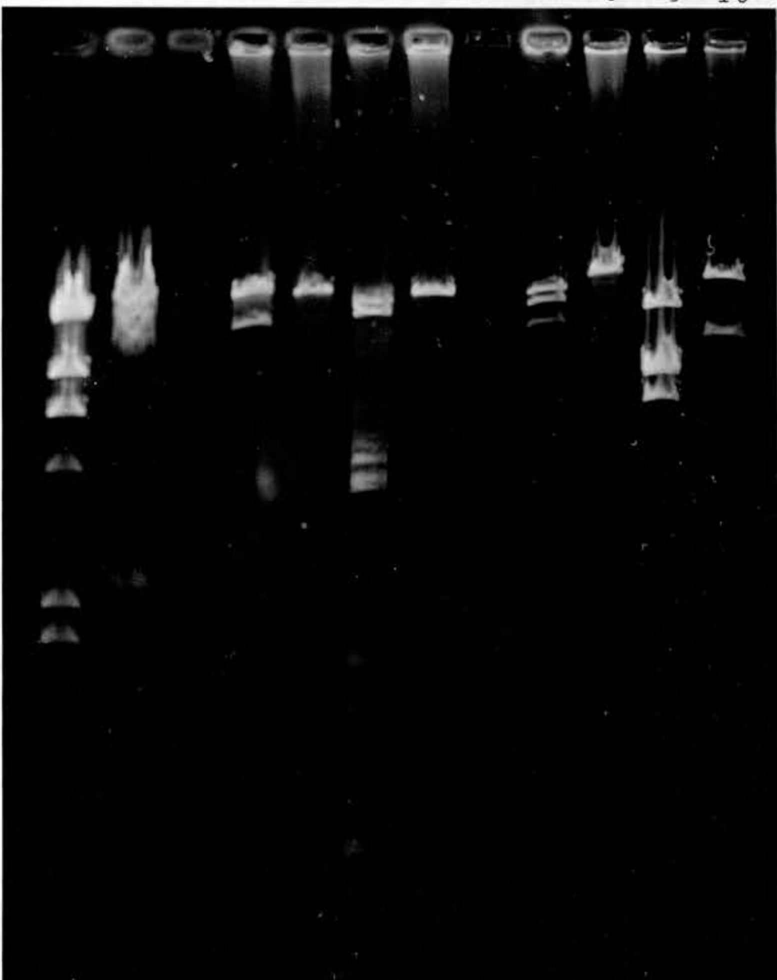
a. BIRNBOIM AND DOLY



b. KADO AND LIU



C. TAKAHASHI AND NAGANO



TRACK 1. Hind III digest of λ DNA
2. Bgl* II digest of λ DNA
3. Hind III digest of R1
4. Hind III digest of RP4
5. Hind III digest of R6K
6. Hind III digest of Sa
7. Bgl* II digest of R1
8. Bgl* II digest of RP4
9. Bgl* II digest of R6K
10. Bgl* II digest of Sa

* Birnboim and Doly DNA digested with Pst I

All gels were run at 140 v on 1% agarose for 3 - 4 hours.

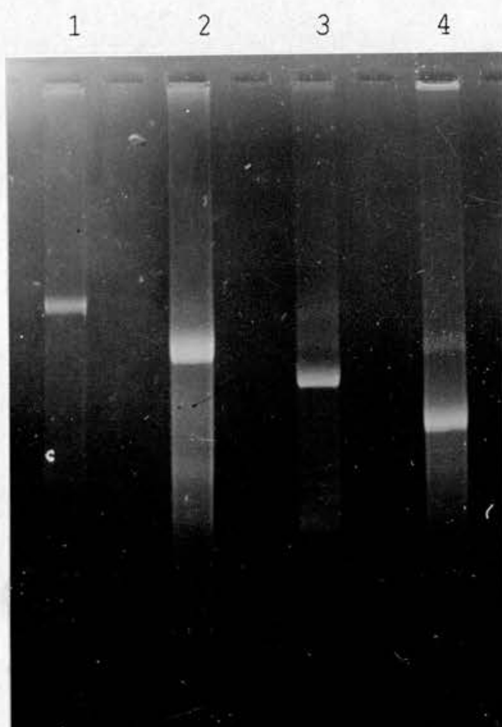
THE EFFECT OF ALTERING CONDITIONS ON THE CLARITY OF RESTRICTION ENZYME DIGESTS.

In order to ascertain the conditions optimal for digestion, various modifications were made both to the preparation of DNA and to the restriction mixture itself. Samples were freed of ethanol by drying under vacuum before resuspending in distilled water, and the ratios of enzyme to DNA were altered. With all the concentrations of enzyme and DNA tested, method II proved most successful (Figure 1.9). A comparison of Figure 1.9a and 1.2c indicates a greater clarity of plasmid bands, with little trailing, and restriction digests (Figure 1.9b) lack the large numbers of small bands, present in figure 1.8c. Therefore, the ratio of enzyme units to quantity of DNA sample, utilised in method II, is approaching that required for optimal digestion and therefore optimal clarity.

FIGURE 1.9. PLASMID DNA PREPARED BY THE TAKAHASHI AND NAGANO METHOD AND RESTRICTED ACCORDING TO METHOD II

a. UNRESTRICTED DNA

1 2 3 4



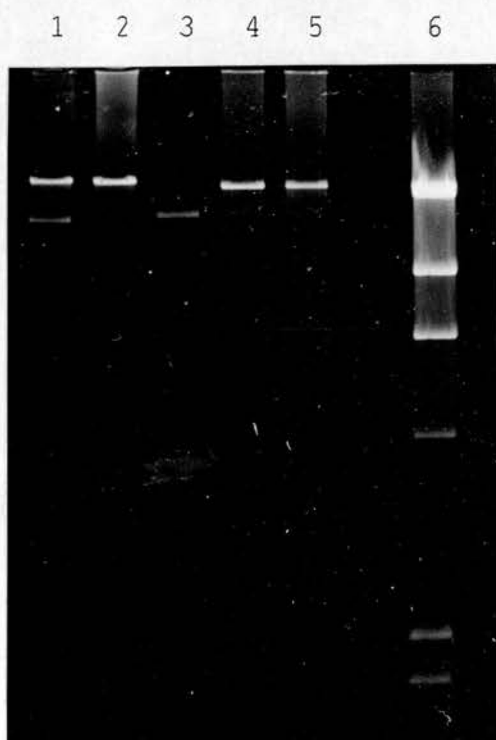
— PLASMID

TRACK 1. R1
2. RP4
3. R6K
4. Sa

0.7% agarose gel run at 80 v for 16 hours

b. HIND III RESTRICTED DNA

1 2 3 4 5 6



— 23.7
— 9.45
— 6.75
— 4.26
— 2.26
— 1.93

TRACK 1. R1
2. RP4
3. R6K
4. Sa-1
5. Sa-2
6. λ DNA

1% agarose gel run at 60 v for 16 hours

DISCUSSION

As more methods become available for the rapid isolation of plasmid DNA there is a need to characterise them according to their use. Most of these methods have been developed in studies with small easily isolatable plasmids but as the applications of recombinant DNA technology become more widespread the need arises to focus attention on the clinical environment, where plasmids are generally larger.

Techniques must be accurate, consistent and easy to carry out if they are to become universally accepted. Horizontal gels were therefore used throughout, despite their disadvantages, as they are much easier to handle than vertical gels and the equipment is more simple to prepare. In addition, vertical systems have their own inherant problems eg. inconsistencies in separation (BRL users manual).

For the rapid preliminary screening of strains the electrophoresis of a total cell lysate is probably adequate. Bacteria are lysed with sodium dodecyl sulphate (SDS) within a well of the agarose gel (Eckhardt, 1978) and whilst the plasmid DNA species will generally migrate as a discrete band, the fate of the chromosomal DNA will depend on the method of preparation. Unfragmented chromosomal DNA will not enter the gel, whereas fragments tend to migrate as a rather broader band of about 10 kb, easily distinguishible from plasmid DNA. Methods like these were initially developed for small high copy number plasmids (Barnes, 1977; Telford _et al_, 1977) but they have no major advantages over current methods that remove the majority of chromosomal DNA prior to electrophoresis.

(Whichever method of rapid preparation is used there will inevitably be some contamination with chromosomal DNA but for most purposes ie. restriction or transformation, trace amounts do not seem to be inhibitory.) The electrophoresis of total cell lysates may, however, have one advantage in that it allows the separation of high molecular weight plasmids that might otherwise be lost, as a result of shearing forces generated during manipulations. This method has resolved plasmids larger than 450 kb (Casse et al, 1979) and because of its simplicity should work for most Gram negative bacterial species. However its use as a preparative method for plasmid DNA suitable for restriction is minimal, as extraction of DNA from the gel would be required and this is difficult to achieve.

A second approach to the separation of plasmid from chromosomal DNA is the cleared lysate technique, first adopted by Clewell and Helsinki (1969) and Klein et al (1980). This involves lysis of the bacteria and removal of chromosomal DNA prior to electrophoresis. Lysis can be effected by detergents such as Triton X-100 and SDS or by EDTA and lysozyme. Some organisms eg E. coli are relatively easy to lyse whereas others require more specialised treatment, such as the use of lysostaphin for staphylococci (Nahaie et al, 1984). The method of Holmes and Quigley (1981) combines lysozyme and detergent treatment with incubation in boiling water; a procedure which releases DNA and permanently denatures non circular DNA. As plasmids are covalently closed circular (ccc) molecules they renature rapidly on cooling and can be separated, by centrifugation, from chromosomal DNA which forms an insoluble clot. Although it is a very rapid method, convincing results could not be obtained with the standard plasmids by the method of Holmes and Quigley, which suggests that this method lacks predictability with large plasmids. The problem arises from the length of the heating period, which is critical, as the DNA can easily be irreparably denatured. Several

modifications (MacNeil, 1986) have, however, been made to the Holmes and Quigley method, enabling its use in the isolation of plasmids from gram positive organisms. Although these methods do generally give good yields with relatively small high copy number plasmids, the recovery of large low copy number plasmids can be very poor, which probably results from their increased susceptibility to heat denaturation. Speed is the main advantage of the cleared lysate methods but the major drawback is that the lysis conditions that give the best yield of plasmid DNA, and least contamination with chromosomal DNA, vary from strain to strain and can even be affected by the plasmid involved.

The most general methods, reported to be applicable to a wide range of bacterial species, involve complete cell lysis, usually with SDS, followed by selective precipitation of chromosomal DNA. This precipitation step has been achieved in a number of ways. Guerry et al (1973) utilised coprecipitation of SDS and chromosomal DNA at high ionic strength, in the cold. Birnboim and Doly (1979) have effected separation by selective alkaline denaturation of high molecular weight chromosomal DNA whilst ccc plasmid DNA remains double stranded, as a result of topological bonding. On neutralization, the chromosomal DNA forms an insoluble clot, leaving the plasmid DNA in the supernatant. This method works well (Fig 2a) for the large plasmids, although chromosomal DNA and RNA are still clearly visible. (The incorrect mobility of Sa and RP4 is probably due to the presence of salts interfering with electrophoresis). It is reported to be applicable to small plasmids also, as the harsh lysis conditions destroy any plasmid - chromosome or plasmid - membrane associations which might decrease the yield of low copy number plasmids. It is virtually independent of the particular bacterial strain or species too, so long as there is suitable enzyme to degrade the cell wall.

Ish-Horowitz and Burke (1981) have modified the Birnboim and Doly method, essentially to remove some of the RNA and decrease the preparation time. However, these alterations do not appear to lead to an improvement (Fig 1.2d). Chromosomal DNA is still present and bands trail although, in this case, it could be attributed to overloading. The mechanism by which DNA fragments are separated during gel electrophoresis is very complex and a number of factors such as DNA concentration, buffer composition, gel composition and chemical modification of the agarose may affect the clarity of separation (Smith et al, 1983). Therefore, poor resolution may not result from the isolation technique itself, but rather from the use of incorrect conditions, such as overloading, during electrophoresis. However, repetition of this method did give inconsistent results, as indicated by the variation in correlation coefficients (Table 1.3).

The method of Kado and Liu (1981) is a modification of the alkaline denaturation method of Birnboim and Doly combined with phenolic extraction. Chromosomal DNA is removed by heating in an alkaline environment and proteins and cellular debris are removed by a phenol chloroform extraction. The lysis step is crucial and small differences in pH affect the plasmid recovery. At pH's lower than 12, the supernatant becomes increasingly more viscous and very difficult to remove from the phenol layer and load onto the gel. However, at pH's approaching the recommended value of 12.6, SDS precipitates. Therefore, a compromise has to be reached to achieve a pH as near to 12.6 as possible but without allowing precipitation of SDS. The resolution of large plasmids by this method is poor (Figure 1.2b) and, as with other workers, difficulty was had in obtaining reproducible results.

The fifth method investigated was that of McMasters et al (1980). This method is also a modification of basic techniques, utilizing high pH, followed by neutralization and phenolic extraction in the presence of sodium chloride to effect separation. Plasmid DNA, however, is precipitated with polyethylene glycol (PEG), as opposed to ethanol. PEG concentrates small amounts of DNA (Humphreys et al, 1975) whilst eliminating small pieces of RNA. Despite the fact that the procedure involves minimal handling of the DNA, with the single step separation of proteins (pellet), chromosomal DNA (particulate interphase) and plasmid DNA, results were difficult to achieve. Attempts to modify the procedure by changing the volume of culture, the length of the precipitation step and the amount of PEG added failed to improve the results. Apart from the inability to isolate large plasmid DNA, this method is time consuming compared with the other methods and involves a phenol chloroform extraction step. Grinsted and Bennett (1984) believe chloroform extraction is unnecessary and the unpleasant involvement of the phenol extraction steps makes these methods less attractive.

Whilst the lack of success with the last three methods may be attributable to the fact that they are not applicable to the plasmids under study, (whilst still being perfectly suitable for other plasmids and/or bacterial species), it is significant to note that in many of the published techniques, cultures which were used to isolate DNA from, were amplified by chloramphenicol challenge. The quantity of DNA present in the starting material is therefore greater. Whilst amplification may be possible in some cases, its use in undefined systems may lead to more problems. Amplification of clinical plasmids is rarely feasible, another factor contributing to the unsuitability of some of these methods for looking at clinical material.

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The final method under investigation was that of Takahashi and Nagano (1984), which combines some of the features of Birnboim and Doly (1979) with others from Kado and Liu (1981) to try and produce a reliable, reproducible, universal technique. As with the method of Kado and Liu (1981), the pH is crucial and it may often be necessary to make up solutions with double glass distilled water to reduce acidity. Chromosomal contamination was minimal with this technique (Figure 1.2c) and it was the most reliable. It gave bands which separated according to their size so that the correlation coefficient was as good as 0.999 + 0.005 (Tables 1.2 and 1.4) and the variation was minimal, compared with the coefficients for Ish-Horowitz and Burke prepared DNA. The accuracy of molecular size determination was further increased by removing RP4 from the calculations. This plasmid was found to be consistently inaccurate (Table 1.4 and Figure 1.7). The conditions which were found to give the least contamination with chromosomal DNA, however were not the same as those published by the original workers. In contrast to the results of Takahashi and Nagano (1984), it was found that the proportion of chromosomal DNA increases as the concentration of sodium hydroxide is raised to 0.56 mM (Figure 1.3). In addition, RNA production was found to increase with increasing sodium hydroxide concentration. However these differences may be attributable to plasmid or strain characteristics. The clarity of results is comparable with those of Birnboim and Doly (1979) but the method is less time consuming. It eleviates the need for complicated steps such as ether extraction of phenol (Klein et al, 1980) and a 5 minute pre-centrifugation of the neutralised mixture was found to increase the yield of supernatant.

It is possible that the resolution of plasmid bands could be improved still further with the use of wedge-gels. Rochelle et al (1986) have

reported that banding patterns are more uniform, there is increased linearity in the relationship between log of molecular size and log 10 of relative mobility, variation about the regression line is decreased and estimation of molecular size is more accurate with this type of gel.

The first stage in restriction enzyme analysis depends on the ability to produce suitable DNA, quickly and easily, and then a consistent protocol is required for the DNA restriction itself. The Takahashi and Nagano (1984) method takes about 1 hour for molecular weight determination and an additional 20 minutes to prepare the DNA for restriction, whilst the Birnboim and Doly (1979) method takes slightly longer. Of these two methods, however (Figure 1.8), it is not possible to determine which, if any, produces DNA more suitable for restriction as conditions were not optimal for digestion. However, digestion of Takahashi and Nagano preparations might be considered slightly better as all the DNA has been restricted. Undigested high molecular weight DNA was found in the restricted samples prepared from DNA extracted by the Birnboim and Doly method.

There are a large number of factors that determine the proper functioning of restriction endonucleases eg the presence of cofactors (Smith, 1979), salt concentration and pH. It is the purpose of the additional steps in the Takahashi and Nagano (1984) method to control the sodium ion concentrations. If the conditions are altered away from the optimum, the specificity of DNA recognition by a restriction enzyme is relaxed i.e. an enzyme recognises a reduced number of nucleotides within the canonical recognition sequence normally characteristic for the enzyme under optimal conditions. This effect was first described for EcoR1

(Polisky et al, 1975) and results in an increase in the number of shorter length fragments. It is believed to result from changes in conformation of the quarternary structure of the active enzyme. The results obtained for both Birnboim and Doly and the Takahashi and Nagano methods are characteristic of relaxed specificity ie the presence of a large number of small fragments (Figure 1.8). The incubation conditions of relaxed specificity are characterised by low salt concentration, high pH values and extremely high enzyme activity and glycerol concentrations. The addition of excess enzyme can therefore produce results as poor as those obtained by adding too little. Thirty-two units of Hind III per 20 ul of DNA sample was used in the above restrictions. However, reducing this amount had no effect on the results which suggested other factors are involved. Relaxed specificity can also result from the addition of organic solvents (Woodhead et al, 1981) eg alcohol; thus a critical step in the preparation of DNA for restriction is the removal of all traces of ethanol by vacuum desiccation. The effect of the inclusion of this desiccation step, on the clarity of plasmid bands, can be seen by comparing Figure 1.8c and Figure 1.9b. The large number of small faint bands, characteristic of non specific cleavage, are not present in Figure 1.9b. The individual bands are noticeably sharper although the smaller fragments are faint. The ratio of enzyme to DNA is identical (16 units to 10 ul) but the alcohol has been removed. In addition the volume was maintained at 20 ul and the buffer volume (2 ul 10x strength) kept constant by altering the amount of distilled water. Such a strict adherance to a constant volume was not maintained in the previous experiment (Figure 1.8) and the ion concentration may well have varied, contributing to the relaxed specificity. The increased sharpness of bands may also be due to a reduction in the amount of DNA loaded; overloading can be a problem in

both restriction and molecular weight determination but altering buffer and gel concentration may help to counteract this (Smith et al, 1983). Spectrophotometric determination of DNA concentrations per sample would help to find the best loading concentration and would also aid in the calculation of how much enzyme to use. These values will vary depending on size and copy number of the plasmid, and therefore a certain amount of experimentation will be required for each case, to find the appropriate amounts needed.

Accuracy and reproducibility are also important when attempting to determine molecular weight by summation of restriction fragments: the loss of small fragments off the end of the gel or nonspecific cleavage will drastically affect the results. The fragments do not sum to the predicted molecular weights in the initial experiments carried out with Takahashi and Negano or Birnboim and Doly prepared DNA (Figure 1.8), indicating not only that conditions are not optimal but also that this is not the best method for measuring molecular weight. However, there are disadvantages in running undigested preparations to determine molecular weight: although conditions are chosen to maximise ccc DNA, open circular or linear DNA may be present in varying amounts depending on the handling conditions and the plasmid. It is therefore not possible to be entirely sure that similar molecular species are being compared and, in the case of transfer studies, whether one or more plasmids are present. The obvious solution is to restrict plasmid DNA with an enzyme that cuts once and this method has been adopted by many researchers. All DNA therefore becomes linear and can thus be compared and there is no longer the problem of losing small fragments. Conditions, however, still need to be suitable for correct cleavage. Although useful, this does not help when dealing with uncharacterised plasmids and transposons. With no knowledge of particular

restriction sites this is not a feasible solution to obtain greater accuracy. Until detailed maps are determined the only option is to run undigested DNA, prepared by a method that minimises the amount of linear and open circular DNA.

In conclusion, only the Birnboim and Doly (1979) and Takahashi and Nagano (1984) methods, of the six studied, proved consistent in preparing plasmid DNA in the range 30 to 90 kb from *E. coli*. Of the two, the latter was the quickest and easiest to perform and resulted in the least chromosomal contamination. The removal of all traces of ethanol is essential to the clarity of bands, particularly of restriction fragments, and every attempt should be made to maximise the conditions optimal for digestion with each enzyme. These conditions will vary with the plasmid size, the preparation method used and thus the DNA concentration, the enzyme used and the amount of sample loaded. It may therefore be necessary to try several preliminary sets of conditions to ensure that the most appropriate one is used .

Most methods have their limitations and as yet there does not appear to be a universal method that accurately and consistently isolates a range of plasmid DNA sizes from a variety of organisms. In organisms like *Lactobacilli* conventional cell lysis and plasmid purification have been reported to be unsatisfactory (Klaenhammer, 1984) and our attempts to successfully isolate plasmid DNA from *Pseudomonas aeruginosa* isolates by the method of Takahashi and Nagano (1984) failed despite the original authors success. Although this latter method would appear to have more widespread use than most, the choice of the best method to use does depend on the organism, the size of the plasmid, the amount of DNA required and the desired purity. However, as a method to begin studies with, the Takahashi

and Nagano technique fulfils most of the essential requirements, and has been successfully used to isolate DNA of plasmids ranging from 30 to 90 kb. This technique was therefore used for all subsequent rapid analysis of R plasmids and Tp resistance transposons.

CHAPTER 2

THE STABILITY OF THE INCOMPATIBILITY GROUP W PLASMID Sa

INTRODUCTION

The plasmid Sa was isolated from Shigella flexneri in 1962 (Watanabe et al, 1968) and was found to be a member of the incompatability group W plasmids. Other members of this group include R388 (Datta and Hedges, 1972) and R7K (Coetze et al, 1972). These plasmids represent the smallest naturally occurring group of R factors and range in size from 29 kb to 38 kb. They are conjugative plasmids, displaying a broad host range [Inc W plasmids have been isolated from different enteric species from around the world (Datta and Hedges, 1971; Datta, 1974)], and carry a variety of drug resistance genes (Jacob et al, 1977; Datta et al, 1979). Gorai et al (1979) have shown there to be extensive homology between the Inc W group plasmids and have examined the physical distribution of genes on the plasmid genomes. Like the Inc P broad host range plasmids (DePicker et al, 1977; Barth and Grinter, 1977; Grinsted et al, 1978; Thomas et al, 1980) the antibiotic resistance genes lie in the region dense in restriction enzyme sites and this is possibly of evolutionary significance (Thomas et al, 1980).

Sa has been reported by Ward and Grinsted (1982) to have a molecular size of 37 kb but there has been a suggestion that Sa may exist in more than one form, with variable molecular size. Gorai et al (1979) utilised a spontaneous deletion mutant of Sa (molecular size 29 kb), which had lost the chloramphenicol resistance determinant but retained the other drug resistance markers (ie Km, Sm and Su), to determine the location of the chloramphenicol resistance gene. These workers also report that Sa has a molecular size of 34 kb as opposed to 37 kb. Other mutants have been found (this laboratory, unpublished results), and Ireland (1983)

identified a spontaneously deleted derivative of the Crown Gall suppressive Inc W plasmid, pSa, which also lacked chloramphenicol resistance, and ranged in size from 26 to 39 kb (Falkow et al, 1974; Meyers et al, 1976; Farrand et al, 1981; Tait et al, 1982). This phenomenon of variable size may not therefore be restricted to Sa, but may be a property of the Inc W plasmids as a whole. R388 has been observed as a 44 kb spieces (personal communication) as well as in it's published 29 kb form (Datta and Hedges, 1972). In some instances, however, different techniques were used for determining molecular size (ie. agarose gel electrophoresis, sucrose density gradients and Electron Microscopy measurements) and this might account for some of the variability observed.

Since the plasmid Sa was to be used in amplification studies and as a 'host' for transposon characterisation, it was imperative to investigate the possibility that the molecular size of this plasmid could vary. Undetermined changes in molecular size, or resistance markers of Sa, would hamper the interpretation of data refering to the size of inserted transposons and /or their amplification. It seemed of particular importance, therefore, to obtain information on the physical and functional differences between the published Sa (Ward and Grinsted, 1982) and chloramphenicol sensitive Sa plasmids isolated during repeated drug testing of E. coli J53(Sa), and strains harbouring trimethoprim-encoding transposons inserted into Sa.

MATERIALS AND METHODS

BACTERIAL STRAINS AND PLASMIDS

The properties and the source of the 'Sa' plasmids utilised in this study are summarised in Table 2.1. The standard strains used were as in Table i. E coli was the host for all plasmids. Resistance to antibiotics was assayed on DST agar (Oxoid) containing appropriate antibiotics at 10 ug/ml, unless otherwise stated.

PLASMID DNA PREPARATION

Plasmid DNA was prepared by the method of Takahashi and Nagano (1984) as described in chapter 1.

RESTRICTION ENZYME DIGESTION

DNA was prepared for restriction (as described in chapter 1) and resuspended in 60 ul of distilled water containing RNase at 50 ug/ml. After incubating at 37°C for 30 minutes, 10 ul aliquots were restricted. Hind III, Bam H1 and Pst I were purchased from NBL Enzymes ltd, along with dilution and reaction buffers. Digestion of plasmid DNA was carried out as described in method II (chapter 1) utilising either 9u Hind III, 6u Bam H1 or 12u Pst 1. Reactions were stopped and analysed on 0.1% agarose gels as described previously (chapter 1). λ DNA (Sigma) was restricted with the above enzymes to act as molecular size markers.

TABLE 2.1: PROPERTIES OF PLASMIDS USED IN THIS STUDY

PLASMIDS	SOURCE	RESISTANCE MARKERS	PUBLISHED MOL. SIZE kb
Sa-1	Spontaneous mutant derivative	Sm Su Ka	-
Sa-2	Watanabe et al, 1968	Sm Su Ka Cm	33

RESULTS

RESISTANCE TESTING

E. coli strains harbouring Sa, plus suitable controls, were tested for their resistance to kanamycin, streptomycin, chloramphenicol and sulphonamide (100 ug/ml). Two resistance patterns were found; some strains carried all four resistance determinants whereas the rest had lost their chloramphenicol resistance. The Sa plasmid carried by these latter chloramphenicol sensitive strains was designated Sa-1 and the chloramphenicol resistant colonies were designated Sa-2.

COMPARISON OF MOLECULAR SIZES OF Sa-1 AND Sa-2

DNA preparations of Sa-1 and Sa-2 were electrophoresed for 16 hours at 70 v in buffer A (chapter 1) on 0.7% agarose gels. Their sizes, compared with known standards, can be seen from Figure 2.1. Sa-2 is larger than Sa-1: 35.84 kb as compared with 30.15 kb for Sa-1. Subsequent repetitions of these preparations revealed that there was often a variation in molecular size, especially for Sa-1, and that this latter plasmid was often present in two forms (Figure 2.2). These forms correspond to molecular sizes of 29 and 44 kb.

FIGURE 2.1: COMPARISON OF THE MOLECULAR SIZES OF Sa-1 AND Sa-2

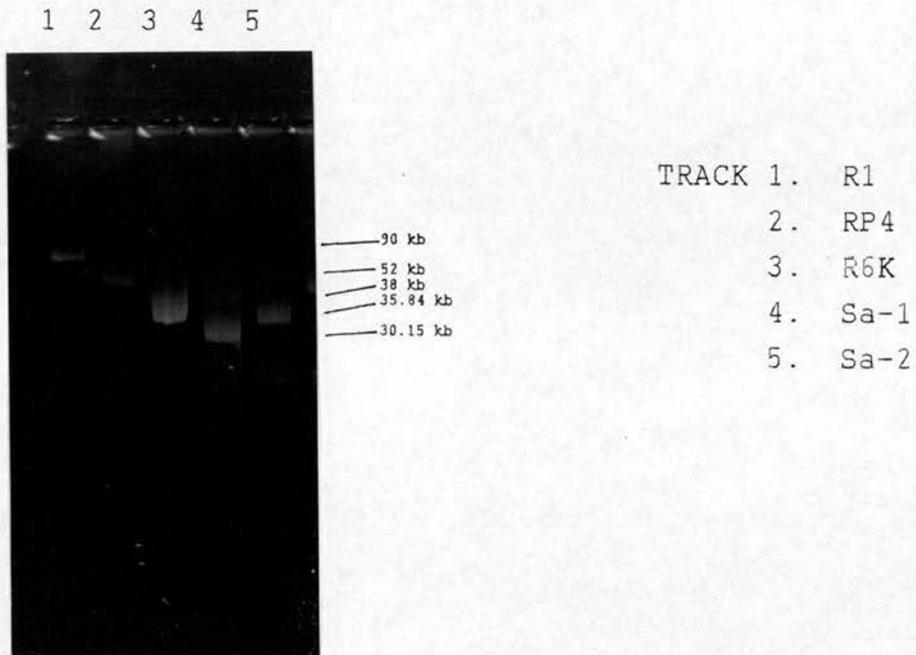
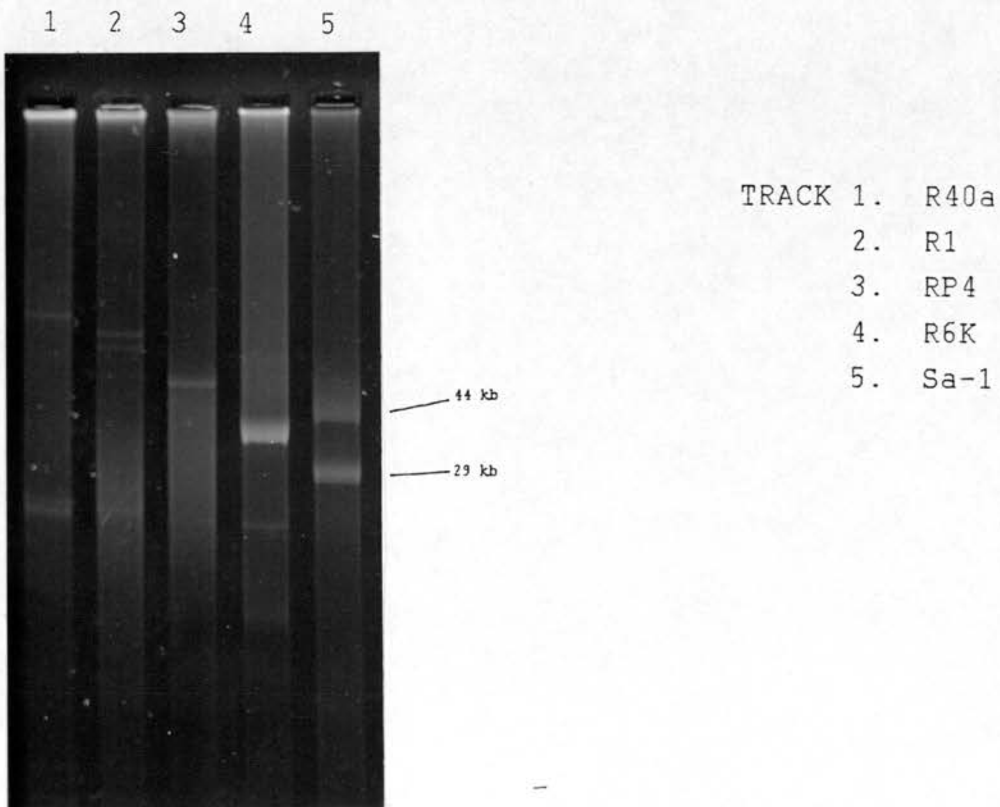


FIGURE 2.2: MULTIPLE FORMS OF Sa-1



RESTRICTION ANALYSIS OF Sa-1 AND Sa-2

In order to discover how the DNA of Sa-1 and Sa-2 differed, restriction analysis of these plasmids was undertaken; the results of which are indicated by Figures 2.3 and 2.4. Initial restriction (Experiment 1) with Hind III and Bam Hl (Figure 2.4) indicated differences between the two plasmids. Hind III was found to restrict Sa-2 three times and Sa-1 once, whilst Bam Hl cut both plasmids twice but the size of restriction fagments was different (Table 2.2). The larger fragment from the Bam HI digest of each plasmid was similar (20.15 kb compared with 20.73 kb), but the smaller fragment of Sa-2 was twice the size of Sa-1. Subsequent repetitions of these digests (Experiment 2: Table 2.2) confirmed the above findings, and further restriction with Pst I (Figure 2.5), which cuts Sa three times (Ward and Grinsted, 1982), served to substantiate the view that Sa-1 and Sa-2 were different. Variations in the number of fragments after Pst I digestion of Sa-1 and Sa-2 were again apparent (Figure 2.5), suggesting that the differences between the two plasmids lie in the region between the two Bam Hl sites. This region is known to contain the chloramphenicol resistance gene of Sa (Ward and Grinsted, 1982).

FIGURE 2.3: HIND III RESTRICTION OF Sa-1 AND Sa-2

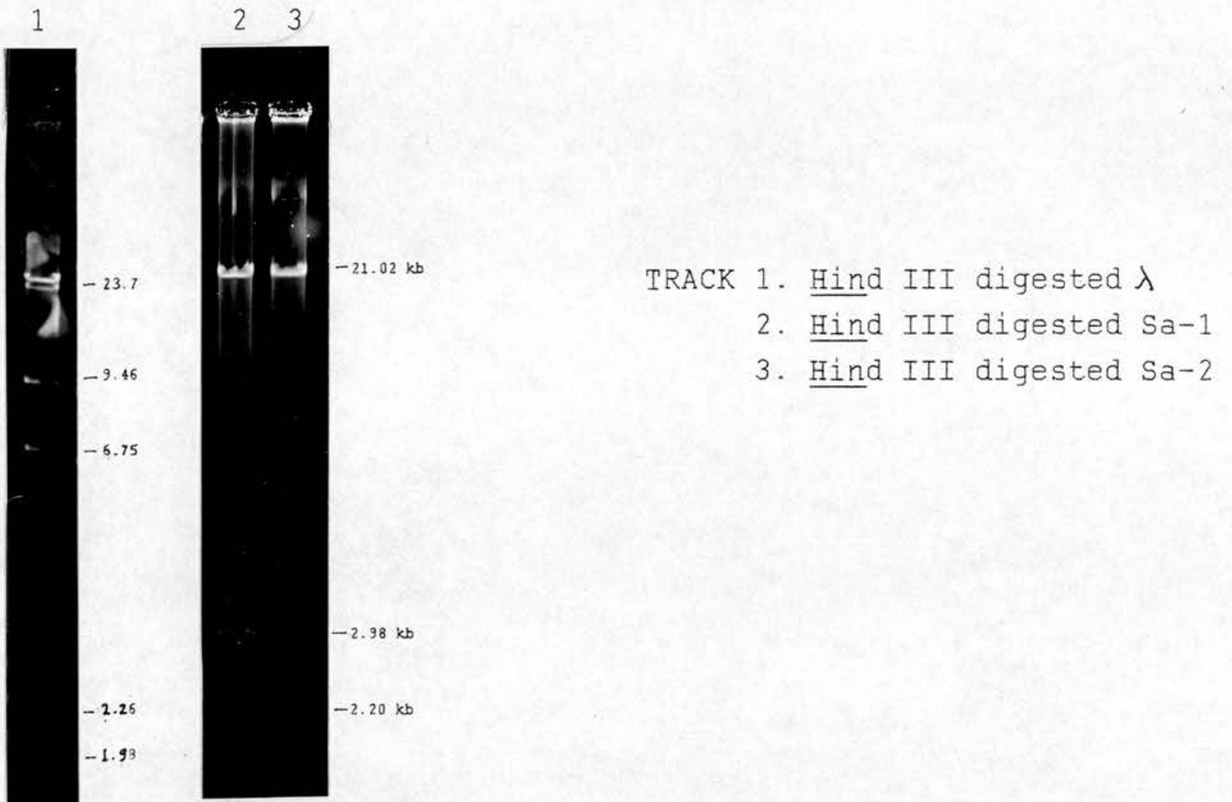


FIGURE 2.4: BAM HI RESTRICTION OF Sa-1 AND Sa-2

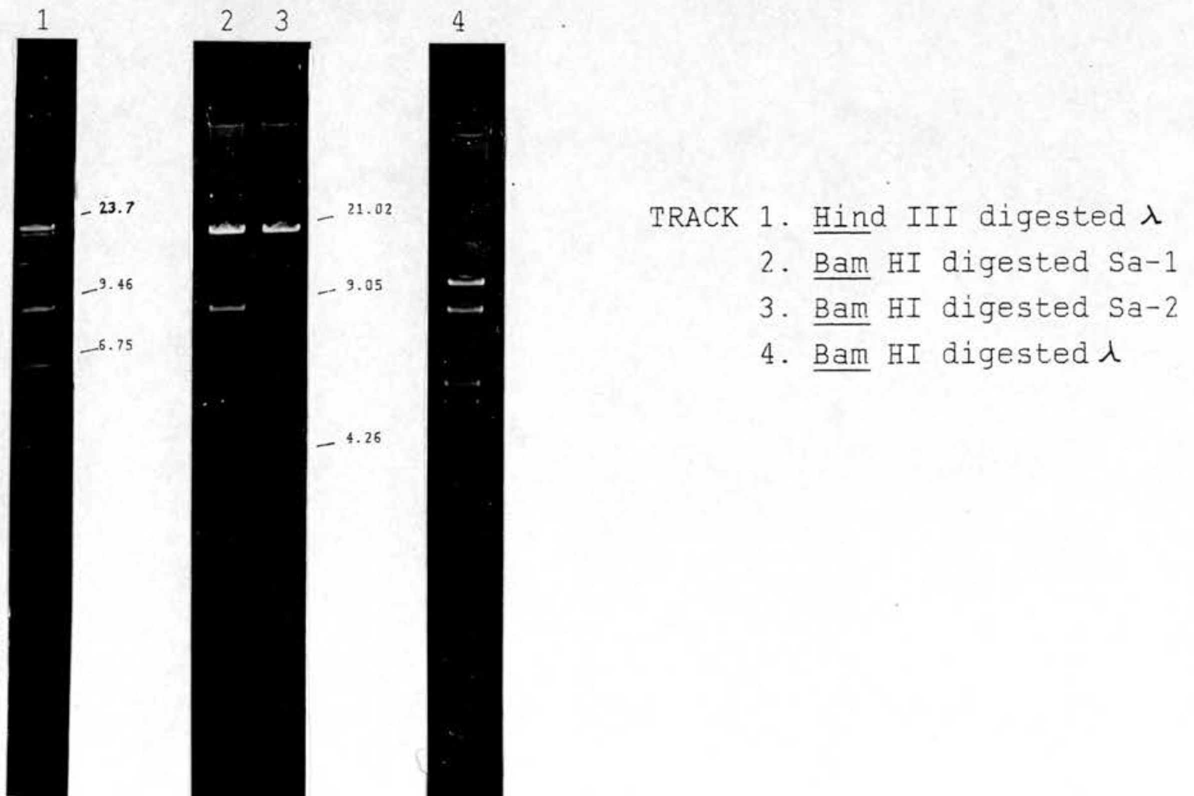
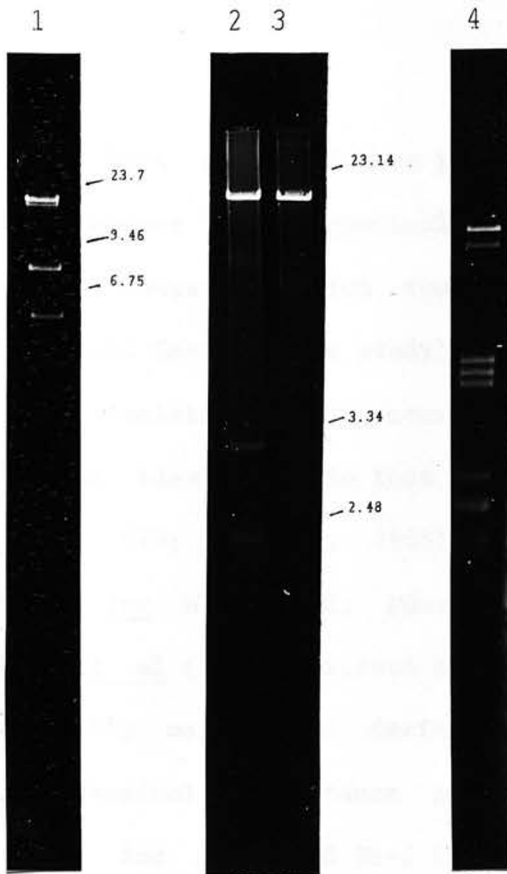


TABLE 2.2: MOLECULAR SIZES OF RESTRICTION FRAGMENTS FROM *Hind* III, *Bam* HI AND Pst I DIGESTION OF Sa-1 AND Sa-2

EXPERIMENT	PLASMID	*Hind* III	*Bam* HI	*Pst* I
1	Sa-1	24.82	20.73 4.02	
	Sa-2	24.82 3.18 2.42	20.15 8.40	
2	Sa-1	21.02	21.02 4.26	23.14
	Sa-2	21.02 2.98 2.20	21.02 9.05	23.14 3.34 2.48

All sizes are expressed in kb

FIGURE 2.5: PST I RESTRICTION OF Sa- AND Sa-2



TRACK 1. Hind III digested λ

2. Pst I digested Sa-1

3. Pst I digested Sa-2

4. Pst I digested λ

DISCUSSION

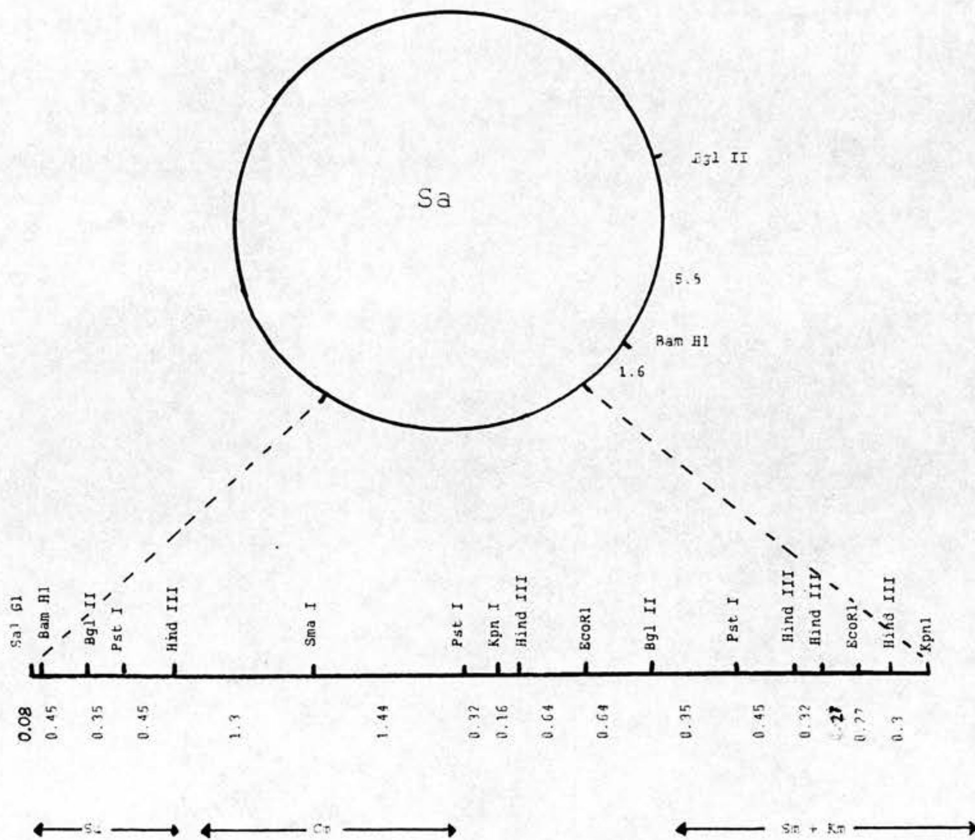
The data presented here permit the preliminary restriction mapping of a spontaneous chloramphenicol sensitive mutant of Sa (designated Sa-1), and its comparison with the published Sa (Ward and Grinsted, 1982) (designated Sa-2 in this study).

The isolation of spontaneous chloramphenicol sensitive mutants, such as those identified in this study, would not appear to be unusual (Gorai et al, 1979; Ireland, 1983). Chloramphenicol sensitive mutants of the protype Inc W plasmid, pSa, have been identified by Ireland (1983), and Gorai et al (1979) utilised an Sa chloramphenicol sensitive mutant plus a genetically manipulated derivative of Sa to establish the location of the chloramphenicol resistance gene. The difference in molecular size observed for Sa-1 and Sa-2 in this study (5 kb) is in agreement with the findings of Gorai et al (1979) and suggests that the lack of resistance to chloramphenicol is due to loss of DNA containing some or all of the chloramphenicol resistance gene, ie. Sa-1 is a spontaneous deletion mutant of Sa-2. Previous evidence has suggested that the chloramphenicol resistance gene of pSa, and therefore possibly other Inc W plasmids, is on a deletable element (Hedges and Datta, 1971; Farrand et al, 1981) but the nature of this element is uncertain. Inc W plasmids carry antibiotic resistance genes (Cm, Ap, Tp and Km) similar to those reported to be on transposons but, despite the high rate of spontaneous deletion (loss of chloramphenicol resistance from pSa has been shown to occur at a frequency of 1% in E coli J53-1 after 8 logs of growth), the chloramphenicol resistance gene of pSa (and probably other Inc W plasmids) would appear not to be identical to the chloramphenicol resistance transposon, Tn9

(Rosner and Gottesman, 1977). A comparison of the restriction map of the chloramphenicol resistance determinant of pSa with that of Tn9 (Ireland, 1983) disclosed major differences and preliminary hybridisation results revealed no homology. In addition attempts to detect transposition of chloramphenicol resistance in rec A strains was unsuccessful. (Transposition of transposons is independent of the rec A function (Kleckner, 1981)).

Preliminary restriction enzyme analysis of Sa-1 and Sa-2 (Figure 2.4) mapped the deletion to the smaller Bam H1 fragment and it is this fragment, according to the map of Ward and Grinsted (1982) (Figure 2.6) that contains the chloramphenicol resistance gene. Hind III restricts Sa five times but the two smaller fragments (Table 2.3) are easily lost from a gel. Restriction of Sa-2 is therefore in agreement with the findings of Ward and Grinsted (1982), but digestion of Sa-1 results in one fragment only (Figure 2.3). This would suggest that the deleted fragment contains at least two Hind III sites. However, the deletion, which lies within the 9 kb Bam H1 fragment and includes some or all of the 2.7 kb chloramphenicol resistance gene region, is only 5 kb in size and the Hind III sites span 6.11 kb. It is therefore possible that the deletion has not removed the Hind III sites but has resulted in fragments too small to detect. In order to locate the deletion more precisely, Sa-1 and Sa-2 were digested with Pst I (Figure 2.5), which cuts the smaller Bam H1 fragment three times - once at either end of the chloramphenicol resistance gene (Figure 2.6). Sa-1 was shown to lack the two smaller Pst I fragments (3.34 and 2.48 kb) of Sa-2, the sum of which is equivalent to the difference in size between the smaller Bam H1 fragments of the two plasmids concerned, and the difference in overall molecular size. It would thus appear (Figure 2.7) that Sa-1 has arisen from Sa-2 by

FIGURE 2.6: PHYSICAL AND FUNCTION MAP OF Sa

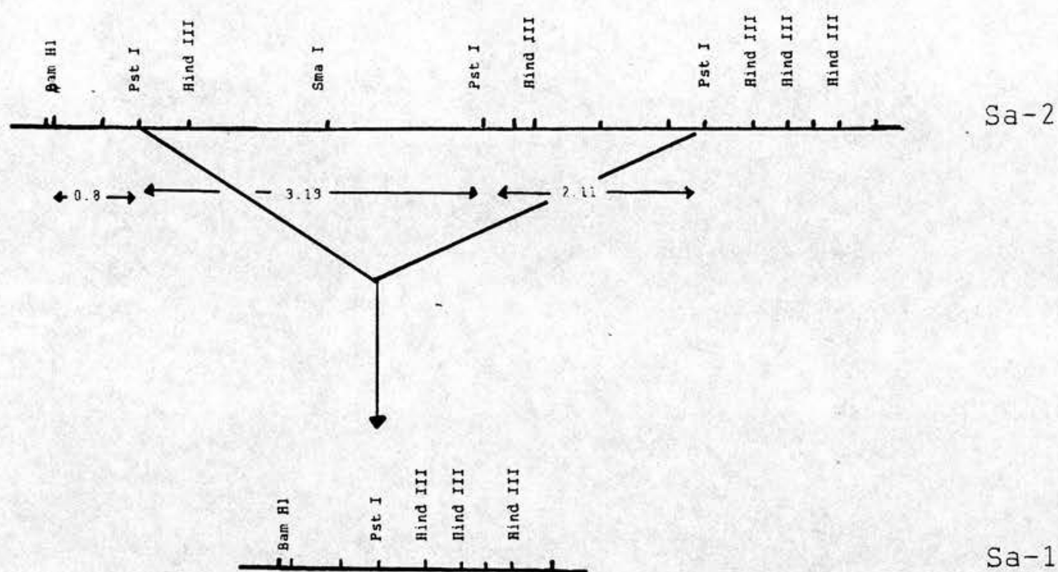


All sizes are in kb

TABLE 2.3: COMPARISON OF THE OBSERVED RESTRICTION FRAGMENT SIZES WITH PUBLISHED RESULTS

RESTRICTION ENZYME	WARD AND GRINSTED (1982)	Sa-1	Sa-2
Hind III	0.32	21.02	2.20
	0.47		2.98
	2.08		21.02
	3.22		
	30.91		
Bam HI	9.26	4.26	9.05
	27.74	21.02	21.02
Pst I	2.11	23.14	2.48
	3.19		3.34
	31.70		23.14

FIGURE 2.7: FORMATION OF Sa-1 DELETION MUTANT



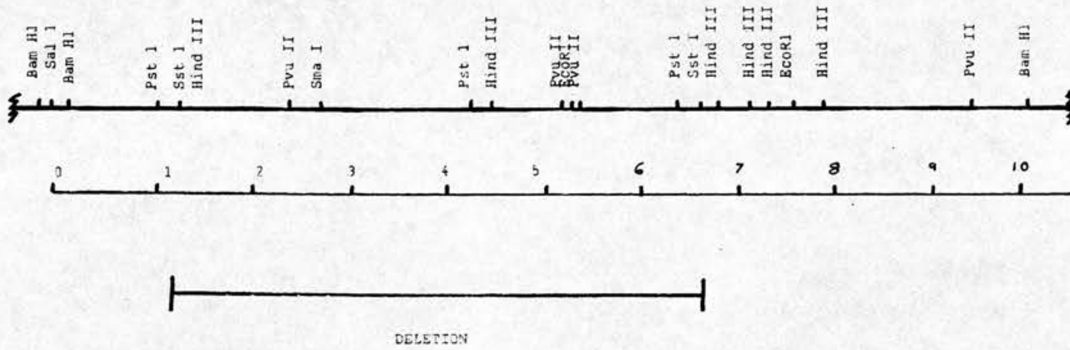
All sizes are in kb

spontaneous deletion of the two smaller _Pst_ I fragments, causing a decrease in size of 5-6 kb. The loss of this DNA would result in the loss of two _Hind_ III sites - leaving _Hind_ III fragments of approximately 0.32, 0.47 and 30.89 kb in size, according to the map of Ward and Grinsted (1982). _Hind_ III digestion of Sa-1 produced one fragment which is in agreement with the above hypothesis, if it is assumed that the two smaller fragments are too small to be detected.

Of possible significance to the spontaneous deletion of DNA carrying the chloramphenicol resistance gene, is the finding that the regions at either end of the deleted DNA have the same pattern of restriction sites (Figure 2.6). Ireland (1983) indicated a similar finding with the chloramphenicol sensitive deletion mutant of pSa (Figure 2.8) and postulated that these direct repeats were responsible for the high frequency of spontaneous deletion. Generalised recombination at these sites, requiring the _rec A_ function, would produce deletions (Sherratt _et al_, 1981). This type of recombination would leave behind one set of the repeated sequences, and this is what is indicated by Figure 2.7: one _Pst_ I site and one _Hind_ III site are lost.

The difference between Sa-1 and Sa-2 is thus due to a spontaneous deletion of an approximate 5 kb _Pst_ I fragment, containing the chloramphenicol resistance gene, brought about by generalised recombination at the site of direct repeats.

FIGURE 2.8: LINEAR RESTRICTION ENZYME MAP OF PLASMID pSa: DETAIL WITHIN 9.9 kb Bam H1 FRAGMENT



From Ireland (1983)

CHAPTER 3

EXAMINATION OF A PORCINE FAECAL ENTEROBACTERIAL STRAIN EXPRESSING TRIMETHOPRIM RESISTANCE

INTRODUCTION

Since the Swann Report (1969) there has been considerable controversy surrounding the use of antibacterial drugs in animal husbandry, because of the potential transfer of resistant bacteria from animals to man (National Academy of Science Report, 1980; Lyons et al, 1980). It was found for instance, that the continuous feeding of pigs on a diet containing Tc's gave rise to large populations of E coli organisms with transferable Tc resistance (Smith, 1980) and that Enterotoxigenic E coli were a common cause of diarrhoeal illness in both humans and animals (Sack, 1978; Soderlind and Molby, 1979; Rowe, 1979). In addition, Tp resistant Salmonella typhimurium strains, isolated from an outbreak in cattle in Britain in 1977, were found by 1979 to cause infections in humans (Threlfall et al, 1980) and a Tp resistant Salmonella krefeld serotype was isolated from both humans and animals in the United States, although it was not common (Mathewson and Murray, 1983). The work of Levy et al (1976), Linton et al (1977a,b) and Davies and Stewart (1978) suggested a flow of resistant E coli between animals and man; an idea that has been reviewed by Feinman (1984). Whilst it was commonly believed that the indiscriminate use of antibacterial drugs contributed to the spread of antibiotic resistance among E coli strains, the Swann report concluded that antibacterial drugs used in medical practise should not be used as growth supplements, but it did not restrict their use in the treatment of infectious diseases in animals. It is this use that still causes concern, as the nature of animal husbandry is such that there is considerable exposure to the same drugs that are used to treat human infections. Studies by Kanai et al (1983), however, indicated the problems facing the

livestock industry, with regards the use of antibiotics. Whilst a large type of housing with automatic devices, such as that used in the broiler industry, simplifies management and leads to efficient mass production, the risks of mass disease are intensified, necessitating the use of antibacterial drugs. Thus, apart from their administration to sick animals, a variety of antibiotics have been given to groups of healthy animals in the hope of protecting them from contracting diseases (Smith, 1980). This policy is much more likely to result in the emergence of antibiotic resistant organisms because the bacterial flora of a much larger number of animals will be exposed to the selection pressure provided by the antibiotics. Despite the implementation of the Swann Report with regard to some antibacterial drugs, eg the prohibition of Tc in 1971 (Smith, 1980) and Cm in 1979 (Jorgensen, 1983) the levels of resistant E coli with conjugative ability remained high (Smith, 1973, 1975). Thus there has been very little demonstrable effect on the genetic constitution of the plasmid family, indicating the profound and lasting ecological changes that can be brought about by administering antibiotics to animals. However the strong discouragement to use antibiotic treatment for Salmonellosis in veterinary practise in Denmark, may have contributed to the lack of multiresistance (Jorgensen, 1986) in this species.

Several studies on the ecology of plasmid - borne antibiotic resistances in gram negative bacteria have been concerned with assessing this potential for exchange of resistance plasmids between humans and animals. Studies of faecal coliforms by Hartley et al (1975) and Bettelheim et al (1976) indicated that E coli from calves and humans shared many common O antigen types and other studies suggested similarities between plasmid populations in man, farm animals and pets on the basis of common resistance patterns (Smith, 1975) and incompatability

groups (Bezanson et al, 1981; Davies and Stewart, 1978). Anderson et al (1975), from molecular weight and DNA homology studies of R-plasmids from enteric bacteria of human and animal origin, indicated that there was a common pool of R-plasmids in man and animals . Studies by Towner and Wise (1983), by Wise et al (1985) and Towner et al (1986) on Tp R-plasmids found in the local Nottingham animal and human populations substantiated this view. The majority of plasmids isolated from animal strains of E coli were found to belong to the same incompatibility groups and carry the same range of antibiotic resistance determinants as those from the human community. Similar results were obtained by Mee and Nikoletti (1983) and Campbell et al (1986) in Australia but work by Amyes (1986) on Tp R-plasmids from human and porcine isolates in the Edinburgh area revealed that most porcine plasmids were unlike their human counterparts. Although contradicting the theory of a common pool of R-plasmids, these findings did not rule out the possibility of genetic exchange altogether.

Possible routes for the interchange of plasmids between humans and animals do exist in the environment (Linton, 1986). It has been clearly shown that contamination of animal carcasses with gut contents is a normal occurance at commercial abattoirs (Howe et al, 1976; Howe and Linton, 1976; Linton et al, 1976) thus providing a possible route for contact with humans. Spread could also be effected through domestic and agricultural effluents into enviromental water bodies (Abdul and Venables, 1986). In addition, it has been shown that a reduction in faecal E coli serotypes occurs in humans fed a sterile diet, suggesting that food is a source of new E coli strains (Bettelheim et al, 1977). Contaminated food as a source of antibiotic resistance bacteria has also been documented by Rolland et al (1985), Cooke et al (1970) and Levy (1984).

The impact of antibiotic usage in animals on the potential spread of resistance may depend on the nature of the antibiotic. According to Smith (1980) the pig population of the UK will remain an enormous reservoir of Tc resistance in E coli with conjugative ability for many years, due to the indescriminate use of Tc. In contrast, the incidence and amount of Ap, Cm, Nm and Fur resistant E coli in faecal specimens appears to be stable. The emergence and persistance of these latter resistances is probably due to veterinary use only and therefore, if this does not increase greatly, the present state will probably remain. The impact of veterinary antibiotic use, however, has been quite different as far as Sm Su resistant organisms are concerned. They have become more prevalent and in the 1979 survey (Smith, 1980) vied with Tc resistance ones as the commonest antibiotic resistant E coli.

In contrast to the progressive increase of Tp R-plasmid - bearing enterobacteria from clinical isolates, (Amyes et al, 1978; Romero and Perduca, 1977; Acar et al , 1977; Towner et al, 1980) the spread of Tp R-plasmids has been slower in animal strains (Smith, 1980). Tp containing products were first introduced for medical and veterinary use in 1968 and 1969 respectively. In the following years there were a number of reports of differing forms of resistance (Jobanputra and Datta, 1974; Towner et al, 1978; Towner et al, 1982) and there were investigations into the Tp resistance found in bacterial isolates of animal origin, particularly pigs (Fleming, 1973; West and White, 1979; Bannatype et al, 1980; Mee and Nikoletti, 1983). It has become clear that plasmids confering Tp resistance have spread throughout a range of coliform isolates in animals and to Klebsislla, Enterobacter, Citrobacter and Acinetobacter species in man (Mee and Nikoletti, 1983). Studies on isolates from animals other than pigs have concentrated mainly on Tp resistant plasmids found in

strains of Salmonella typhimurium (Richards et al, 1978; Ward et al, 1982; Threlfall et al, 1983). Both West and White (1979) and Wise et al (1985) found a high prevalence of Tp resistance in bovine isolates from farms in England, suggesting that calves were a major resevoir of Tp resistance amongst farm animals. Whilst levels of resistance in E coli from pigs and lambs were lower, they still constituted significant resevoirs of Tp resistance. The examination of faecal specimens from pigs, sheep and cattle entering the Edinburgh city market, on the other hand, indicated that the only significant emergence of Tp resistant faecal enterobacteria in farm animals occurred in pigs (Amyes, 1986). This difference in the major resevoir of Tp R-plasmids is reflected in the intensity of farming in different areas and thus the potential for infection and the economics of antibacterial drug use (Amyes, 1987). (The effects of different management policies on antibiotic resistance levels has been documented for other antibiotics (Hinton et al, 1985).). Whilst cattle in Scotland are grazed more often and less densely than in England , pigs are generally confined, leading to the rapid spread of infection. Whilst Amyes (1986) found that a much larger proportion of the porcine resistant population were resistant to high levels of Tp, compared with clinical strains from a concurrent study, a much lower percentage of these strains possessed transferable Tp resistance. This revealed a more pronounced movement of genes away from plasmids to the bacterial chromosome.

Although the Tp resistant plasmids of human and porcine strains in the Edinburgh area appeared different (Amyes, 1986), the transposons responsible for the Tp resistance were very similar. The ubiquitous Tn7, was prevalent in pig isolates (Amyes, 1986) and a few carried a smaller transposon, Tn4132, previously found in some human strains (unpublished

observations). This transposon is thought to be closely related to Tn7 (Young and Amyes, 1985a), and it's finding in an animal isolate implicates the transfer potential of resistance genes, as opposed to the whole R-plasmid, from animal and human resevoirs. Tn7 was first described in human isolates of enterobacteria (Barth et al, 1976) and was also reported in a Salmonella typhimurium strain of bovine origin (Richards et al, 1978). Due to the possibility that the animal population may harbour more clues as to the evolution of Tp resistance genes and their spread, Tp resistant porcine isolates were studied further.

MATERIALS AND METHODS

RESISTANCE TESTING

Resistance testing was carried out on Diagnostic Sensitivity Test agar (DSTA) (Oxoid, Basingstoke) containing antibacterial drugs at the above concentrations. A 10^{-4} dilution of overnight broth cultures (10^5 cfu/ml) was prepared in single strength Davis Mingioli media (Davis and Mingioli, 1950) without supplements (DM base) and multiply inoculated on to appropriate plates with a Mast Multiple Inoculator. Growth at the point of inoculation defined resistance to the included compound.

PLASMID DNA PREPARATION AND RESTRICTION

Plasmid DNA was prepared by the method of Takahashi and Nagano (1984) and analysed by agarose gel electrophoresis, as described in Chapter 1. Single digests were performed with *Hind* III (9u), *Bam* HI (6u) and *Pst* I (12u). The restriction procedure was that utilised in the restriction mapping of Sa-1 and Sa-2 (Chapter 2). Molecular sizes of the smaller fragments were estimated by comparison with those obtained for lambda DNA following digestion with *Hind* III (Murray and Murray, 1975).

STANDARD CONJUGAL MATING PROCEDURE

Nutrient broth cultures (4.5 ml) of donor and recipient were grown up overnight. The donor culture (0.1 ml) was mixed with 1 ml of recipient in 4.5 ml of prewarmed nutrient broth and incubated statically at 37°C for between 1 and 5 hours. After vortexing, the mating mixture was centrifuged (2544 g for 15 minutes) and resuspended in 5.6 ml of DM base. Neat, 10^{-1}, 10^{-2} and 10^{-3} dilutions (0.1 ml) were plated onto appropriate selective plates and incubated at 37°C for up to 72 hours. The donor culture was diluted 10^{-6} in DM base and 0.1 ml plated on Maconkey agar (Oxoid) for a viable count. Donor and recipient cultures, centrifuged (2544 g for 15 minutes) and resuspended in 4.5 mls of DM, were utilised as controls. An aliquot (0.1 ml) of each was plated neat on selective plates, which were incubated at 37°C for upto 72 hours. Transconjugants were purified by restreaking on selective plates. The frequency of plasmid transfer was expressed per viable donor cell, as described by Amyes (1974).

PREPARATION OF SELECTIVE PLATES

Selective plates for transfer studies were prepared using DM base and antibacterial drugs at the following concentrations, unless otherwise stated: ampicillin, chloramphenicol, kanamycin, nalidixic acid, streptomycin, tetracycline and trimethoprim - 10 ug/ml; rifampicin - 25 ug/ml; sulphamethoxazole and spectinomycin - 100 ug/ml. For the selection of E. coli strain J53 transconjugants, 1 ml proline (5 mg/ml), 1 ml methionine (5 mg/ml), 1.4 ml glucose (20%) and the appropriate amount of antibiotic were added to 50 mls of double strength DM media and the volume made up to 60 mls with distilled water. This was added to 40 mls of hot bacteriological agar (Oxoid) and plates poured immediately. Media for the selection of E. coli J62 were prepared in a similar way except methionine was replaced with 1 ml histidine (5 mg/ml) and 2.5 ml tryptophan (2mg/ml).

BACTERIAL TRANSFORMATION

A 4.5 ml culture of E.coli C600 was grown up overnight in Luria Broth (LB) (5g yeast extract, 10g sodium chloride, 10g tryptone made up in 1 litre of distilled water, to which was added 1 ml of 20% filter sterilised glucose per 100 ml broth). One hundred ml of fresh LB broth was inoculated with 1 ml of overnight culture and incubated at 37°C (shaking) for 2 hours. Forty mls of this culture was transferred to a 50 ml polypropylene centrifuge tube and cooled on ice before spinning down the cells at 5211 g for 5 minutes (Sorvall: Dupont Rc-5B superspeed). The supernatant was poured off and the cells resuspended in 4 ml of cold 0.1 M calcium chloride. After leaving on ice for 20 minutes the cells were pelletted as before, and resuspended in 0.8 ml of 0.1 M cold calcium chloride. With a sterile 1 ml pipette, 0.2 ml aliquots of cell suspension were placed in sterile glass bottles. Fifty microlitres of DNA was added to each bottle and left on ice for 30 minutes before transferring to a waterbath at 42°C for two minutes. The bottles were cooled on ice before adding a 2 ml aliquot of fresh LB broth to each. Cultures were grown, shaking, for 2 hours at 37°C to allow time for plasmid establishment. A 1 in 10 dilution of each culture was prepared in sterile distilled water and for each sample, diluted and undiluted suspensions were plated on LB plates containing appropriate selective antibiotics. Plates were incubated overnight at 37°C.

PREPARATION OF DNA FOR ELECTROELUTION

Small Scale Method

DNA was prepared by the method of Takahashi and Nagano (1984) (see Chapter 1) from 2 x 9 ml nutrient broth cultures, per sample. The two tubes of each sample were combined and alcohol precipitated again, before resuspending in 100 ul of Buffer C (10 mM Tris acetic acid; 2 mm disodium EDTA, pH 8.0).

Large Scale Method

Plasmid DNA was purified by a modification of the method described by Birnboim and Doly (1979). Cells were grown in 150 ml of Luria Broth Base (Gibco Labs, Madison) and pelleted in a Beckman JA - 14 rotor at 11100 g for 10 minutes. Cells were resuspended in 2.5 ml of solution I (50 mM glucose, 10 mM EDTA, 0.25 M Tris, pH 8.0 and 2 mg/ml lysozyme - made fresh daily). After incubation on ice for 30 minutes, 5 ml of solution II (0.2 N NaOH, 1% SDS) was slowly mixed in. Following a further 5 minute incubation on ice, 3.75 ml of solution III (3 M sodium acetate, pH4.8) was added. The lysate was incubated on ice for 1 hour, followed by centrifugation for 15 minutes at 18900 g (Beckman JA - 14 rotor). The supernatant was ethanol precipitated by the addition of two volumes of cold 95% ethanol, and kept at -20°C overnight. DNA was recovered by centrifugation at 13300 g for 10 minutes. The pellet was resuspended in 9 ml of TE buffer (50 mM Tris, pH 8.0; 10 mM EDTA) and treated with RNase at a final concentration of 50 ug/ml for 20 minutes at 37°C.

The plasmid DNA was purified in CsCl ethidium bromide density gradients using a single banding step. Ten grams of 99.9% pure //CsCl (Terochem Ltd., Edmonton, Canada) was added to each sample and gently

mixed, before transferring to a Beckman quick seal cetrifuge tube (Beckman, California). In semi-darkness, 150 ul of EtBr (10 mg/ml) was added per tube and the tubes filled up to the top with CsCl blank (1g/ml), taking care to remove all bubbles. After sealing, the tubes were loaded (still in semi-darkness) into a Beckman TI-70 fixed angle rotor. The gradients were spun at 3 1139 g for 20 - 24 hours at 20°C. The plasmid DNA was viewed under a UV light source and removed from the gradients, in semi-darkness, using a elongated pasteur pipette. The DNA was extracted 3 to 4 times with iso-amyl alcohol and precipitated at -20°C for 20 - 30 minutes (maximum) with 70 % ethanol. After centrifugation the pellet was resuspended in 100 ul of TE buffer and washed with 200 ul (2 x volume) of 95% ethanol. The precipitate was collected by centrifugation, after freezing at -20°C for 30 minutes, and dried under vacuum. The DNA was resuspended in an appropriate volume of TE buffer.

ELECTROELUTION

Large and small scale preparation were loaded on to 0.7% agarose gels and electrophoresis was carried out at 50 V for 16 hours. Gels were stained in EtBr before eluting fragments by a technique derived from the methods of Winberg and Hammarskjold (1980) and Dretzen et al (1981). A strip of wetted DEAE membrane (Schleicher and Schuell, NA- 45) was placed in an incision just ahead of the band of interest. Electrophoresis was continued at twice the standard running voltage until binding was complete, as judged by ethidium bromide fluorescence using long wave UV. The strip was freed of residual agarose by thorough shaking in a tube containing 1.5 ml of TE buffer (10 mM Tris; 1 mM EDTA). After repeating this washing the membrane was totally immersed in 300 ul of high salt solution (1 M NaCl; 1 mM EDTA; 20 mM Tris , pH 8.0) in an eppendorf tube. The tube was gently shaken, and after ensuring the strip was still totally submerged, the tube was incubated at 65°C for 45 minutes. The buffer was then removed to a fresh tube and a further 300 ul of high salt solution was added to the strip. The process was repeated. The DNA was precipitated from both tubes of removed buffer with 95% ethanol. The precipitate was dissolved in 50 - 100 ul of TE and reprecipitated with 70% ethanol before resuspending in a final volume of 30 - 50 ul of TE, depending on the amount of DNA.

RESULTS

PRELIMINARY CHARACTERISATION OF THE PIG ISOLATE, P-20

In order to determine the plasmid profile of the original pig isolate DNA was isolated from overnight broth cultures by the method of Takahashi and Nagano (1984) (Chapter 1). (The P-20 isolate had been stored 'frozen' in a glycerol/broth solution at - 70°C, prior to experimentation). Agarose gel electrophoresis was carried out at 70 V for 16 hours on a 0.7% gel; the results of which are indicated in Figure 3.1. The P-20 isolate possesses 5 (and possibly 6) plasmids. The sizes of the smaller bands can not be determined accurately, as the standard plasmids run concurrently with this experiment are no smaller than 38 kb. The larger plasmid has a molecular size of 107 kb. P-20 was also tested for its resistance to various antibiotics and results indicated that this strain was resistant to Tp and Tc only.

TRANSFERABILITY OF PLASMIDS FROM THE PIG ISOLATE - P-20

In order to separate the P-20 plasmids and assess their transferability , 5 minute and 5 hour standard matings were set up between the pig isolate, P-20, and E. coli J62, selecting on DM plates containing the J62 supplements, plus rif (25 ug/ml) and Tp (10 ug/ml). The transfer frequencies were low: 2.37×10^{-7} transconjugants per donor cell after a 5 minute mating and 9.02×10^{-6} after 5 hours. Transconjugants were tested for their resistance to unselected markers (Table 3.1) and their DNA was examined by agarose gel electrophoresis (Figure 3.2).

FIGURE 3.1: PLASMID PROFILE OF THE ORIGINAL PIG ISOLATE - P-20

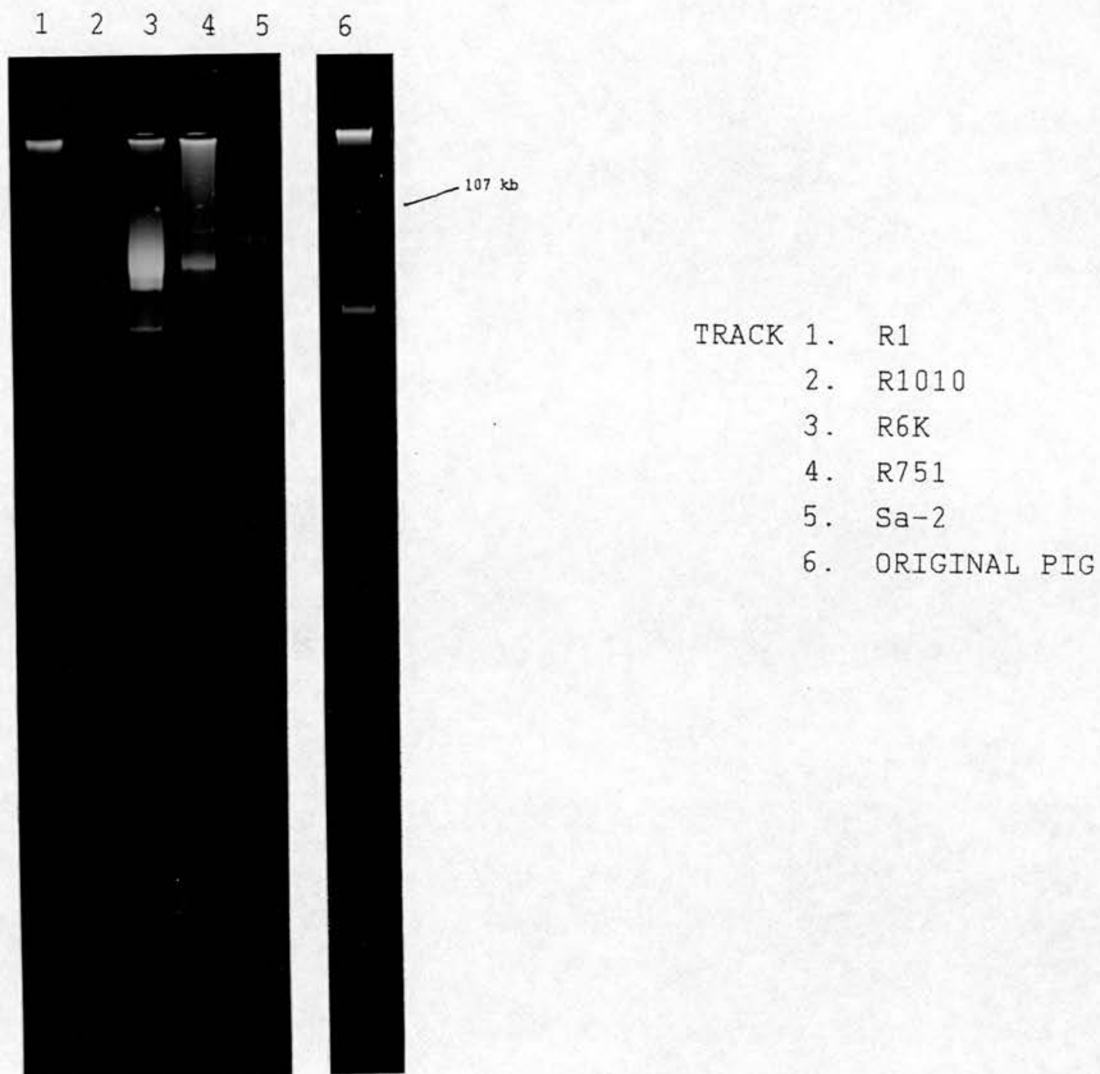
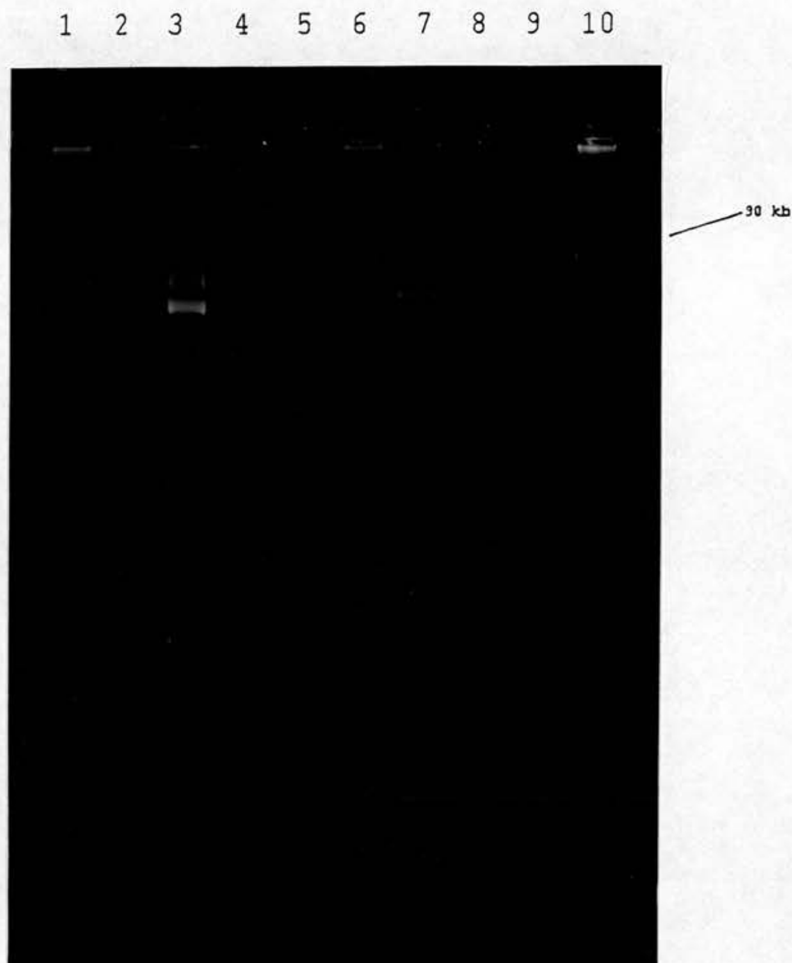


TABLE 3.1: RESISTANCES OF E COLI J62-2 TRANSCONJUGANTS CONTAINING PLASMIDS FROM THE ORIGINAL PIG ISOLATE, P-20

TRANSCONJUGANT	LENGTH OF	RESISTANCES
HI	5 minutes	Tp
H2	5 hours	Ka Tp (Sp/Sm)
H3	5 hours	Ka Tp (Sp/Sm)
H4	5 hours	Ka Tp (Sp/Sm)
H5	5 hours	Tp

Resistances present, but not always expressed are given in parenthesis

FIGURE 3.2: PLASMID PROFILES OF TRANSCONJUGANTS FROM THE CONJUGATION OF THE PIG ISOLATE - P-20, WITH E COLI J62-2 (BEFORE STORAGE)



TRACK				
1.	R1		6.	H1
2.	RP4		7.	H2
3.	R6K		8.	H3
4.	Sa-1		9.	H4
5.	R1010		10.	H5

All transconjugants were resistant to Tp but 3 transconjugants displayed additional resistance to Ka; which was not expressed in the original pig isolate, P-20. Subsequent retesting revealed that transconjugants H2, H3, and H4 sometimes expressed Sp/Sm resistance also.

Initial examination of DNA from the above transconjugants indicated that a 5 minute mating allowed the transfer of the large plasmid band but not the small plasmids (Figure 3.2) whilst a longer mating time (5 hours) resulted in the appearance of more than one plasmid band in the transconjugants. Reexamination of the DNA of these transconjugants after storage at -70°C, however, revealed discrepancies from the initial findings, suggestive of instability. After 1 months storage DNA from H1, H3 and H5 was analysed (Figure 3.3) and only one large plasmid band was evident in each transconjugants. H3 would appear to have lost the smaller plasmid bands but acquired a plasmid slightly smaller than the single plasmid of H1 and H5. These 3 transconjugants were restored at -70°C and reexamined along with the original stored transconjugants (H1 - H5) a further month later (Figure 3.4). Variation in the numbers and sizes of bands was again apparent and the sizes of the small plasmid bands present in the transconjugants differed from the sizes of the small plasmids found in the original pig isolate, P-20.

FIGURE 3.3: PLASMID PROFILES OF TRANSCONJUGANTS H1, H3 AND H5 AFTER ONE MONTHS STORAGE

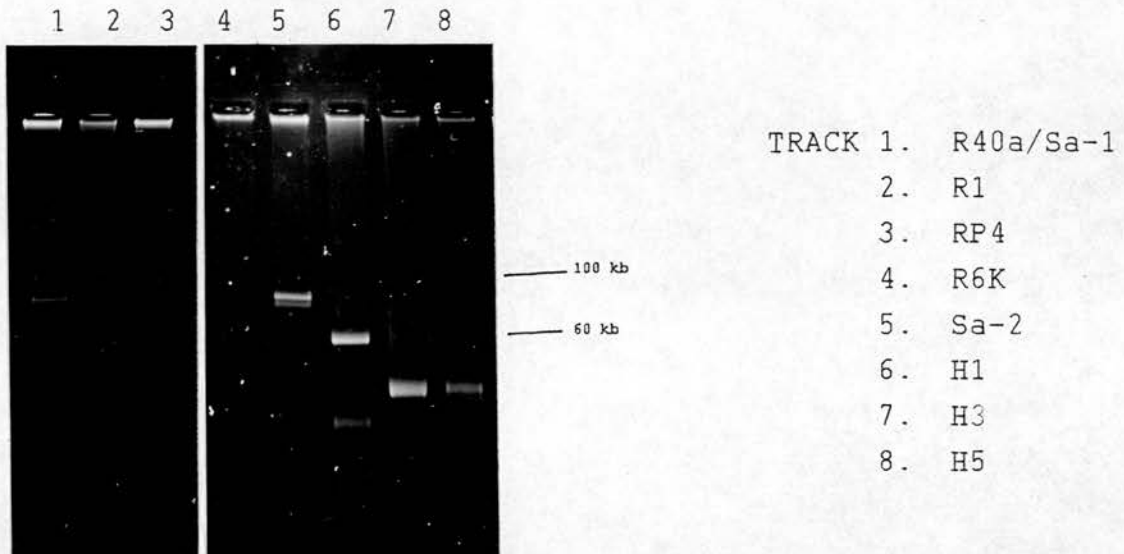
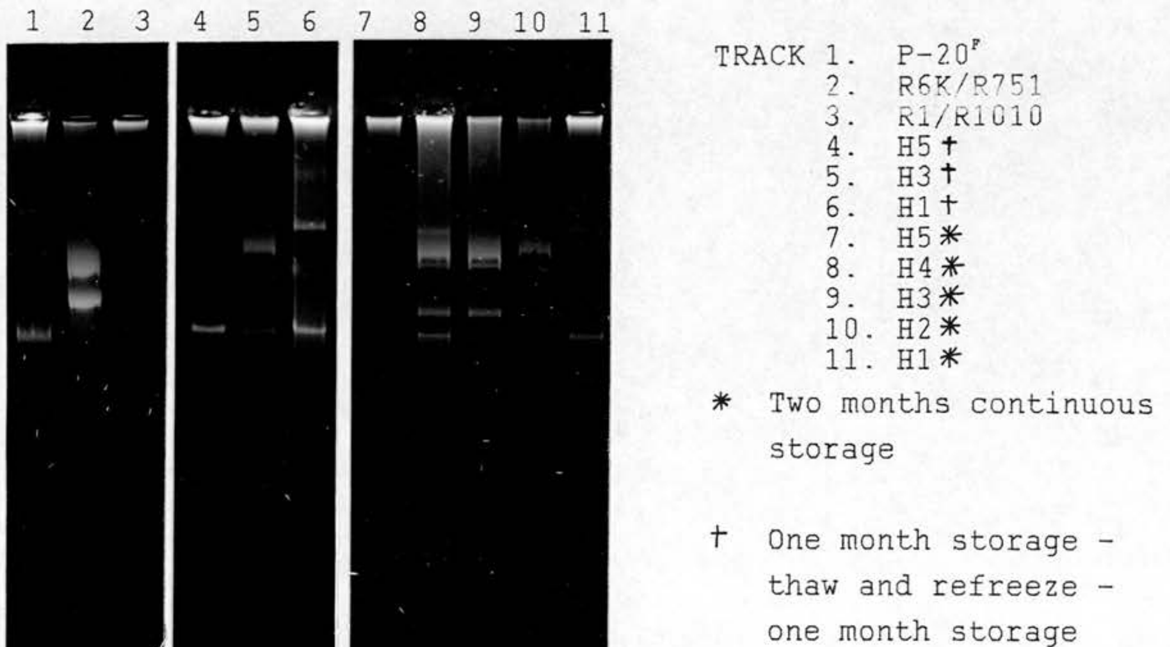


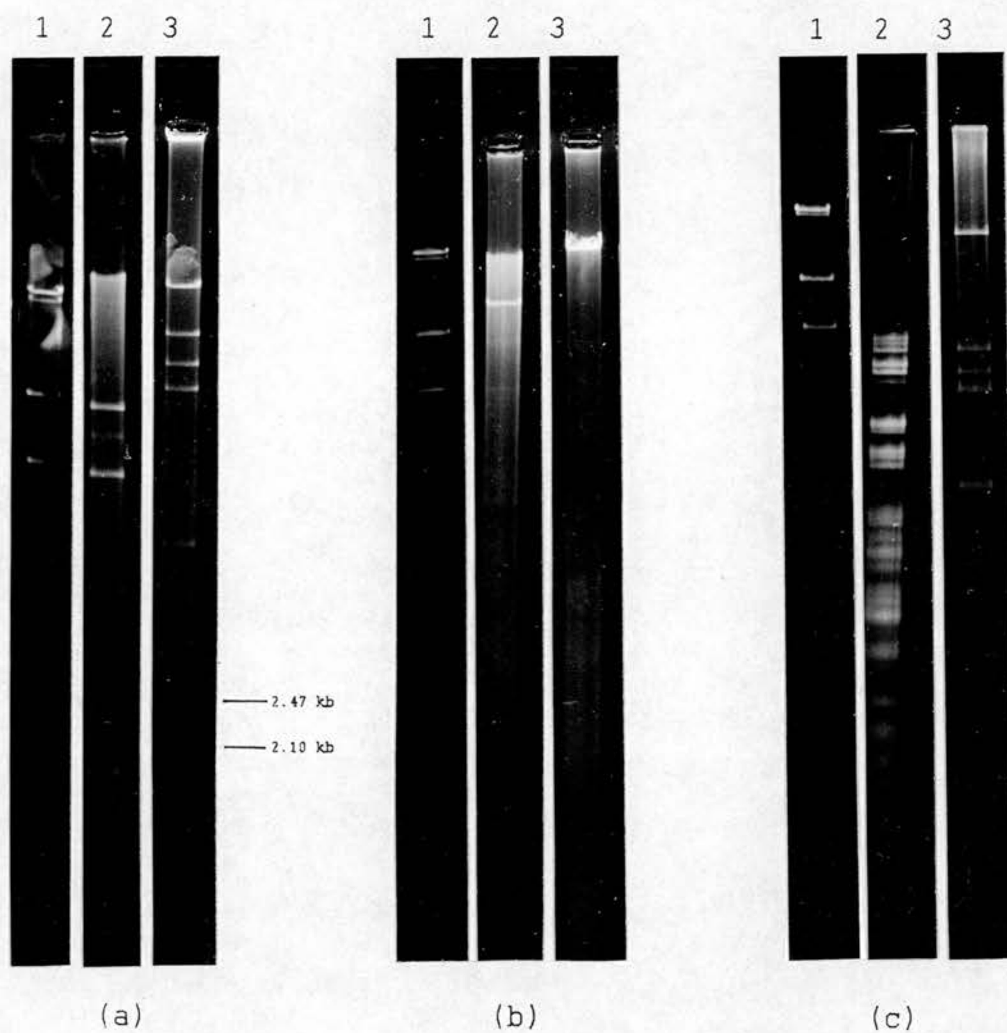
FIGURE 3.4: PLASMID PROFILES OF TRANSCONJUGANTS H1 - H5 AFTER TWO MONTHS CONTINUOUS STORAGE AND AFTER FREEZING AND RESTORAGE



RESTRICTION ANALYSIS OF THE LARGE PLASMID OF TRANSCONJUGANT H1 AND IT'S COMPARISON WITH THE PLASMID R483

In order to determine whether the Tp resistance of H1 was due to a *Tn7* like transposon, restriction enzyme analysis of the plasmid of transconjugant H1 and the Inc Ia plasmid, R483, was carried out utilising *Hind* III, *Bam* H1 and *Pst* I. As can be seen from Figure 3.5 the restriction patterns are very different. The characteristic 2.47 and 2.10 kb *Hind* III fragments of *Tn7* (Datta *et al*, 1979) are absent from the digest of transconjugant H1.

FIGURE 3.5: RESTRICTION DIGESTS OF PLASMID DNA ISOLATED FROM E COLI J62(R483) AND TRANSCONJUGANT H1



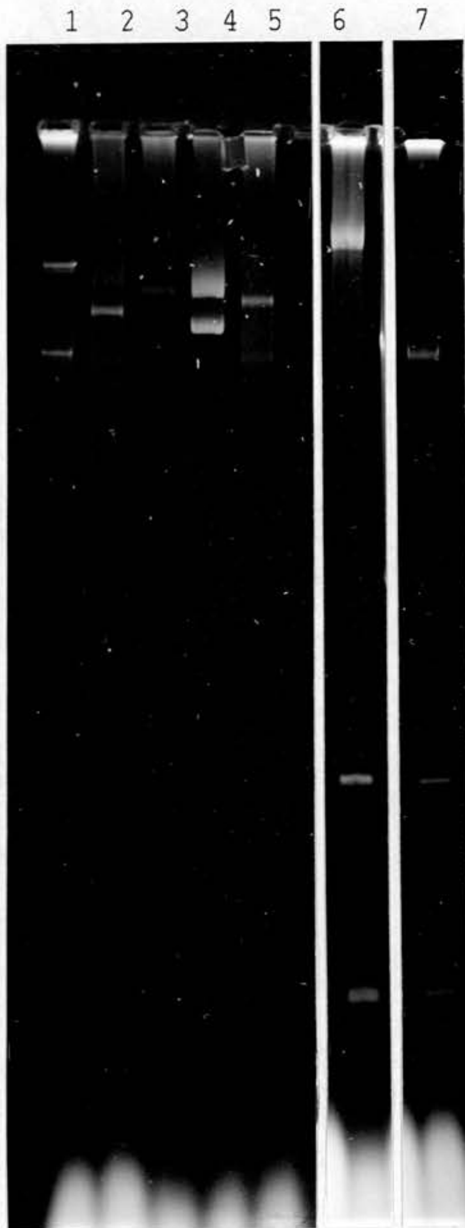
(a) DNA digested with Hind III
(b) DNA digested with Bam HI
(c) DNA digested with Pst I

TRACK 1. Hind III digested λ DNA
2. Transconjugant H1 DNA
3. R483 DNA

EFFECT OF STORAGE CONDITIONS ON P-20 ISOLATES

The previous transfer results indicate that storage of isolates at -70°C may affect plasmid stability (eg. the storing of transconjugant H3 appeared to result in the loss of the multiple bands and the appearance of one plasmid band not present in the original P-20 isolate.). Therefore, the resistance markers and plasmid profiles of the original P-20 isolate, stored in glycerol/broth at - 70°C- designated $P\text{-}20^F$ (where F = frozen) - and on agar slopes - designated $P\text{-}20^S$ (where s = slope)- were analysed and compared. Resistance testing indicated that whilst $P\text{-}20^F$ expressed resistance to Tp and Tc, $P\text{-}20^S$ lacked Tc resistance. The MIC's of Tp for the two strains were found to be similar ie greater than 1000 ug/ml. Examination of DNA prepared by the method of Takahashi and Nagano (1984) (see Chapter 1) revealed differences between the two differently stored isolates (Figure 3.6). The P-20 culture stored frozen, prior to investigation, had an additional plasmid band, which could be responsible for this strains resistance to Tc, compared with $P\text{-}20^S$.

FIGURE 3.6: EFFECT OF STORAGE CONDITIONS ON THE PLASMID PROFILE OF P-20



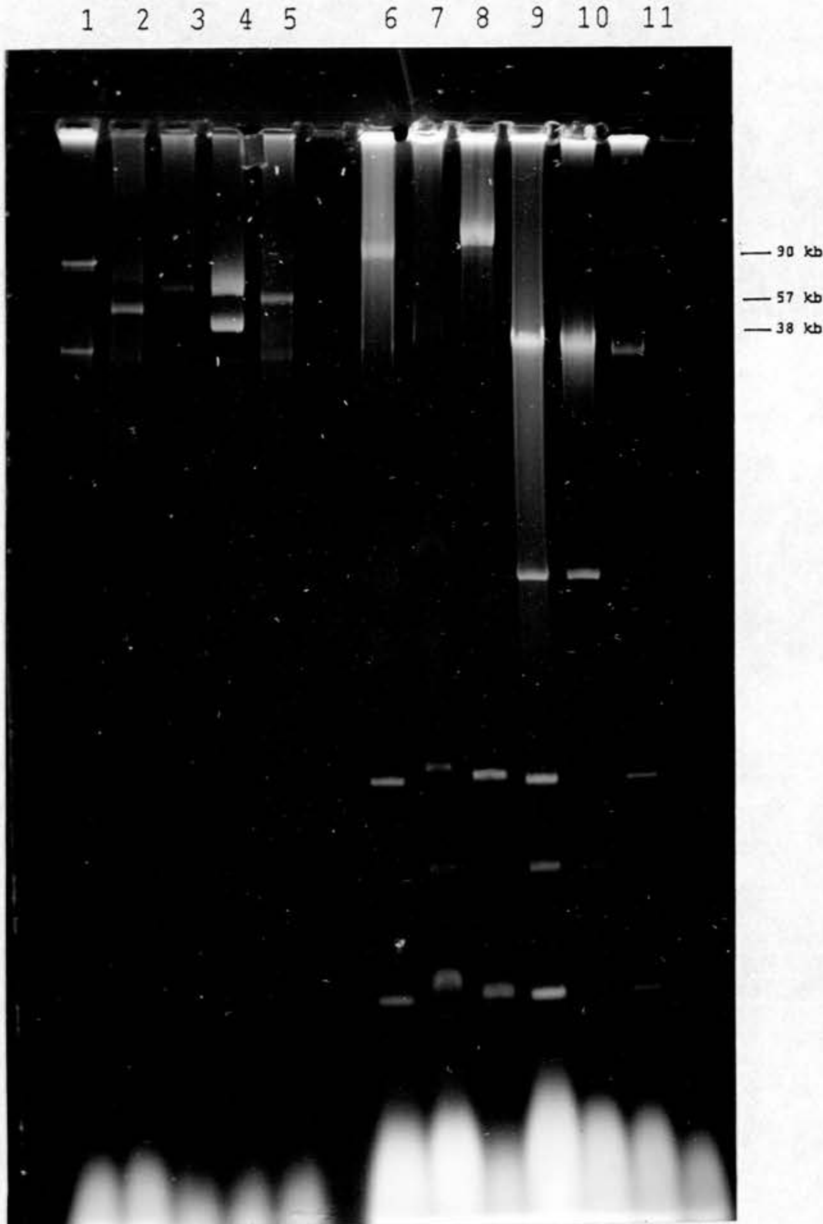
TRACK 1. R1
2. R1010
3. RP4
4. R6K
5. R751
6. P-20^{s}
7. P-20^{F}

EFFECT OF MEDIA ON PLASMID PROFILES

Due to the finding that some transconjugants from the transfer of P-20^{F} plasmids to J62 expressed Ka resistance, whilst P20^{F} itself appeared to be sensitive to Ka, overnight cultures of P20^{S} and P20^{F} in Isosensitest broth, Isosensitest broth + Ka at 20 ug/ml and Isosensitest broth with Tp at 500 ug/ml were analysed for plasmid DNA content. (Cultures grown in nutrient broth, as before, were used as controls). Whilst both cultures failed to grow in the presence of Ka the results of DNA analysis (Figure 3.7) indicated a media affect on plasmid number and possibly size. Growth in Isosensitest broth resulted in loss of the large plasmid band, or possibly the splitting up of this band, (Tracks 7 and 10 compared with 8 and 11, respectively), whilst the small plasmids of P20^{S}, grown in Isosensitest broth (Track 7) appear to be slightly larger than the corresponding plasmids in nutrient broth (Track 8). All strains, irrespective of growth media were resistant to Tp and the P20^{F} cultures were additionally resistant to Tc.

In order to quantify the effects of isosensitest broth on the stable maintenance of these plasmids, the two strains P20^{F} and P20^{S} were grown up overnight in 4.5 ml of nutrient broth, Isosensitest broth, isosensitest broth + Tp at 500 ug/ml and Isosensitest broth containing Thymidine at 50 ug/ml. The growth of these two strains in the different culture media again had no effect on drug resistance, but the results of analysis of plasmid DNA (Figure 3.8) appeared to differ from the initial findings. All tracks show evidence of the large plasmid whilst two of the smaller bands appear to be very faint or missing. Growth of P-20^{F} in Isosensitest broth, as compared with nutrient broth, has caused a reduction in the intensity of the large plasmid band, although not

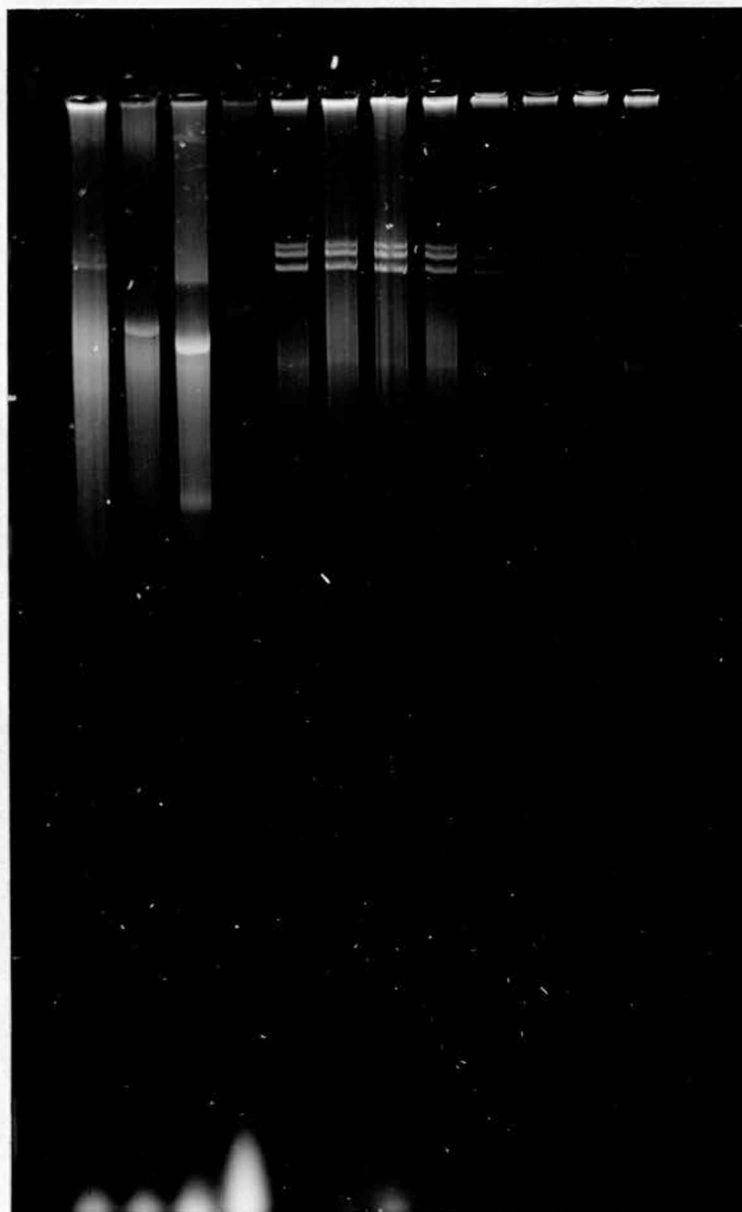
FIGURE 3.7: EFFECT OF MEDIA ON PLASMID PROFILES



TRACK			
1. R1		6.	P-20^{s} Tp
2. R1010		7.	P-20^{s} Iso
3. RP4		8.	P-20^{s} NB
4. R6K		9.	P-20^{r} Tp
5. R751		10.	P-20^{r} Iso
		11.	P-20^{r} NB

FIGURE 3.8: EFFECT OF ISOSENSITEST BROTH ON PLASMID PROFILES

1 2 3 4 5 6 7 8 9 10 11 12



TRACK			
	1. R1	5. P-20^{s} NB	9. P-20^{F} NB
	2. R1010	6. P-20^{s} Iso	10. P-20^{F} Iso
	3. R6K	7. P-20^{s} Iso + Tp	11. P-20^{F} Iso + Tp
	4. R751	8. P-20^{s} Iso + Thy	12. P-20^{F} Iso + Thy

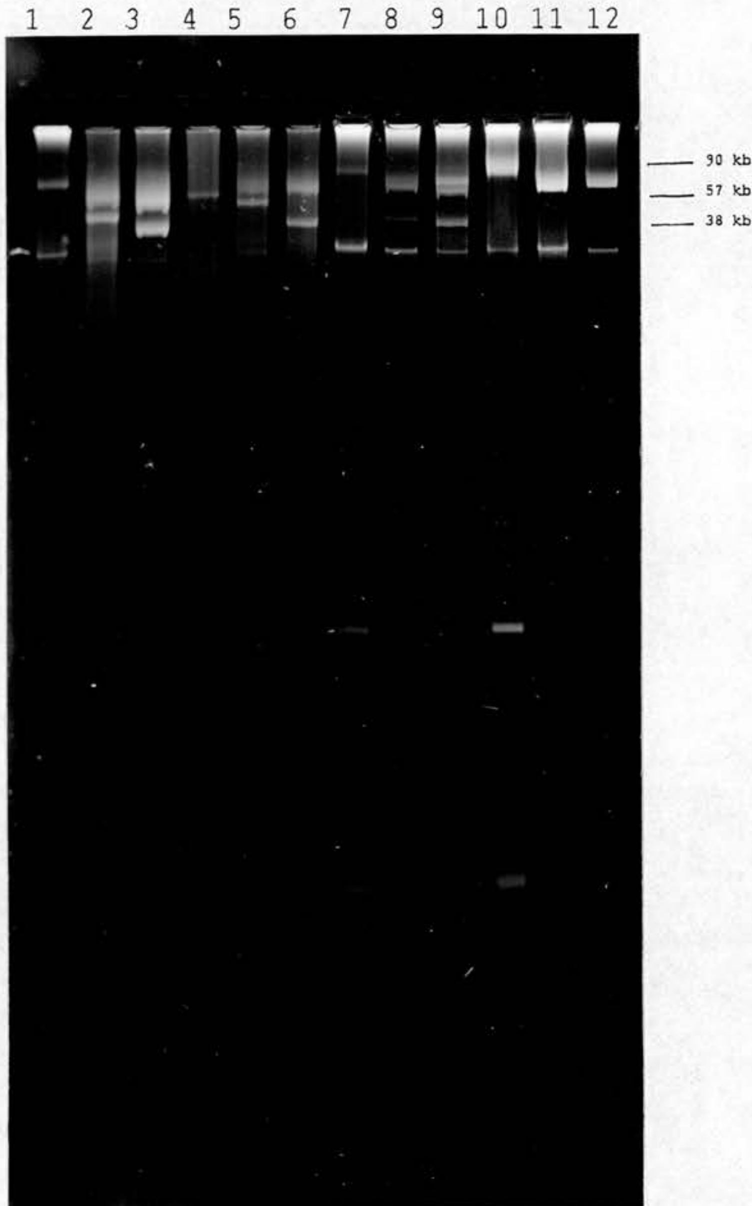
eliminating it all together, as previously found. Growth in isosensitest broth containing Tp (Tracks 7 and 11) would appear to enhance the intensity of the smallest band, suggesting that this small plasmid may encode a Tp resistance gene, that is amplified during growth in medium containing Tp.

MOBILISATION OF THE SMALL PLASMIDS OF P-20 UTILISING THE INC W PLASMID Sa

Sa-2 was introduced into the H1 transconjugant, from E coli J53, in order to determine whether the small plasmids present in transconjugants H2, H3, H4 and the original pig isolate, P20, were present in an integrated form in transconjugant H1. Selection was carried out on DM plates containing J62 supplements and Cm at 10 ug/ml. Sa-2 transferred into E coli J62 (H1) with a frequency of 2.02×10^{-4} per donor cell. Examination of the DNA from these transconjugants (Figure 3.9) indicated that the small plasmid bands, absent in H1, had reappeared, but they did not correspond to the plasmids in the original pig isolate.

To test for the possible integration of the small plasmids into the E coli chromosome, $P20^S$ and $P20^F$ were used as donors in 5 hour matings with a Rec A strain, E coli PB 1150, that expresses high level Sm resistance. Selection was made on DSTA plates containing Tp at 20 ug/ml and Sm at 2000 ug/ml. No transconjugants were obtained in this experiment, nor in a similar transfer study carried out between these strains for 16 hours.

FIGURE 3.9: MOBILISATION OF THE SMALL PLASMID BANDS



TRACK
1. R1
2. R1010
3. R6K
4. RP4
5. R751
6. Sa-2
7. Original Pig isolate - P-20^F
8. (Sa-2;H1)^F
9. (Sa-2;H1)^F
10. Original Pig isolate - P-20^s
11. E coli J62(Sa-2, original pig plasmid)^s
12. E coli J62(Sa-2, original pig plasmid)^s

^{F} This strain was formed by conjugating P-20^F with E coli J62 and introducing Sa-2 into the resulting transconjugant

^{s} This strain was formed by conjugating P-20^s with E coli J62 and introducing Sa-2 into the resulting transconjugant

CHARACTERISATION OF THE MOBILISED PLASMIDS

A J62(Sa-2, H1) transconjugant, now containing small plasmids + Sa-2, was used as a donor in a 5 minute and a 5 hour mating with E coli J53, to see if these smaller Sa-2-mobilised bands could be separated and characterised. Selection was made on DM plates with J53 supplements plus Tp, Cm or Tp and Cm. In addition , the same donor transconjugant was subcultured twice, before carrying out a 5 minute mating with E coli J53. The transfer frequencies of these matings are indicated in Table 3.2.

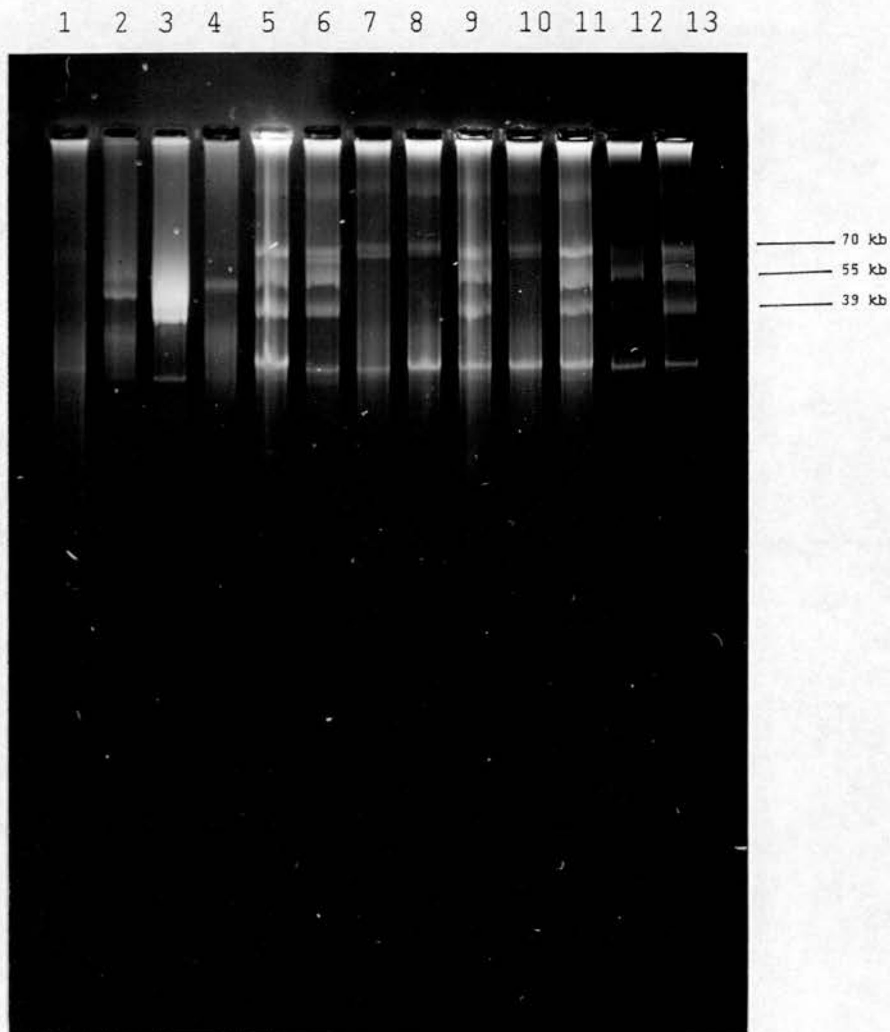
Examination of the resistance markers of transconjugants from these matings revealed three types of colony. The predominant colony type from all three transfers was resistant to Ka, Sm, Sp, Cm and Tp, whilst the 5 minute mating after subculture produced one colony resistant to Tp and Tc only, and the 5 minute and 5 hour matings, prior to subculture, both generated one or two colonies resistant to Tp only

Examination of the DNA of representative transconjugants from matings, prior to subculture (Figure 3.10), indicated that all strains possessed a large plasmid of approximately 70 kb, a small plasmid of approximately 4 - 5 kb and a very much smaller plasmid. Those expressing Cm, Ka, Sm, and Sp resistance also had plasmid bands characteristic of Sa-2. Examination of DNA of transconjugants from the transfer of subcultured J62(Sa-2, H1) to E coli J53, (Figure 3.11), indicated that, whilst most samples possessed bands indicative of Sa-2, and the large and small plasmids of the H1 family of transconjugants, one colony (Track 11) possessed only a very small plasmid band. This band corresponded to the small plasmid of the original pig isolate, P20, and since this transconjugant was found to be Tp resistant, there is the suggestion that this band may carry an additional Tp resistance gene.

TABLE 3.2: TRANSFER FREQUENCIES FROM MATINGS BETWEEN E COLI J62(Sa-2, HI) AND E COLI J53

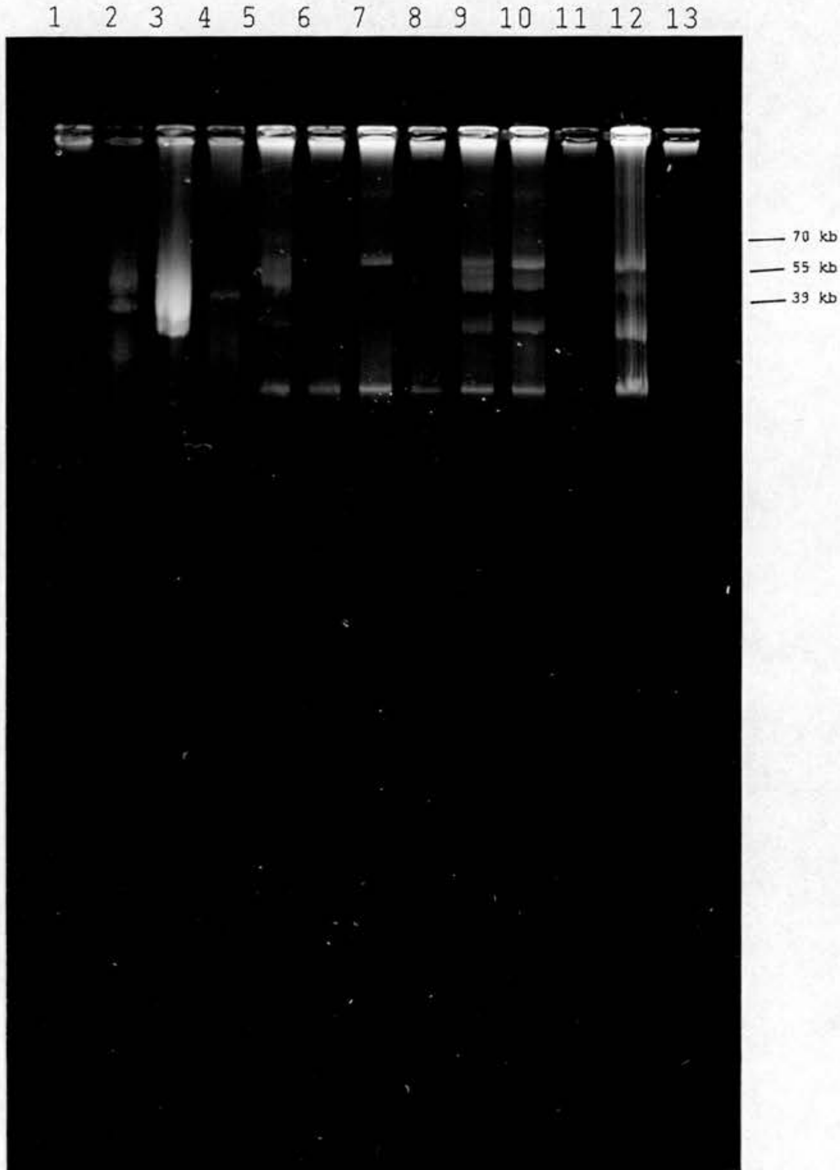
TRANSFER TIME	TRANSFER FREQUENCY PER DONOR CELL AFTER SELECTION ON		
	Tp	Cm	Tp Cm
5 minutes	1.60×10^{-4}	1.09×10^{-4}	5.38×10^{-7}
5 hours	8.60×10^{-2}	6.46×10^{-5}	1.07×10^{-6}
5 minutes (after subculture)	2.39×10^{-4}	1.47×10^{-4}	2.21×10^{-6}

FIGURE 3.10: AGAROSE GEL ELECTROPHORESIS OF TRANSCONJUGANTS FROM THE TRANSFER OF (Sa-2, H1) TO E COLI J53 (WITHOUT SUBCULTURE)



TRACK						
1. R1	5.			9.		
2. R1010	6.	5 hour		10.	5 minute	- Tp
3. R6K	7.	transfer	- Tp only	11.	transfer	
4. R751	8.		- Tp only	12.		
				13.		

FIGURE 3.11: AGAROSE GEL ELECTROPHORESIS OF TRANSCONJUGANTS FROM THE TRANSFER OF (Sa-2, H1) TO E COLI J53 AFTER SUBCULTURE



TRACK 1. R1
2. R1010
3. R6K
4. R751
5. Sa-2
6. Original Pig - P-20[F]
7. H5
8. H1
9.
10. 5 minute
11. transfer - Tc Tp only
12.
13.

CHARACTERISATION OF THE PIG ISOLATE PLASMIDS BY TRANSFORMATION

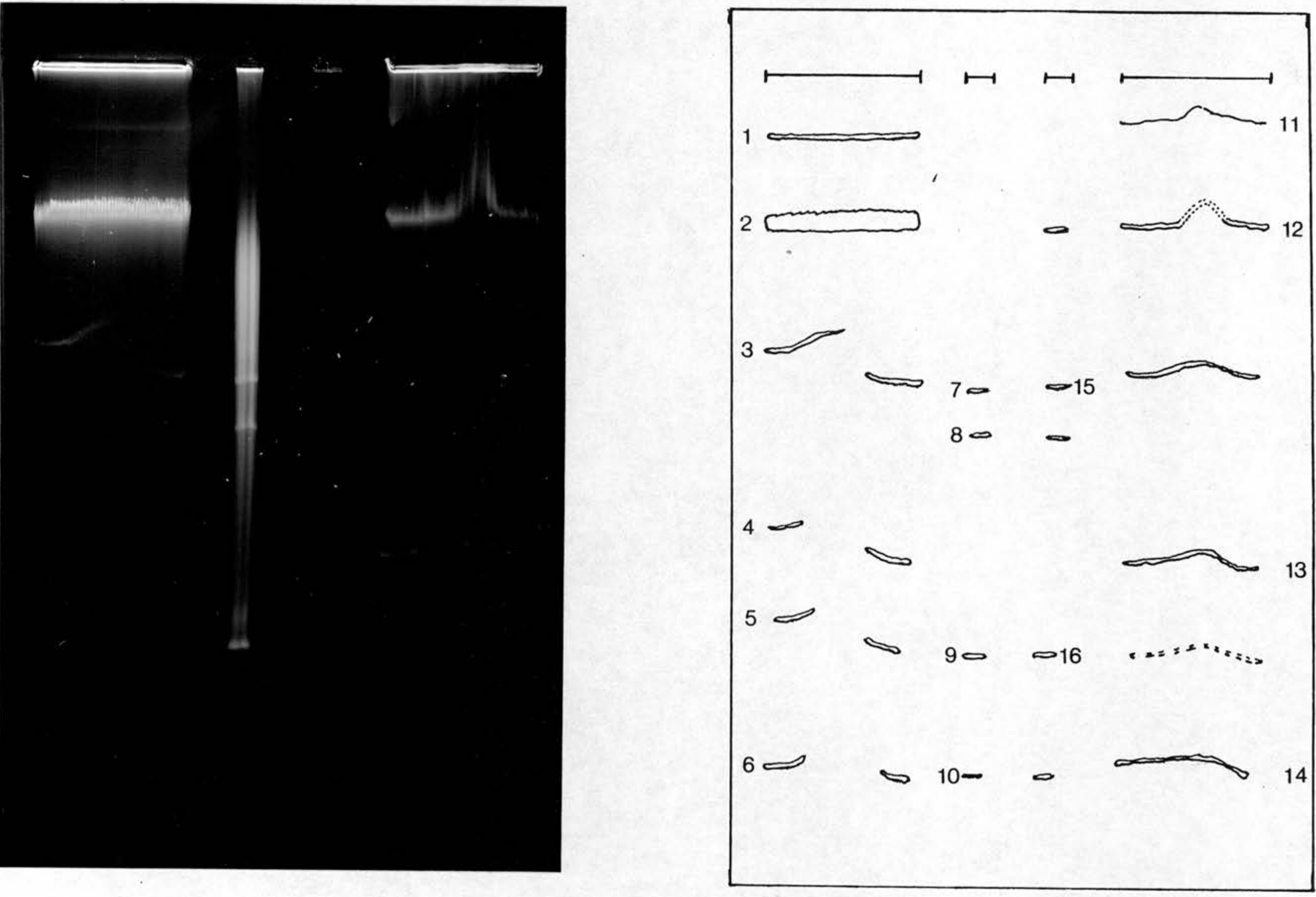
Due to the possibility that the smaller plasmid bands may be responsible for Tc and Ka resistance, and may also encode Tp resistance genes, further experiments to separate the individual bands were set up. C600 was transformed directly with DNA from the original pig isolates and also with DNA from individual plasmid bands eluted from an agarose gel.

Direct transformation with P20 DNA resulted in one transformant, resistant to Tp only. This transformant did not possess either the large or the small plasmids, thought to be responsible for Tp resistance, which have been found in the original pig strain and other transconjugants (results not shown).

Electroelution resulted in the extraction of 16 individual bands (Figure 3.12) and 30 ul of each sample was used to transform E coli C600, selecting for Tp and Tc. The resistances of purified colonies transformed with this DNA are given in Table 3.3. DNA was isolated from 8 different transformants resistant to Tc only, and compared with DNA from P20 to ascertain whether the plasmid band was the same size as that used to transform E coli C600. No plasmid bands were observed after electrophoresis at 70 v for 16 hours (Figure 3.13, suggesting that this piece of DNA may have inserted into the chromosome.

Further examination of transformants substantiated the view that the plasmids of the original pig isolate were unstable (Figure 3.14). Transformants 1, 6 and 7 (Figure 3.14: tracks 4, 7 and 8 respectively) were all transformed with DNA from single bands of differing sizes, yet each appears to possess a band of identical size. This may correspond to a plasmid band in the original pig isolate (Tracks 2 and 3) although it's position is indicative of chromosomal DNA. These three transformants are

FIGURE 3.12: AGAROSE GEL ELECTROPHORESIS OF LARGE AND SMALL SCALE PREPARATIONS OF THE P-20^r AND P-20^s ISOLATES



Numbers represent plasmid bands extracted from the gel

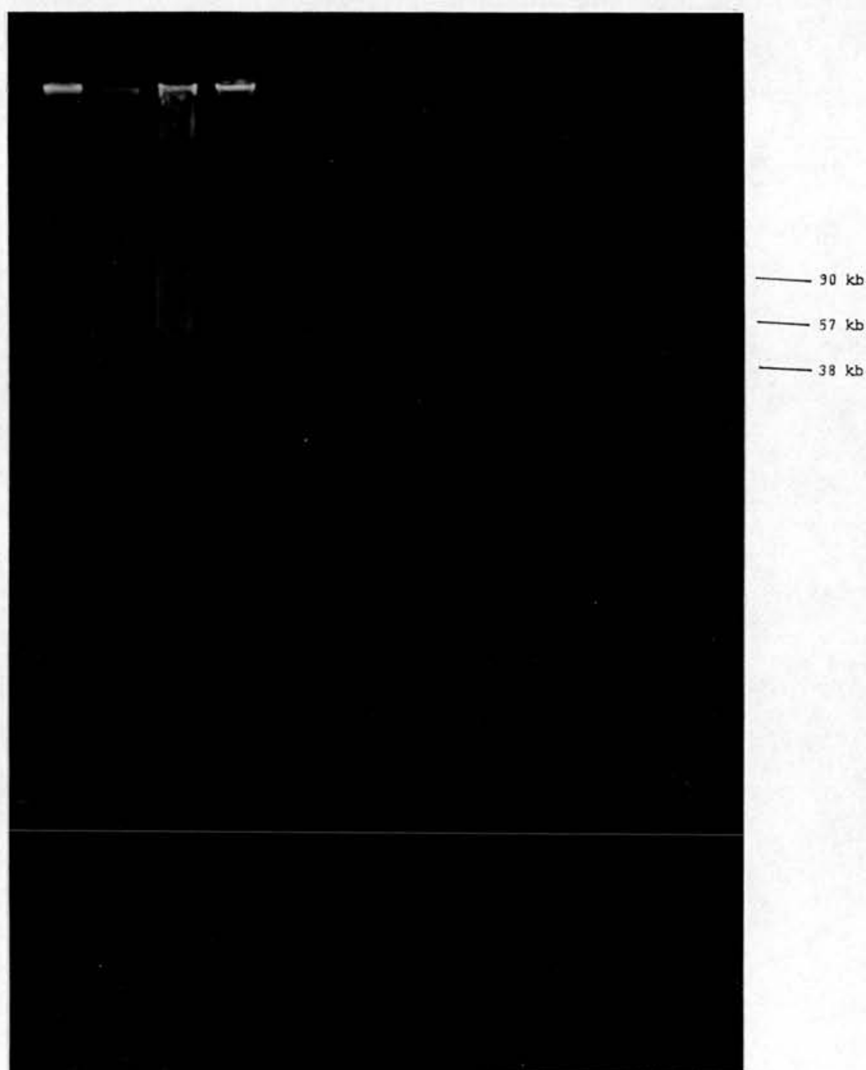
TABLE 3.3: RESISTANCE MARKERS OF TRANSFORMANTS

PLASMID* BAND NO	PIG ISOLATE	DNA PREPARATION METHOD	Tp	Tc	Sm/Sp
1	P-20^{F}	small	-	+	-
2	P-20^{F}	small	-	+	
3	P-20^{F}	small	-	+	-
4	P-20^{F}	small	+	+	+
5	P-20^{F}	small	-	+	+
			+	-	+
6	P-20^{F}	small	-	+	
7	P-20^{F}	large	-	+	
8	P-20^{F}	large	+	-	-
			-	+	-
9	P-20^{F}	large	+	-	
10	P-20^{F}	large	+	-	-
			-	+	-
11	P-20^{S}	small	+	-	
12	P-20^{S}	small	+	-	
13	P-20^{S}	small	+	-	
14	P-20^{S}	small	+	-	
15	P-20^{S}	large	+	-	
16	P-20^{S}	large	+	-	

^{F} - Frozen
^{S} - Slope
* - numbers correspond to plasmid bands (Figure 3.12)

FIGURE 3.13: AGAROSE GEL ELECTROPHORESIS OF Tc RESISTANT TRANSFORMANTS

1 2 3 4 5 6 7 8 9 19 11 12

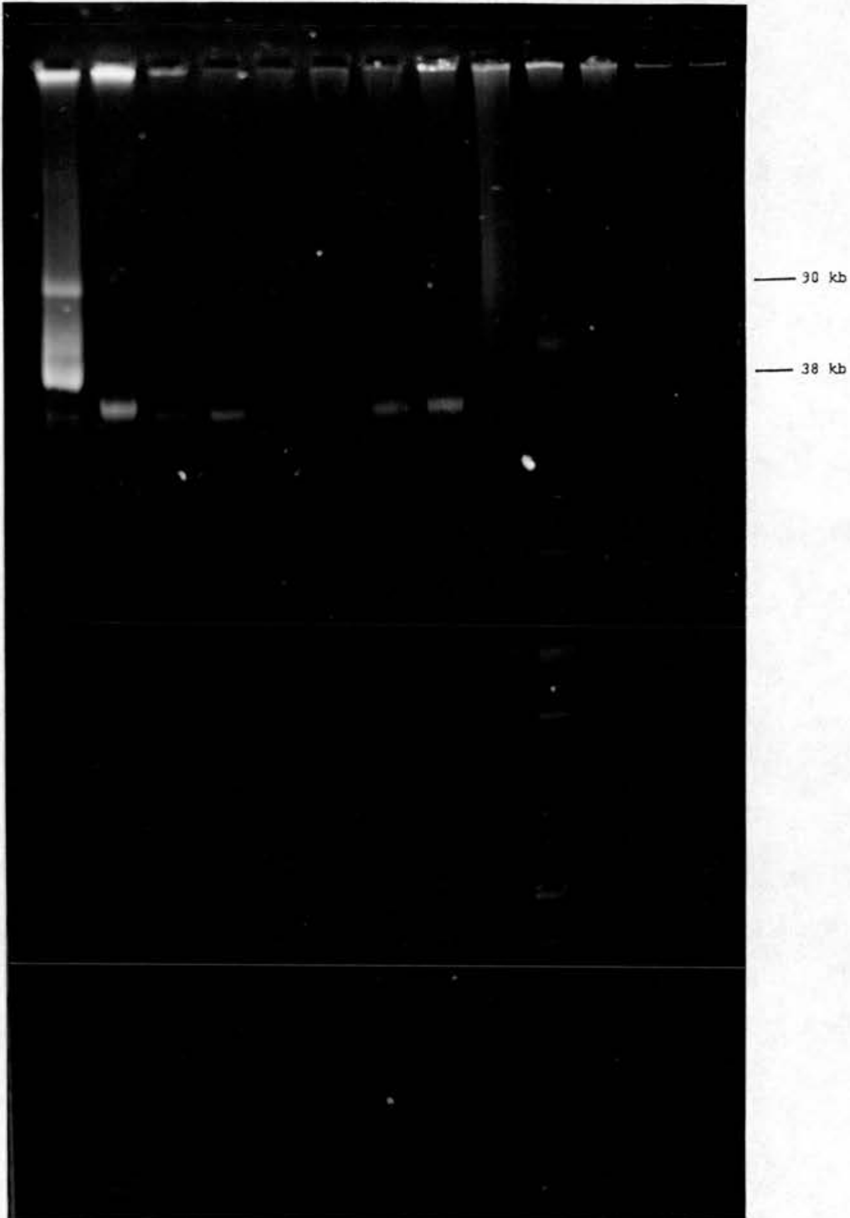


TRACK			
	1. R1	5. Tc 5	9. Tc 7
	2. RP4	6. Tc 6	10. Tc 9
	3. R6K	7. Tc 6	11. Tc 9
	4. P-20^{F}	8. Tc 7	12. Tc 10

Numbers refer to plasmid bands extracted from the gel

FIGURE 3.14: AGAROSE GEL ELECTROPHORESIS OF TRANSFORMANTS

1 2 3 4 5 6 7 8 9 10 11 12 13



TRACK

1. R1/R6K
2. P-20^{F}
3. P-20^{S}
4. T-1 Tc
5. T-4 Tc Tp
6. T-5 Tc
7. T-6 Tc
8. T-7 Tc
9. T-8 Tc Tp
10. T-9 Tp
11. T-10 Tc Tp
12. T-11 Tp
13. T-12 Tp

Numbers refer to plasmid bands extracted from the gel (Figure 3.12)

all resistant to Tc only, suggesting this plasmid band is responsible for expression of Tc resistance. Track 10 contains DNA from plasmid band no 9. Despite the extraction of a single plasmid band from the original gel, this transformant possesses atleast 7 discernable bands. The same result was obtained after transformation with plasmd bands equivalent to band 9 ie. band 16, indicating that this single band may have split up to give a mumber of smaller fragments.

DISCUSSION

It has been suggested for some time that there is a linkage between drug resistance in animal bacteria and clinical strains (Anderson et al, 1975; Bezanson et al, 1981; Mee and Nikoletti, 1983). Although there is some doubt as to the similarity and therefore relatedness of plasmids from animal and human sources, the levels of Tp resistance occuring in both environments follow similar trends (Amyes, 1987) and the transposons responsible for the resistance are very similar (Richards et al, 1978). The finding of both Tn7 and a smaller Tp resistance transposon, Tn4132, in both animal and human isolates from the Edinburgh area (Amyes, 1986) prompted the further examination of porcine isolates for evidence of the continueing evolution of the Tp resistance gene.

As with the majority of porcine isolates from the Edinburgh area (Amyes, 1987), P-20 expressed high level Tp resistance ($>$ 1000 ug/ml), normally indicative of Tp resistance plasmids or transposons (Towner et al, 1983). Despite the fact that only a small percentage of pig isolates from the survey of Amyes (1987) were shown to harbour Tp resistance plasmids and were thus able to transfer Tp resistance, initial examination of P-20 by agarose gel electrophoresis indicated an array of plasmids, and Tp resistance was found to be transferable. The largest of these plasmids was similar in size, 107 kb, to the previously isolated plasmid, pUK555 (Amyes, 1987), which was found to encode both Tp and Tc; the resistances expressed by P-20. (Subsequent molecular weight analysis revealed that this plasmid had a size range of 90 - 110 kb). Conjugation studies showed this large pUK555-like plasmid to be transferable to E coli J62-2 (after a

5 minute mating), resulting in a transconjugant (H1) resistant to Tp only. (For flow diagram of experimental stages in the characterisation of P-20 see Figure 3.15). The lack of the small plasmids, present in P-20, and the lack of Tc resistance suggested that this latter resistance marker was encoded by one of the smaller plasmids. Multiple bands were however, visible in DNA preparations of transconjugants H2, H3 and H4 from longer transfers (5 hours), but these additional bands were not indicative of the small plasmids of P-20^F. It is possible that they were fusion products of the smaller bands, a phenomenon described by Bennett et al (1986). However, this coupled with the observation that the large pUK555-like plasmid in these latter three transconjugants was of reduced intensity, suggests that the plasmids of P-20 may be unstable. Wilshaw et al (1979) have indicated the possibility of fragmentation, cointegration and other DNA rearrangements accompanying DNA transfer, and Berthold et al (1986) indicated the potential for rearrangments in the multiresistant plasmid pBP16.

This instability was further evidenced when the DNA of these transconjugants was analysed after storage. Whilst bands similar to those initially present in H2, H3 and H4, and therefore indicative of plasmid instability, were apparent, some of the transconjugants appeared to possess plasmids of a size just larger than the smaller bands of the original P-20^F isolate. These same bands also appeared in transconjugant H5, after storage, which was originally believed to have possessed only one plasmid. Their presence can not be accounted for by fragmentation of the approximately 100 kb large plasmid as this is still present; however, it is possible that these small plasmids may have arisen from the chromosome. They may therefore have the capability of integrating and reexcising themselves from the bacterial chromosome.

Stuy (1980) reported that the chromosomal integration of conjugative plasmids was common in antibiotic resistant Haemophilus influenzae. Their variability in size from those of the P-20^F isolate could be explained by the fact that excision is not a precise process (Kleckner, 1981) and may result in deletion and insertions. The fact that all five transconjugants initially possessed the large plasmid, irrespective of the number or size of smaller plasmid bands also present, would suggest that the absence of these latter plasmids in transconjugants H1 and H5 could be a result of their integration into the chromosome. Their existence and potential for mobilisation from the chromosome is exemplified by their reappearance in transconjugant H1 after the introduction of Sa-2. The sizes of these bands, whilst different from those of P-20^F, were similar to those in the initial transconjugants after storage, indicating the likelyhood of aberrant excision from the chromosome. An attempt to qualify the hypothesis that these small plasmids were integrated into the chromosome of transconjugants H1 and H5 , by carrying out transfers from P-20 to a recombinant deficient strain, was unsuccessful .

The variability in plasmid size and number is not confined to E coli J62-2 transconjugants: repeated examination of the P-20 isolate stored under different conditions indicated similar anomalies. The appearance of an additional plasmid band, probably encoding Tc resistance in P-20^F as compared with P-20^S, could be accounted for by contamination on storage. (After collection isolates were initially stored on slopes before transfer to - 70°C.). However, subsequent reexamination of DNA revealed that this band was not always absent in the P-20^S isolate (although Tc resistance was never expressed), indicating the possibility for movement of the smaller plasmids in and out of the chromosome. The effects of

media on the plasmid profiles of P-20^S and P-20^F, while difficult to interpret, suggest that growth medium may affect plasmid stability. The effect of medium, in particular thymidine, on bacterial sensitivity to Tp is well documented (Koch and Burchall, 1971; Amyes and Smith, 1974; Amyes and Smith, 1978a,b; Escamilla _et al_, 1986). The apparent loss of the large pUK555-like plasmid after growth of P-20 in Isosensitest broth, which lacks thymidine - a potent antagonist of Tp and sulphonamide action (Amyes and Smith, 1976) - and its reappearance in the presence of thymidine, could be explained in a number of ways. This large plasmid may require thymidine for its replication and stable maintenance, and therefore the lack of it in isosensitest broth, whilst not preventing the growth of the bacterial host, would after a number of generations, lead to the loss of this plasmid. Alternatively, the P-20 culture may consist of two populations of cell; one harbouring this large plasmid and one lacking it. The absence of thymidine in the culture media may preferentially select the strain lacking this plasmid which will then become dominant in the culture. This latter hypothesis is favoured by subsequent repetitions of the above experiment which indicated a marked reduction in the number of copies of this large plasmid in P-20^F cultures grown in isosensitest broth, not a total loss. The fact that this result was not mirrored in the retesting of the P-20^S culture is not clear, but may further indicate the unstable nature of this system and the coincidental loss of the large plasmid from cultures grown in Isosensitest broth, as opposed to any metabolically determined loss.

Characterisation of the individual plasmids of P-20, to determine the location of resistance markers, proved difficult. The additional expression of Ka resistance, and occasionally Sm/Sp resistance, by H2, H3 and H4 could be accounted for by the presence of the additional bands in

these three transconjugants. Alternatively the splitting up of a plasmid, leading to DNA rearrangements, or just its transfer to another strain could result in derepression of resistance genes and their subsequent expression. Differential expression of resistance genes in different organisms has been reported by Smith (1969), who found that the level of expression of penicillinase in P mirabilis was one twentieth that in E coli K12. It is known that pieces of DNA can act as novel switches by integration and excision (Kleckner, 1981) and this could explain the absence of Tc resistance in transconjugant H1 and its reappearance, along with the small plasmid bands, on the introduction of Sa-2. Alternatively, the Tc resistance determinant may be capable of conjugal transfer in the absence of a plasmid, in a similar manner to that reported in Streptococcus faecalis (Franke and Clewell, 1981). However, continued analysis of resistance markers of P-20 and its transconjugants indicated that their presence was generally irrespective of plasmid profile: the appearance of the smaller bands was not always accompanied by expression of resistance genes.

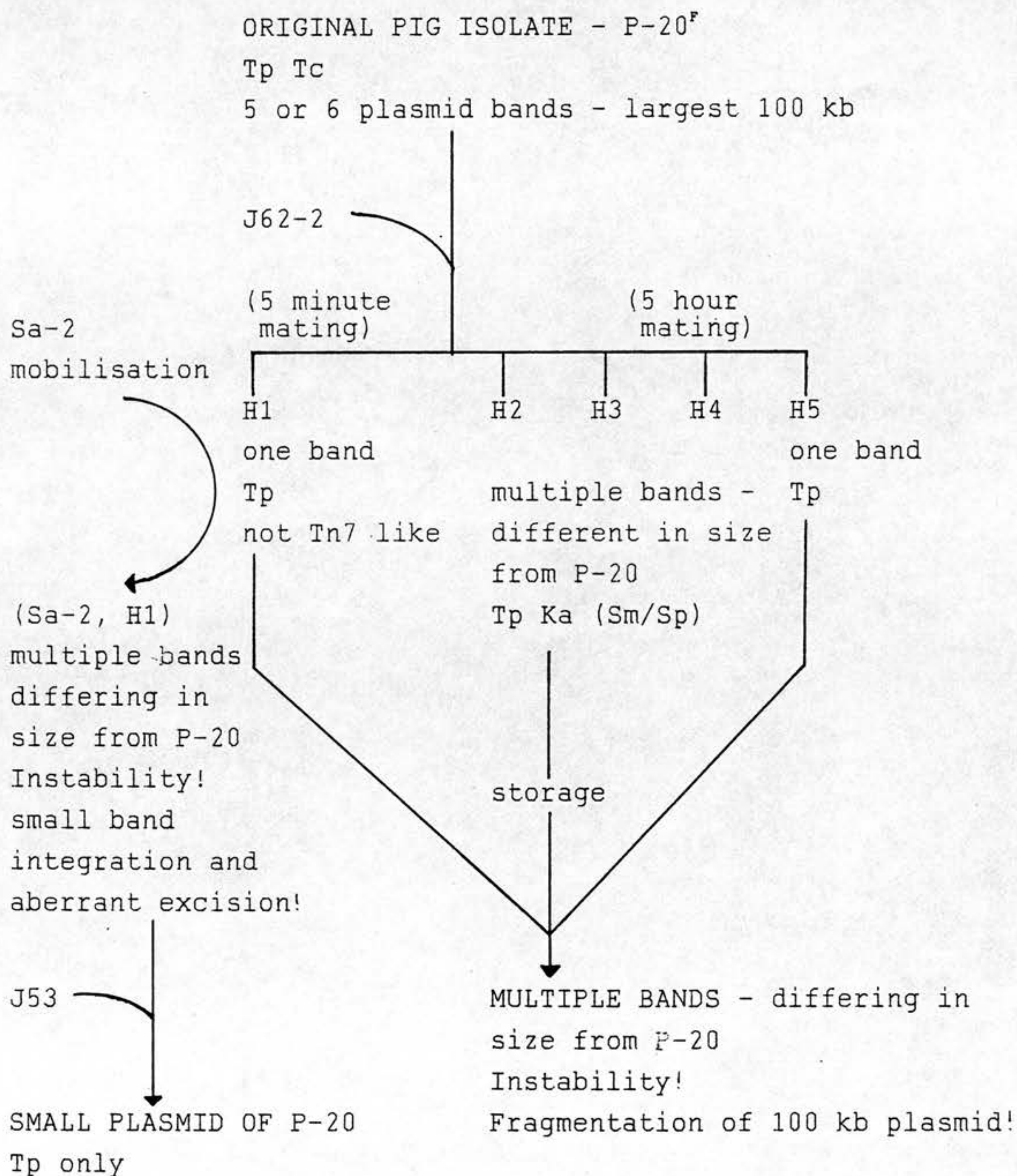
Although conjugation studies (after mobilisation with Sa-2) to separate individual plasmid bands indicated that the smallest plasmid band of P-20^{F} may encode a Tp resistance gene, the carriage of Tp resistance by the large PUK555-like plasmid can only be assumed. (The finding of Tp resistance on a small non conjugative plasmid was suggested by Kraft et al, 1983). Restriction enzyme analysis indicated that the large plasmid did not carry a Tn7-like transposon, as the two characteristic internal Hind III fragments of Tn7 were missing. The results, however, do not rule out the possibility of carriage of other Tp transposons. Alternatively, Tp resistance may be due to a plasmid encoded resistance gene, similar to that of R388 (Amyes and smith,1976). The ability of the small plasmids to

integrate into the chromosome (or another plasmid) coupled with the continued expression of any resistance markers once integrated, hampered any analysis of the determinants of the pUK555-like plasmid. (Strains apparently harbouring the large plasmid only, could owe their Tp resistance to a gene on this plasmid or to chromosomally located genes, as a result of the integration of the small plasmids). The shift of resistance genes, notably Tp resistance, from resistance plasmids to the bacterial chromosome is not uncommon. Recent investigations have indicated an increase in high level non self transferable Tp resistance in both porcine and clinical strains (Towner, 1981; Steen and Skold, 1985; Amyes, 1987). Studies with transformed DNA were unable to clarify the situation, but only reiterated the capabilities of the small plasmid bands to integrate, and the instability of the system. No plasmid DNA was isolated from Tc resistant transformants, and E coli C600 bacteria, transformed with a single plasmid band, carried a diversity of plasmid bands.

In conclusion it would appear that the original pig isolate consists of one large plasmid of approximately 90- 110 kb similar to pUK555 and 3 to 4 smaller plasmids which are unstable and capable of integrating into the chromosome (or another plasmid) and possibly fusing together. These plasmid insertions into the chromosome may be mediated by transposons or by other insertion sequences. These integrated forms can be excised from the chromosome by mobilisation (with Sa-2) or by conjugational transfer, but their excision may not be precise. Regions of chromosomal DNA may be excised with the original insert, leading to plasmids of slightly larger size than those of the original P-20 isolate. The expression of resistance markers carried by these smaller replicons, ie Tc, may or may not be affected by this integration. A transposon (or transposons) may

provide the region of homology required to link some of these smaller plasmids together, giving rise to the observed variation in band size. The intermittant expression of Sm/Sp along with Tp suggests the presence of a Tn7-like transposon. Richards and Nugent (1979) reported that the presence of the Tp resistance gene in a wide range of bacterial groups came about by the dissemination of the Sp/Sm Tp transposon, Tn7, and the finding of Tp resistance within P-20 may be due to a similar transposon. Tp resistance would appear to be encoded by the smallest of the P-20 plasmid bands, and probably also by the largest plasmid, although this latter resistance, if present, is not due to a Tn7-like transposon. The location of the Tc resistance gene is uncertain although its presence on many of the small bands used for transformation could be explained by multiple transposition.

FIGURE 3.15: FLOW DIAGRAM ILLUSTRATING THE EXPERIMENTAL STAGES IN THE CHARACTERISATION OF THE TRIMETHOPRIM RESISTANCE OF P-20



CHAPTER 4

CHARACTERISATION OF THE TRIMETHOPRIM RESISTANCE TRANSPOSON Tn4135

INTRODUCTION

Insertion sequences (IS) and transposons, along with the transposing phages Mu and D108 (class III), are collectively known as transposable elements, and were first described by Mclintock in 1951. Transposons are discrete pieces of DNA that can insert at many different, non-homologous sites in the genomes of prokaryotic and eukaryotic cells, promoting deletions, inversions and fusion of replicons (Galas and Chandler, 1981; Shapiro, 1983). According to Kleckner (1981), transposons can generally be divided into two main classes: class II elements, like Tn*3* and Tn*21*, with short inverted repeats at their ends, and composite class I transposons which carry IS elements at their flanks, in either direct or inverted orientation, such as Tn*9*, Tn*10*, Tn*5* or Tn*903*. IS sequences only encode determinants relevant to their own transposition (Kleckner, 1981) whilst transposons encode functions such as antibiotic and metal resistance, enterotoxin synthesis or novel metabolic enzymes favourable to the host under certain environmental conditions. These latter elements are larger (2.5 kb to 40 kb) than IS elements ($\leq$ 2 kb) (Schmitt *et al*, 1985) and are therefore able to carry sufficient genetic information for several additional proteins.

Current interest in transposable elements can be attributed to (i) their ubiquity (ii) the novel DNA sequence recognising, cleavage and joining reactions that their movement entails (iii) the insertion mutations, genome rearrangements and changes in gene expression they cause (iv) their role in genome evolution and (v) their usefulness in the genetic analysis and manipulation of many different organisms (Berg *et al*, 1984).

Considerable attention has focused on the structure of transposons in order to understand the DNA rearrangements generated by transposition (Schmitt et al, 1979; Berg, 1983; Arthur and Sherratt, 1979) and their evolution. Three structural features are common to essentially all transposable elements: (i) they have inversely repeated ends (IR) of up to 40 bp (Ptashne and Cohen, 1975; Berg et al, 1975; Heffron et al, 1975; Kopecko and Cohen, 1975) eg. the IR of Tn7 are 28 bp in length (Gosti-Testu and Brevet, 1982); (ii) these IR's flank a central region containing transposition genes and, for transposons, additional determinants (eg drug resistance); and (iii) upon transposition, they generate a short duplication of target DNA that flanks the inserted element as direct repeats (Kleckner, 1981; Schoffl et al, 1981). For the Tn3 family the transposition genes include tnpA, the structural gene for the transposase enzyme, and tnpR, the resolvase enzyme (Heffron et al, 1979; Altenbuchner and Schmitt, 1981). However, work by Hassan and Brevet (1983) and Hauer and Shapiro (1984) suggest that Tn7 does not have a resolvase activity analogous to that encoded by members of the Tn3 family.

The inverted orientation of the repeats found at the ends of most transposons, possibly constituting recognition sites (Berg et al, 1984), was thought to be essential to transposition. Transposons have, however, been isolated with repeats in a direct orientation eg. Tn1525 (Labigne-Roussel et al, 1983), Tn2680 (Iida et al, 1982) and Tn2440 (Nies et al, 1985). In most instances where transposons have been flanked by direct repeated copies of IS elements, IS1 has been identified as the element providing the transposition functions (Nies et al, 1985). However, since IS sequences themselves terminate in inverted repeats, a transposon flanked by direct repeats of an IS element will still be flanked by inverted repeat sequences.

The mechanisms by which transposition takes place are complex and vary with transposon family. The Tn3 family (Kleckner, 1981) transpose through the obligatory formation of a cointegrate structure. However, Ouartsi et al (1985) suggested that the transposition of Tn7 was different, as no cointegrates could be found. Other transposons encoding trimethoprim resistance may also fall into this latter category, depending on their evolutionary origins. Since there is no in vitro system for studying transposition, models of the molecular mechanisms must rely on interpretation of in vivo experiments and they therefore remain rather speculative. Two mechanisms have been postulated to intepret the experimental data: conservative transposition and replicative transposition. Conservative transposition probably results from a concerted set of reactions that include recognition of the element and target DNA by transposase, cleavage of the target, and cutting of one or both strands at each element-vector junction. Tn5, Tn10, IS elements and a number of moveable elements of eukaryotic cells appear to transpose in this way (Berg, 1983). Replicative transposition, as illustrated by the Tn3 family, involves cutting one or both ends of the transposon, replication of the element, and fusion to the donor and target molecules at the site of insertion. A copy of the transposon appears at each junction between the donor and target DNA (Kleckner, 1981). This cointegrate structure is stable in recA cells unless an element-encoded protein, resolvase, is present. The site, res, at which resolvase acts to catalyse site specific recombination, is located between the two divergently transcribed genes involved in the transposition reaction (Altenbuchner and Schmitt, 1981). Resolution of the cointegrate by site specific recombination results in donor and target both with a copy of the transposon (Bennet et al, 1976; Feenegald and Shapiro, 1979; Klaer et al,

1980; Read et al, 1980; Reed, 1981). It was often assumed that all transposition was replicative: the evolutionary argument for this being the concept that transposons were selfish pieces of DNA driven to develop transposition mechanisms to increase their copy number relative to all other genomic sequences (Sapiena and Doolittle, 1981). Two alternative models, symmetric and asymmetric, have been proposed for replicative transposition; depending on the use of both IR's simultaneously, or on a processive mode starting at one IR (Hershey and Bukhari, 1981). The findings that only one of the IR present may be active at any one time, that one end is more competent at initiating transposition than the other, possibly because of unequal binding of transposase to either end, and that truncated single ended derivatives of Tn1721 and Tn21 (Motsh and Schmitt, 1984, Schmitt et al, 1984) can still transpose, all favour the model of as ymetrical replicative transposition. The latter finding was contrary to the work of Heffron et al (1979); Foster et al (1981a) and Isberg and Syvanen (1981), who indicated that deletion of one or both ends produced a non-complementable transposition defect. Galas and Chandler (1981) suggested that the original models proposed by Shapiro (1979) and Arthur and Sherratt (1979) were inadequate to explain the behaviour of some transposons. They therefore proposed a rolling circle type model to accommodate all known properties of the transposition process, including the apparent dichotomy between the conservative and replicative pathways.

The ubiquitous behaviour of transposons is well documented, but this potential for spread poses problems for those trying to combat the dissemination of bacterial drug resistance. Not all replicons, however, can act as transposon acceptors (Barth et al, 1976) and not all transposons posess the same specificity of insertion. Tn3 possesses a low specificity of insertion (Rubens et al, 1976), and is thus capable of

inserting into the plasmid RSF1010 at a minimum of 12 distinct sites. In contrast, Tn7 can only insert once into the chromosomes of E coli (Barth et al, 1976; Lichtenstein and Brenner, 1981, 1982) and of Caulobacter crescentus (Ely,1982) and inserts preferentially into the ilv gene cluster of the chromosome of Vibrio species (Thomson et al, 1981). This limited potential of Tn7 to insert into chromosomal sites may prevent any increase in trimethoprim resistance by multiple transposition, but may also preferentially select for the evolution of gene amplification as an alternative means of increasing resistance. However, Tn7 does have the potential to insert into many plasmid sites (Fennewald et al, 1979), notably RP4 (Barth and Grinter, 1977). The specificity of Tn7 insertion is also different from other transposons, in that Tn7 always transposes into a particular replicon in the same orientation (Barth and Grinter, 1977; Moore and Krishnapillaie, 1982; Lichtenstein and Brenner, 1981). Although there are many potential sites of insertion for a transposon (Cohen, 1976, Cohen and Kopecko, 1976; Kleckner, 1977) the distribution of these sites is not random: there are often regions with many sites ('hot spots') and other regions where the frequency of transposition is much lower (Grinsted et al, 1978). Thus although Tn5 inserts into many sites some regions are preferential (Berg et al, 1984). Such regional specificity has been shown for the insertion of Tn10 into the lacZ gene (Foster,1977), of Tn3 into the R plasmid R6-5 (Kretschmer and Cohen, 1977), of Tn2 into Col E1 (So et al, 1975), of Tn1 into Col E1 (Dougan and Sherratt, 1977) and of Tn7 into RP4 (Barth and Grinter, 1977). This specificity of insertion may occur through recognition of some relatively common, but specific DNA sequence, although there is the suggestion that selection depends solely on the specificity of transposase for target DNA and not on the sequence of the vector. Base pairs at the ends of

duplicated segments may play a role. Although a particular sequence of DNA may have potential sites for the insertion of a particular transposon, it does not follow that that sequence will be used for transposition at high frequency. Other factors such as transposition immunity may influence transposition. This phenomenon was defined by Robinson *et al* (1977) as the inability of a plasmid already carrying one copy of a transposon to acquire a second one by transposition. It has been described for Tn*3* (Robinson *et al*, 1977), Tn*501* (Stanisch *et al*, 1977) and Tn*1721* (Schmitt *et al*, 1984) but Tn*10* (Bennet *et al*, 1977) and Tn*7* (Hassan and Brevet, 1983) have not been shown to exhibit transposition immunity. Transposon immunity does not result from the instability of plasmids carrying two copies of Tn*3* (Robinson *et al*, 1977), as plasmids with two copies have been isolated. It is the inability of a plasmid to obtain copies of the same plasmid sequentially, as opposed to together. Immunity takes time to establish and it is this delay in the establishment of the first Tn*3* which allows the transposition of a second Tn*3* to occur at low frequency. The molecular mechanisms involved in this process are unclear. Insertion frequencies may also be affected by the size of sequence between the inverted repeats (Chandler *et al*, 1982). The observation that the 11 kb tetracycline resistance region of the plasmid N3 does not transpose at a detectable frequency ($<$ 3x 10^{9}; Brown *et al*, 1984) is consistent with this view. However, Ouartsi *et al* (1985) report that the transposition frequency of Tn*7* derivatives is independent of the size of the transposable DNA. (Derivatives from 8.4 to 23 kb were analysed).

Much interest has centred arround the evolution of transposons but it is not known whether these elements evolved in bacteria or were transmitted to them from another type of organism. They may have evolved as natures tool for genetic engineering, because of their ability to

rearrange other DNA sequences, or the reason for their existance may lie solely in their ability to overreplicate in a host. Schmitt et al (1984) have looked at possible ways in which genes could have been incorporated in to transposons. The finding that transposons were flanked by IS sequences (Ptashne and Cohen, 1975) suggested that transposons were the end result of insertion of two identical IS sequences bracketing a sequence of DNA, which contained in part, an antibiotic resistance gene (Rubens et al, 1976). Chromosomal (non-transposon) markers could thus be flanked by duplicate copies of an IS element in direct or inverted orientation. Such composite elements could subsequently undergo modification, by deletion or point mutation, to eliminate unnecessary or deleterious portions and to refine the expression and regulation of the determinants required for transposition, and accessory genes. However, Tn3 was found to be flanked by very short IR (140 bp) which were found not to correspond to any known IS sequence (Rubens et al, 1976). It is possible that Tn3 was initially flanked by much larger inverted repeats which have subsequently become deleted, leaving only a 140 bp region. The time scale in which these steps have shaped present-day transposons is not known. In view of the highly efficient recombination mechanisms that govern the incorporation and rearrangement of resistance markers, these processes can be rapid and it is conceivable that such events may have occurred recently and are still active in shaping new transposons.

Just as transposons are thought to have evolved by the occurance of IS sequences flanking potentially useful genes, so it is reasonable to presume that bacterial genomes have evolved by transposition of a transposon, encoding drug resistance, onto a plasmid lacking any other resistance markers. Transposons can alter both the organisation and the expression of prokaryotic genes at a frequencey equal to or greater than

mutation events affecting a single or a few base pairs. They are capable of affecting the expression of neighbouring genes by virtue of the transcriptional start and stop signals they carry (Calos and Miller, 1980; Berg et al, 1980) eg insertion of Tn3 at a site adjacent to the origin of replication causes an increase in plasmid copy number (Rubens et al, 1976). Transposon induced alterations can occur in several ways (Kleckner, 1981; Schmitt et al, 1985). Insertion of a transposable element into a gene is an immediate event, frequently resulting in the inactivation of the gene. It has been reported (Chao and Mcbroom, 1985) that insertion of a transposon (in this case Tn5 or Tn10) can infer a selective growth advantage on E coli in chemostat competition, possibly by enhancing the mutation rate of the host bacterium, thus increasing the chances of a favourable mutation. The evolutionary role of transposons as mutator genes is more appealing than allocating them the role of parasitic DNA (Sapienza and Doolittle, 1981). The insertion of a transposon is a precise mechanism with identical sequences inserted each time and generally with no loss of recipient DNA: although small deletions have been reported after transposon insertion into RP4 (Barth et al, 1978; Datta et al, 1979; McCombie et al, 1983). Excision on the other hand is rarely precise and often results in the deletion of DNA. Excision of Tn5 and Tn10 has been examined in some detail (Botstein and Kleckner, 1977; Berg, 1977; Ross et al, 1979; Foster et al, 1981b; Egner and Berg, 1981). Most if not all excision by these elements occurs by pathways which are independent of recA function and of transposon encoded functions. Two elements inserted in opposite orientation into a chromosome, frequently leads to inversion of intervening DNA and duplications occur when two elements flank the region of interest in direct orientation. Translocation can occur when a DNA fragment is flanked by two identical

transposons and the whole unit acts as a compound transposon. It is therefore possible for transposons to take adjacent chromosomal or plasmid genes across with them to recipient plasmids. It is because of this ability of transposons to rearrange DNA sequences in a variety of ways that interest has focused on them as in vivo genetic engineering tools (Kleckner et al, 1977).

The accretion of useful genes, such as antibiotic resistance genes (Campbell, 1981; Hartl et al, 1984), by transposons on plasmids adapted to a particular host, is likely to be a continous process leading to widespread dissemination of generally useful genes. Transposons provide an efficient mechanism for incorporating resistance determinants into new vectors which can transfer to and stably replicate in diverse hosts.

Many different antibiotic resistance genes have been found to reside on transposons eg. Ap (Hedges and Jacob, 1974; Heffron et al, 1975; Kopecko and Cohen, 1975; Bennett and Richmond, 1976), Tc resistance (Kleckner et al, 1975), several forms of Ka resistance (Berg et al, 1975) and Cm resistance (Gottesman and Rosner, 1975), so the implication of these genetic elements in the carriage of Tp resistance genes is not suprising. The most extensively studied Tp resistance transposons have been Tn7 (Barth et al, 1976) and Tn402 (Shapiro and Sporn, 1977). Tn7, isolated from R483, is a 12 - 14 kb transposon (Barth et al, 1976; Gosti-Testu et al, 1983) mediating resistance to streptomycin/ spectinomycin, as well as Tp, and has accounted for a large proportion of the high level, non-transferable resistance to Tp. Tp resistant bacteria, isolated since 1978 in Finland (Fling et al, 1982; Elwell et al, 1979), Sweden (Steen and Skold, 1985) and the United Kingdom (Datta et al, 1979; Richards et al, 1978; Towner et al, 1982; Richards and Nugent, 1979), owed their Tp resistance to chromosomal or plasmid located Tn7. Tn402, on the

other hand is smaller than Tn7, 7 kb, and is resistant to Tp only. It has not been transposed from other specimens except R751 and there is no genetic evidence for relatedness to Tn7, although some early studies on enzymic properties have indicated that Tn402 and Tn7 were related. One transposon may therefore be the ancestor of the other as suggested by Shapiro and Sporn (1977). In addition to these two transposons mediating Tp resistance, transposons that are identical to Tn7 - Tn1527 (Goldstein et al, 1986), Tn1824 (Tietze et al, 1982), Tn71 and Tn72 (Barth and Datta, 1977a) and Tn79 and Tn80 (Richards and Nugent, 1979; Palenque et al, 1983) - and transposons that have evolved from Tn7 - ie. Tn78 (Datta et al, 1981) - have been described. Preliminary evidence suggested that the Tp resistance gene of R388 may also reside on a transposon (Amyes and Smith, 1977), although this, and also whether Rss28 and Rss42 (Amyes and Smith, 1978) contain transposons, has not been proven.

The majority of Tp resistance transposons so far isolated have encoded linked Tp/Sp resistance and only a few have been found to be resistant to Tp only ie. Tn402, Tn78 and transposons from four clinical E coli isolates in the Nottingham area (Towner et al, 1982). These latter transposons were thought to be related to Tn78 (which may in turn be related to Tn7), as they were capable of transposing to other plasmids and the bacterial chromosome as efficiently as Tp/Sm encoding transposons. In 1983, an additional transposon, Tn4132, encoding Tp only, was isolated from the Edenhall Hospital, near Edinburgh during a three year survey (Young and Amyes, 1983). This transposon, like Tn402, was found to be smaller than Tn7 (approximately 3 kb) but studies of its gene product (Young and Amyes, 1985a) suggested it was related to Tn7.

In a concurrent study of enterobacteria in farm animals, Tp resistance transposons were isolated from porcine strains (Amyes, 1987) by

mobilisation with the Inc P plasmid, RP4. The ubiquitous Tn7 was prevalent and a few strains (ie. original pig strain P-20) carried a smaller Tp only transposon. This smaller transposon, Tn4135, was thought to have evolved from Tn7 by deletion of the region encoding Sm/Sp resistance. In order to ascertain the relationship between this smaller transposon and Tn7, its properties were studied, initially in the RP4 plasmid background.

MATERIALS AND METHODS

TRANSFER OF TRANSPOSONS BETWEEN PLASMIDS

In order to transpose a gene from one plasmid to another, the following approach was adopted. A suitable strain was selected which contained the donor plasmid harbouring the transposon. A recipient plasmid was introduced into this bacterium and selection was made both for a genetic marker on this recipient plasmid and a marker on the transposon. After selection and in order to promote transposition the transconjugant containing both plasmids was subcultured twice by transferring one colony into 10 ml of nutrient broth, incubating overnight at 37°C and transferring 0.01 ml of this to another 10 ml of nutrient broth. This culture was incubated overnight at 37°C, before streaking out on the same selective plates as above. A single colony was used as a donor in a standard mating with a plasmid-free strain; selecting for the genetic marker on the transposon-recipient plasmid, the genetic marker on the transposon and the two markers together. Transconjugants from this mating were subcultured twice and restreaked on the same selective plates. If necessary the plasmid and transposon were transferred back into a suitable bacterial strain in a standard mating. All transconjugants were tested for unselected markers to confirm that the recipient plasmid had indeed only picked up the markers of the required plasmid and transposon.

RESISTANCE TESTING

The procedure used to test bacteria for their resistance to various drugs has been described in Chapter 3.

DETERMINATION OF TRIMETHOPRIM MIC's

Overnight 4.5 ml nutrient broth cultures were diluted 10^{-4} in DM base, to give approximately 10^5 organisms per ml, and multiply inoculated on to DSTA plates containing doubling concentrations of Tp (from 2.5 ug/ml to 1280 ug/ml).

PLASMID DNA PREPARATION AND RESTRICTION

Plasmid DNA was prepared by the method of Takahashi and Nagano (1984) and analysed by agarose gel electrophoresis, as described in Chapter 1. Single digests were performed with *Hind* III (9u), *Bam* HI (6u), *Pst* I (12u) and *Pvu* I (4u) and a double digest was carried out in *Hind* III buffer (NBL Enzymes Ltd) with *Hind* III and *Pvu* I. The restriction procedure was the same as that used in the restriction mapping of Sa-1 and Sa-2 (Chapter 2). Molecular sizes of the smaller fragments were estimated by comparison with those obtained for lambda DNA following digestion with *Hind* III (Murray and Murray, 1975).

RESULTS

TRANSFER OF Tn7 FROM R483 TO RP4

For comparative purposes, Tn4135 and Tn7 were required in the same plasmid background. Therefore Tn7 was transferred to RP4 using the exclusion property of Proteus mirabilis NC6197 toward the Inc Ia plasmid R483. No Inc Ia plasmids are maintained within P. mirabilis, therefore by conjugating this species with a culture of E coli harbouring a mixture of plasmid forms (RP4, RP4::Tn7, and/or R483::Tn7) and selecting on Tp containing plates, only those Proteus cells to which RP4::Tn7 has transferred, will be viable.

The plasmid RP4 was introduced into E. coli J53(R483::Tn7) in a 5 hour standard mating, selecting for Tp and Tc resistances. (The transfer frequency is shown in Table 4.1, mating 1). Transconjugants were purified and their resistance markers checked. After promoting transposon transfer by subculture, a single transconjugant colony carrying the resistance determinants to Ap, Ka, Tc, Sm, and Tp, was used as a donor in a standard mating with a rifampicin resistant mutant of P. mirabilis NC 6197. Selection was carried out on DSTA plates containing Tp and Rif (Table 4.1, mating 2). Resistances of transconjugants were again checked and a single colony, resistant to Ap, Ka, Tc, Tp, Sm amd Rif, was identified. This strain was used as a donor in a standard mating with E. coli J53. Selection was carried out on DM plates containing the supplements for strain J53 plus Tp and Ka (Table 4.1, mating 3). A colony was identified and purified which conferred resistance to Ap, Ka, Tc, Tp and Sm and this strain was used as a donor to transfer RP4::Tn7 back to J62-2. Selection

TABLE 4.1: TRANSFER FREQUENCIES FOR THE TRANSFER OF Tn7 FROM R483 TO RP4

MATING	SELECTION MEDIA	TRANSFER FREQUENCY PER DONOR CELL
1	DM^{J53} Tp Tc	2.24×10^{-2}
2	DSTA Tp Rif	5.46×10^{-4}
3	DM^{J53} Tp Ka	1.30×10^{-5}
4	DM^{J62-2} Tp Tc	8.30×10^{-4}
	DM^{J62-2} Tp Rif	3.63×10^{-3}
	DM^{J62-2} Tc Rif	3.73×10^{-3}

DM^{J53} - DM base with J53 supplements
DM^{J62-2} - DM base with J62 supplements

was performed on DM plates with J62 supplements to which had been added Rif and either Tp, Tc or Tp and Tc together (Table 4.1, mating 4). Transconjugants were purified and found to confer resistance to Ap Ka Tc Tp Sm/Sp and Rif. This inferred the presence of plasmid RP4 into which had been inserted Tn7.

DETERMINATION OF THE RESISTANCE PATTERN OF E COLI J62(RP4::Tn4135)

Characterisation of Tn4135 initially involved the determination of its resistance pattern. E coli J62(RP4::Tn4135) was tested for its resistance to Tp, Su, Cm, Ka, Ap, Tc, Rif and Sp. Initial tests indicated that this strain was resistant to Ap, Ka, Tc and Tp and sensitive to the remaining drugs. However, subsequent retesting indicated that E coli J62(RP4::Tn4135) sometimes expressed additional resistance to Sm, and when this occurred, the strain was also resistant to Sp.

MINIMUM INHIBITORY CONCENTRATIONS

Tn4135 was further characterised by analysing the level of Tp resistance conferred by this transposon on E coli J62. The MIC of Tp was determined for J62(RP4::Tn4135) and compared with MIC's for E. coli J62 strains harbouring RP4, RP4::Tn7, R751::Tn402 and R388 and for the original pig isolate, P-20. All strains, except J62(RP4), were found to be resistant to levels of Tp greater than 1280 ug/ml.

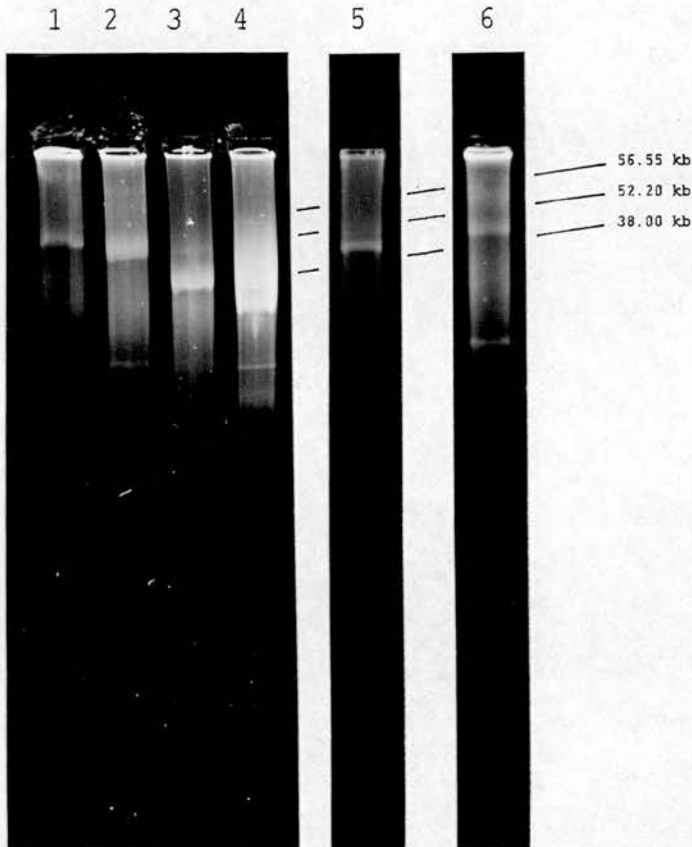
MOLECULAR SIZE OF Tn4135 IN RP4

The molecular size of Tn4135 was determined by comparing the DNA of RP4::Tn4135 with the DNA of known standards, including RP4. Samples were electrophoresed at 60V for 16 hours on 0.7 % gels (Figure 4.1) and the increase in molecular weight of RP4, after transposition of Tn4135, was taken as representative of the size of the Tp resistance transposon. RP4::Tn4135 was found to have a molecular size of 56.55 kb, indicating that Tn4135 has a molecular size of 4.35 kb. (The molecular size of RP4 alone is 52.2 kb.) This compared with 12 - 14 kb for Tn7.

COMPARATIVE RESTRICTION PATTERNS OF Tn4135 AND Tn7

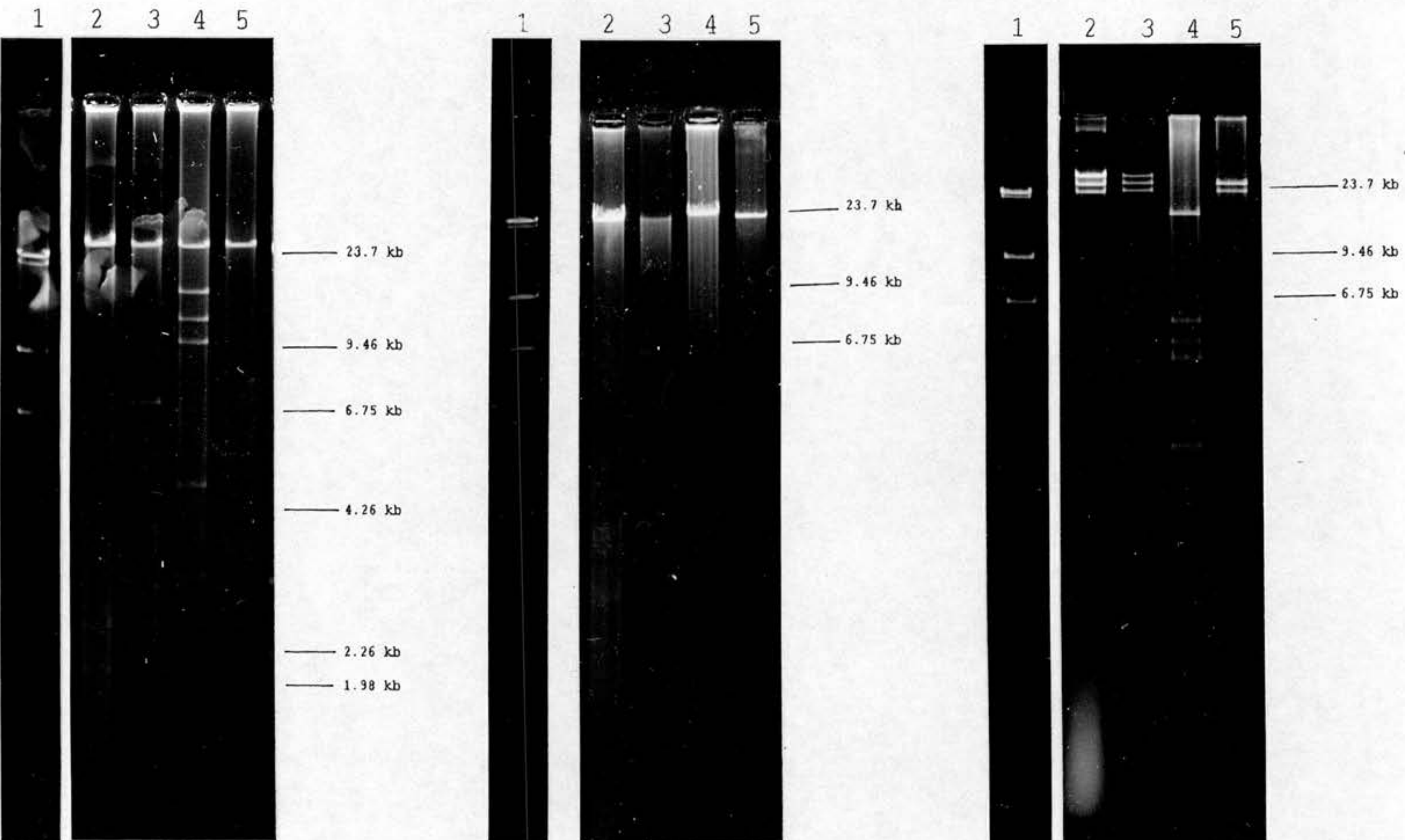
In order to assess the relatedness of Tn4135 to Tn7, restriction enzyme analysis was carried out. Figure 4.2 indicates the results of restricting RP4, RP4::Tn4135 and RP4::Tn7 with Hind III, Bam HI and Pst I. RP4 was cut only once by Hind III, but the insertion of either Tn7 or Tn4135 increased the number of Hind III fragments, indicating that both these transposons contain Hind III sites. The number of sites varied however: RP4::Tn7 was cut 4 times by Hind III whilst RP4::Tn4135 was cut only 3 times. Tn7 thus appeared to contain an extra 6.63 kb fragment (Table 4.2) in addition to the 2.47 and 2.10 kb fragments found in Tn4135. Results of the Bam HI digestion (Figure 4.2b) suggest that this enzyme does not cut Tn4135: both RP4 and RP4::Tn4135 possess only one Bam HI fragment, compared with the two of RP4::Tn7. Restriction with Pst I (Figure 4.2c) substantiates the view that Tn4135 and Tn7 are different: the latter transposon having an additional 7.26 kb fragment.

FIGURE 4.1: MOLECULAR SIZE DETERMINATION OF RP4::Tn4135



TRACK 1. R1 4. Sa-1
2. RP4 5. RP4::Tn4135
3. R6K 6. R483::Tn7

FIGURE 4.2: A COMPARISON OF THE RESTRICTION PATTERNS OF Tn7 AND Tn4135 DNA



(a) *Hind* III digested DNA. (b) *Bam* HI digested DNA. (c) *Pst* I digested DNA.
TRACK 1. DNA. 2. RP4::Tn4135. 3. RP4::Tn7. 4. R483. 5. RP4

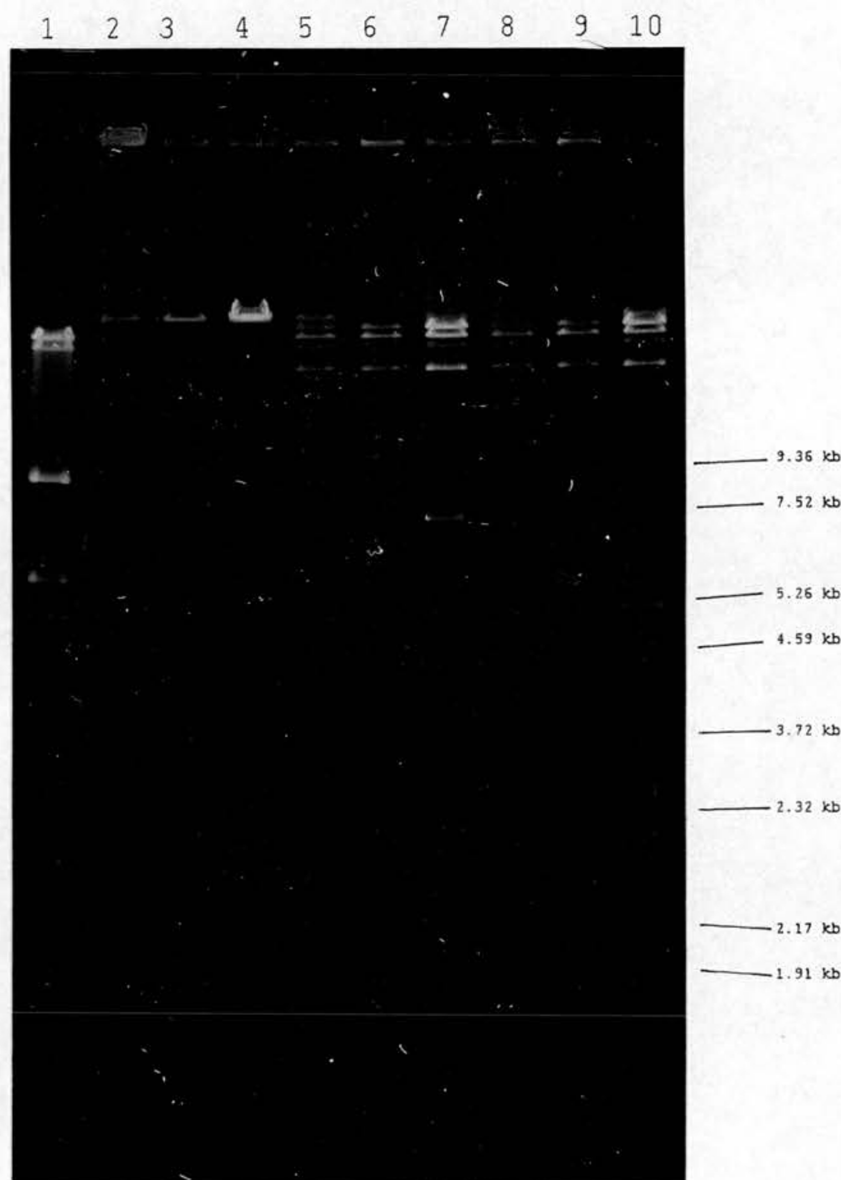
TABLE 4.2: RESTRICTION FRAGMENT SIZES FROM HIND III, BAM HI AND PST I DIGESTION

PLASMID TRANSPOSON	*Hind* III	*Bam* HI	*Pst* I
RP4	23.52	24.07	21.90
			20.59
			6.09
			3.00
RP4::Tn*4135*	23.52	24.07	22.98
	2.47		21.92
	2.10		20.59
			6.09
			3.00
RP4::Tn*7*	23.52	24.07	22.98
	6.63	20.88	21.92
	2.47		20.59
	2.10		7.26
			6.09
			3.00

All sizes are expressed in kb

The three plasmids were additionally digested with Pvu I, and Pvu I and Hind III together (Figure 4.3), in order to characterise further the difference between Tn7 and Tn4135. Pvu I restricts both transposons, but the sizes of the fragments differ (Table 4.3). In addition to the small Pvu I fragments attributable to RP4 (5.26 kb, 4.59 kb and 2.17 kb) RP4::Tn7 possesses three fragments of 9.36 kb, 2.32 kb and 3.72 kb, whilst the equivalent fragments of Tn4135 are only 7.52 kb, 1.91 kb and 3.72 kb. The largest fragments probably correspond to some unrestricted DNA, in linear, open circular and covalently closed circular form, whilst the 24 kb band is indicative of the large restriction fragment of RP4. Double digestion further emphasizes the differences between the two transposons.

FIGURE 4.3: SINGLE AND DOUBLE DIGESTS OF RP4, RP4::Tn4135 AND RP4::Tn7 DNA WITH Pvu I AND Hind III



TRACK					
1.			8.	RP4	
2.	RP4		9.	RP4::Tn7	<u>Pvu</u> I +
3.	RP4::Tn7	<u>Hind</u> III	10.	RP4::Tn4135	<u>Hind</u> III
4.	RP4::Tn4135				
5.	RP4				
6.	RP4::Tn7	<u>Pvu</u> I			
7.	RP4::Tn4135				

TABLE 4.3: RESTRICTION FRAGMENT SIZES FROM SINGLE AND DOUBLE DIGESTS WITH HIND III AND PVU I

PLASMID TRANSPOSON	Hind III	Pvu I	Hind III/Pvu I
RP4	32.46	30.29	30.29
		28.27	28.27
		24.62	24.62
		22.98	22.98
		19.11	19.11
		5.26	5.26
		4.59	4.59
		2.17	2.17
RP4::Tn7	32.46	28.27	28.27
	6.78	24.62	24.62
	1.98	19.11	19.11
		9.36	9.36
		5.26	8.15
		4.59	5.26
		3.73	4.59
		2.32	3.73
		2.17	2.32
			2.17
			1.98
RP4::Tn4135	32.46	30.29	30.29
	1.98	28.27	28.27
	1.59	24.62	24.62
		22.98	22.98
		19.11	19.11
		7.52	7.79
		5.26	6.48
		4.59	5.26
		3.73	4.59
		2.17	3.73
		1.91	1.98

All sizes are expressed in kb

DISCUSSION

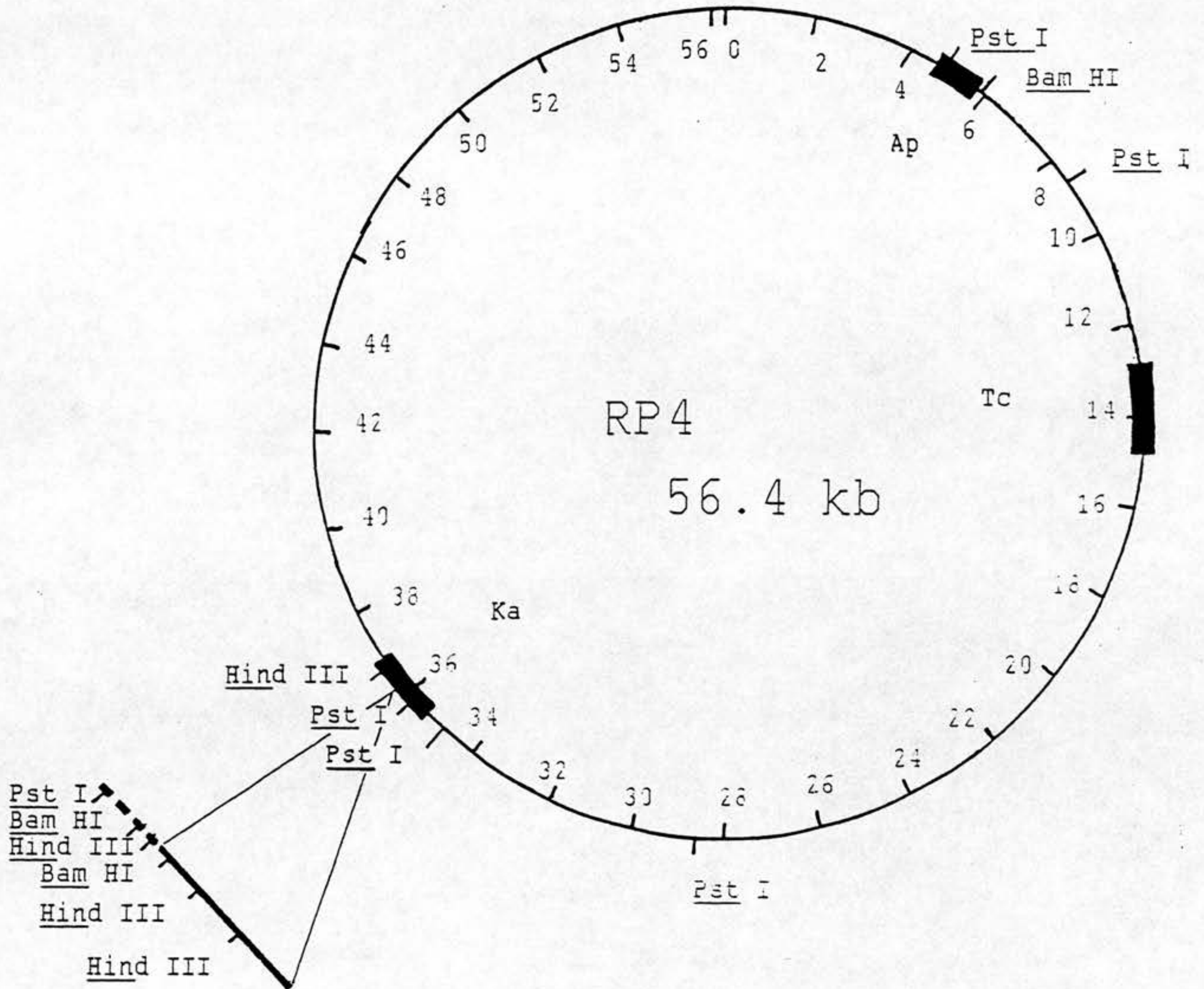
Since the first report of the 12 - 14 kb transposon, Tn7, encoding Tp and Sm/Sp resistance (Barth et al, 1976) a number of other Tp resistance transposons have been isolated, both from human and animal sources (Barth and Datta, 1977a; Shapiro and Sporn, 1977; Towner et al, 1982; Young and Amyes, 1983; Palenque et al, 1983). Whilst some of these isolates have proved to be identical to Tn7 (Tietze et al, 1982; Goldstein et al, 1986) or have evolved from Tn7 (Datta et al, 1979) there appears to be an emergence of small transposons, apparently resistant to Tp only. Young (1984) and Young and Amyes (1983) isolated three such transposons: Tn4132, Tn4133 and Tn4134, all of about 3 kb that were resistant to Tp only. Molecular size analysis estimated Tn4135 to have a size equivalent to these three transposons, approximately 3 - 6 kb, which is considerably smaller than Tn7. In one or two experiments RP4::Tn4135 was found to be smaller than RP4 alone: a phenomenon experienced when other small Tp transposons were inserted into RP4 (Young, 1984). The apparent variation in the size of Tn4135, as determined by an alteration in size of an RP4 transposon-containing derivative, may be due to small deletions, caused by the transposition process. Previous reports have shown that the insertion of Tn7 (Barth and Grinter, 1977) and other small transposons (Barth et al, 1978; Datta et al, 1979; McCombie et al, 1983) into RP4, can cause small deletions at the site of insertion, making the accurate determination of molecular size difficult. Weisberg and Adhya (1977) have indicated that deletions occur by spontaneous loss of fragments during or immediately after the insertion process.

Initial studies with E coli J62(RP4::Tn4135) indicated that this transposon lacked Sm/Sp resistance, suggesting that it may have evolved from Tn7 by loss of the region encoding these resistant determinants. As with Tn4132, Tn4133 and Tn4134 (Young, 1984), but unlike Tn78 (Datta et al, 1979), subsequent retesting of resistances indicated that Sm/Sp resistance could be expressed. This indicates that Tn4135 has not evolved from Tn7 by deletion of the Sm/Sp encoding region, but a deletion in the controlling region of these resistance determinants could explain the lack of continous expression of these genes. Tn4135 could therefore carry Sm/Sp resistance genes in a dormant, switched off state. It is conceivable that the insertion of Tn4135 in RP4 may have occurred, in some instances, adjacent to a resident IS element that could act as a 'switch' for the expression of Sm/Sp resistance, complementing the loss of control caused by deletion. The role of IS elements as a mobile promotor resulting in the switching on or off of bacterial genes has been suggested previously by Glansdorff et al (1980).

Restriction enzyme analysis has been used to ascertain the evolutionary relatedness of Tp transposons to Tn7, by virtue of the fact this transposon possesses two charactertistic internal Hind III fragments of aproximately 1.9 and 2.3 kb (Barth and Grinter, 1977). Tn78 (Datta et al, 1979) was found to contain these two fragments whilst Young (1984) could demonstrate only the smaller of the two fragments in the small Tp-only transposons, Tn4132, Tn4133 and Tn4134. Hind III digestion of Tn4135 generated two fragments of 2.47 and 2.10 kb indicating a greater similarity of this transposon with Tn78 than with the other small transposons. The fact that the Tp transposons so far studied all appear

to possess the smaller of the two *Hind* III fragments, may be significant to evolution. This 2.2 kb region is thought to encode a gene required for transposition (Smith and Jones, 1984) and has thus been conserved whilst other less essential regions have been deleted. The finding of small Tp only transposons with differing restriction patterns may be indicative of the potential of transposons to mediate their own evolution. It is conceivable that Tn*7* (and probably other transposons) has the potential to delete different regions of its DNA, possibly by virtue of possessing homologous 22 base pair long regions in the same orientation at several locations along its length (Lichtenstein and Brenner, 1981), resulting in a family of smaller transposons. Tn*4132*, Tn*4133*, Tn*4134*, Tn*4135* and Tn*78* may all have arisen from Tn*7* by deletion of different regions of DNA. However, although Tn*4135* was found to have these two internal fragments, the number of restriction fragments produced by *Hind* III digestion of RP4::Tn*4135* was one less than digestion of RP4::Tn*7* with the same enzyme. The deletion of a *Hind* III site would explain this finding, but would also result in the loss of one of the characteristic *Hind* III fragments observed. Insertion of Tn*4135* into the single *Hind* III site of RP4 (Grinsted *et al*, 1977) would also reduce the number of *Hind* III fragments but would result in the loss of Ka resistance. (RP4::Tn*4135* still expresses Ka resistance). An alternative explanation is that Tn*4135* has in fact, lost a *Hind* III site by deletion, but this has been complemented by insertion close to the *Hind* III site of RP4 (Figure 4.4). Tn*4135* may therefore be similar to Tn*4132*, Tn*4133* and Tn*4134* and the variable restriction patterns may be attributable to the site of insertion into RP4.

FIGURE 4.4: POSSIBLE INSERTION SITE OF Tn4135 IN THE PLASMID RP4

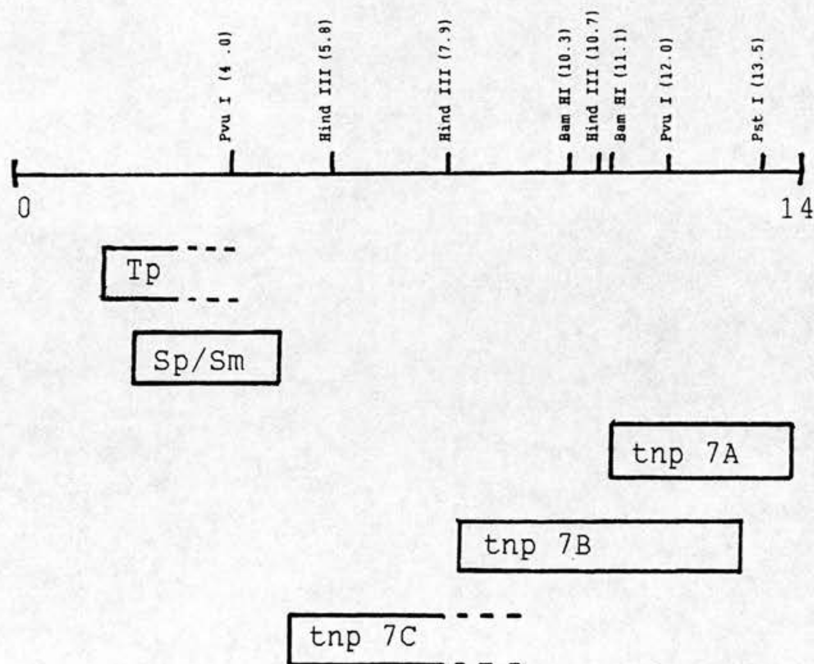


The location of relavent RP4 restriction sites and genes are as specified by Barth and Grinter (1977). The solid line represents Tn4135 DNA whilst dotted lines correspond to regions of the Tn7 genome thought to be deleted in the evolution of Tn4135. For the localisation of Tn7 restriction sites see Gosti-testu et al (1983). All coordinates are expressed in kb.

Digestion with Bam HI, substantiates the view that a deletion has occurred at the right hand end of Tn7. Bam HI cuts Tn7 on either side of the right hand Hind III site (10.7 kb) (Figure 4.5) to give an internal fragment of less than a kb (probably too small to observe by the method employed) and cuts RP4 once (Barth and Grinter, 1977). RP4::Tn7 digestion yielded two large fragments, the sizes of which are not accurate, because of the inability to measure fragments of molecular weight greater than 11 kb by this method (Grinsted et al, 1977). Digestion of RP4::Tn4135 with Bam HI yielded only one fragment suggesting that one or both of the Bam HI sites of Tn7 had been deleted to give Tn4135. Depletion of both Bam HI sites would result in a linear molecule (ie. one fragment) similar to the digestion of RP4 alone with this enzyme. Loss of one restriction site, however, would give rise to two fragments, the sizes of which would depend on the site of insertion of Tn4135. The Bam HI site of RP4 is directly opposite that of Hind III, so if it is assumed that Tn4135, with one Bam HI site, inserts close to this Hind III site of RP4 (Figure 4.4), two fragments of approximately equal size would be produced. Closer examination of the Bam HI fragments of RP4 and RP4::Tn4135, separated by agarose gel electrophoresis (Figure 4.1b), does reveal differences between the single plasmid bands present in each digest, suggesting that the thicker band of RP4::Tn4135 may be due to two fragments of identical size.

Pst I digestion of plasmid DNA reiterated the view that the right hand end of Tn4135 and Tn7 differ: Tn4135 appeared to lack the Pst I cut site of Tn7. The fact that this right hand region of DNA is outwith the region coding for the resistance determinants (Barth and Grinter, 1977a; Fling and Richards, 1983; Smith and Jones, 1984) substantiates the view

FIGURE 4.5: RESTRICTION ENDONUCLEASE AND FUNCTIONAL MAP OF Tn7



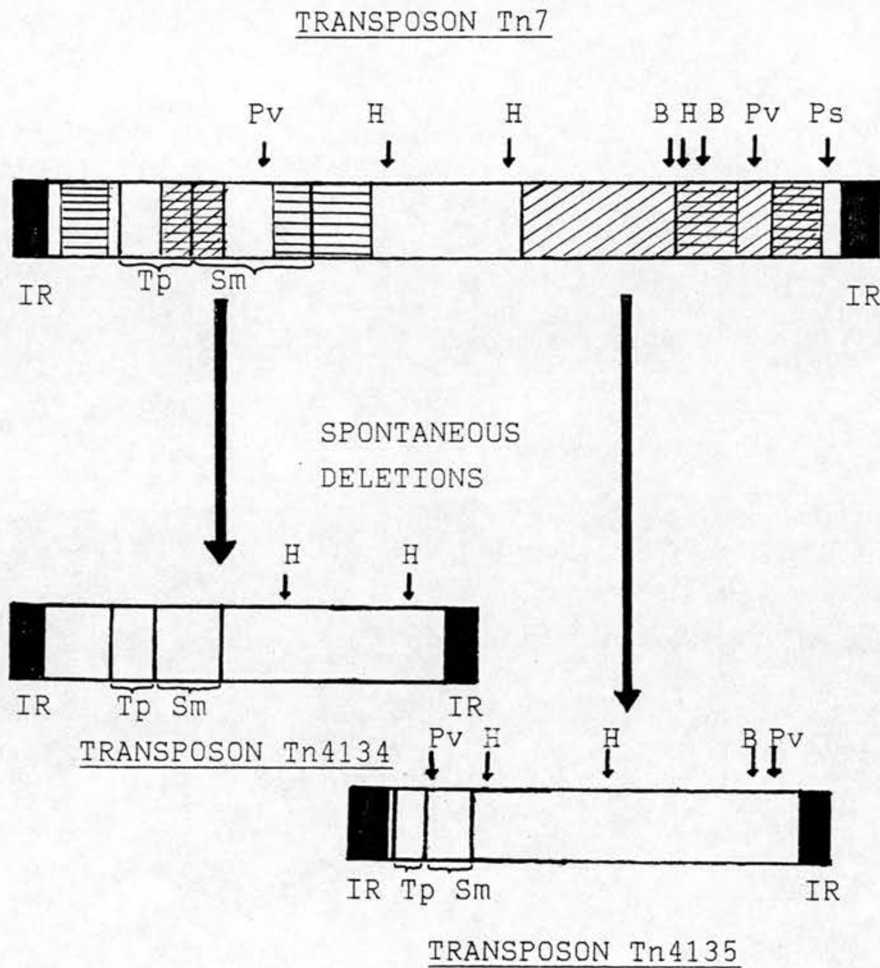
Restriction enzyme cleavage sites are in kb and are located as indicated by Gosti-Testu et al (1983) and Hauer and Shapiro (1984). Functions are located according to Hauer and Shapiro (1984). Tnp 7A, tnp 7B and tnp 7C indicate regions encoding transposition functions.

that a control region, perhaps encoding an inducer enzyme for the expresion of Sm/Sp, may have been deleted, not the genes themselves. Deletion of this right hand end alone , however, would only account for a reduction in size of a few kb, since it is unlikely that the 28 base pair terminal repeats, essential for transposition (Kleckner, 1981), would have been deleted as well. Work with deletion mutants of Tn7 (Ouartsi et al, 1985) has indicated that a fragment larger than the 42 base pair terminal sequence, containing this 28 base pair IR at its end, is required for transposition, and have located functions essential to transposition in a region, greater than 2.5 kb, on the right hand end of Tn7. (The Pst I site of Tn7 lies outside the 42 base pair terminal region.) It is therefore likely that these regions are still intact in Tn4135.

Pvu I digestion was utilised to define the deletion at the right hand end and to locate any deletions elsewhere in the Tn7 genome that could account for the size differences observed. Pvu I restricts Tn7 once between the Bam HI and Pst I sites, thought to be deleted in Tn4135, and once in the left hand end of Tn7, giving an internal fragment of approximately 8 kb. The number of fragments generated by Pvu I digestion of RP4::Tn4135 and RP4::Tn7 are identical indicating that the Pvu I cut site at the right hand end of Tn7 is still present in Tn4135. This site lies within the proximal one third of the four 22 base pair contiguous direct repeats (Ouartsi et al, 1985) thought to play an important cis acting role in transposition. This finding again reiterates the importance of certain regions in transposition and thus their conservation in DNA evolution. It also indicates that more than one deletion is involved in shaping the right hand end of Tn4135, since the two restriction sites, Pst I and Bam HI, on either side of the intact Pvu I site are thought to be missing.

Young (1984) postulated that the small transposon Tn4134 had evolved from Tn7 as a result of deletion in the right hand end of the transposon, leading to loss of a Hind III restriction site, and a deletion in the resistance determinant region at the left hand end. Evidence presented here for Tn4135 suggests this transposon is genetically similar to Tn4134 and further defines the deletion of the right hand end of the transposon (Figure 4.6), giving possible reasons for the conservation of surrounding regions of DNA. These results, however, do not rule out the possibility that Tn4135 and Tn4134 have evolved independently from each other via separate deletions of the Tn7 molecule.

FIGURE 4.6: PROPOSED MODEL FOR THE EVOLUTION OF THE SMALL TRIMETHOPRIM-ONLY TRANSPOSONS FROM Tn7



IR = inverted repeat; Tp = trimethoprim resistance gene; Sm = Sm/Sp resistance gene; H = Hind III site; B = Bam HI site; Pv = Pvu I site; Ps = Pst I site; [diagonal hatching] = suspected deletions giving rise to Tn4134; [horizontal hatching] = possible deletions giving rise to Tn4135.

Note - diagrams not to scale.

CHAPTER 5

BEHAVIOUR OF TRIMETHOPRIM RESISTANCE TRANSPOSONS DURING TRANSFER TO AND FROM DIFFERENT REPLICONS

INTRODUCTION

The evolutionary response of bacteria to the selective pressures imposed by the medical and veterinary use of antibiotics has been witnessed over the past four decades and studies on the emergence and dissemination of drug resistance genes have vividly illustrated the genetic flexibility of bacteria. The introduction of each new group of antibiotics has lead to the emergence of resistance plasmids conferring resistance to these drugs (Anderson, 1968; Mitsuhashi, 1969). However, it has become clear that a number of organisms contained transferable plasmids long before the antibiotic era and these have provided the resevoir of plasmids available for the acquisition of resistance genes (Datta and Hughes, 1983).

Trimethoprim was introduced, in combination with sulphamethoxazole (cotrimoxazole), in the United Kingdom in 1969 and in France in 1971. Initial resistance to the combination remained low: less than 3% (Gruneberg, 1976) in E coli isolated from urinary tract infections two years after the introduction of cotrimoxazole, but after three years, R factors confering high levels of resistance were detected in clinical strains of bacteria (Fleming et al, 1972; Datta and Hedges, 1972). The first R factor of this type was designated R388 (Amyes and Smith, 1976) and was found to be resistant to Su as well. When cotrimoxazole was introduced there was already a high percentage of bacteria harbouring R plasmids within the clinical population and most of these R plasmids contained determinants for sulphonamide resistance (Datta, 1969).

Trimethoprim was marketed in Britain as a single agent in September 1979, after 10 years of use in combination with sulphamethoxazole. This was despite views that its use alone in the treatment of urinary tract infections would lead to the rapid development of aquired resistance once the protective sulphonamide moiety was removed (Bushby and Hitchings, 1968; Barry and Pattishall, 1983; Rich and Mee, 1985). However, the possibility of devising an adequate scientific test to prove or disprove these views would be almost impossible (Hamilton-Millar, 1984) because of different practices in drug prescription, the clinicians desire to have a choice of drugs and because of difficulties in making allowances for the selection of Tp resistance with other drugs eg. Ap (Amyes et al, 1981). This misstrust of Tp as a single drug was based partly on the ready production of resistance to Tp, following exposure of heavy inocula, of initially sensitive bacteria, to increasing concentrations of Tp in vitro (Darrell et al, 1968), but was challenged by a number of reports (Anderson et al, 1974; Brumfitt and Hamilton Millar, 1979; Greenwood, 1979; Amyes et al, 1981). These fears of increased resistance were not unequivocally substantiated by reports from Finland, where Tp alone has been used since 1973 (Kasanen and Sandquast, 1982): the frequency of resistant E coli, causing urinary tract infections, remained fairly constant in Finland at about 10% between 1972 and 1977. This was despite the use of Tp alone (Kasanen et al, 1978), although the levels of Tp resistance were higher than in Sweden (Huovinen and Toivanen, 1980) where Tp alone was not commercially available, and Helsinki where the drug had been used less. Additionally the levels of resistance in Edinburgh , where the combination of Tp and Su had been used exclusively since 1969, were found to be approaching those in Finland (Amyes et al, 1981), suggesting there was little difference in the use of Tp alone or in combination with Su. It

would appear, however, that Tp resistant organisms other than Enterbacteriaceae (eg Pseudomanas and Acinetobacter species) emerged more often in patients (58%) receiving Tp alone than in those on cotrimoxazole (22%) (Brogden et al, 1982). Lacey (1982) and Brumfitt et al (1983) reported little rise in the overall incidence of Tp resistant bacteria since the intoduction of Tp alone, although the incidence of high level Tp resistance had increased (Brumfitt et al., 1980; Towner, 1981; Kraft et al, 1984). Huovinen et al (1986) , in contrast, support the view that emergence of Tp resistance is linked to changes in the consumption of both Tp and cotrimoxazole, although Skold et al (1986) found more than one third of Tp resistance carrying patients, from a study in Jamtland, never to have been exposed to Tp. A similar finding of spread to individuals not being treated with antibiotics was encountered by Rydberg and Cederberg (1986).

Resistance to Tp has been reported from many parts of the world (Table 5.1) and has involved many bacterial species (Rowe and Threlfall, 1981) (Table 5.2). Resistance is now extending from E coli to other gram negative bacteria such as Vibrio cholerae (Threlfall et al, 1980; Goldstein and Acar, 1985; Gerbaud et al , 1985; Goldstein et al, 1986; Young and Amyes, 1986b), Acinetobacter sp (Goldstein et al, 1983), Pseudomonas aeruginosa (Moilleau-batt et al, 1987)* and even phytopathogenic bacteria such as Pseudomonas syringae (Leary and Trollinger, 1985).

The prevalence of resistance to Tp varies from species to species, from country to country (Table 5.3) and may depend on local epidemiological factors (such as the type of patient, antibiotic pressure), location and time (Dornbusch and Toivanen, 1981; Huovinen et al, 1982; Pulkinen et al, 1984; Kraft et al, 1985; Huovinen et al, 1986).

* Manuscript in press

TABLE 5.1: REPORTS OF TRIMETHOPRIM RESISTANCE IN DIFFERENT COUNTRIES

COUNTRY	% OF STRAINS RESISTANT TO Tp	REFERENCE
BRITAIN	5 - 20	Amyes *et al* (1978); Brumfitt *et al* (1983); Hedges *et al* (1972); Datta *et al* (1981)
FRANCE	17 - 36	Goldstein *et al* (1986)
BRAZIL	44.4 (Enteric)	Tiemans *et al* (1984)
BANGLADESH	36 (*Cholerae*)	Threlfall *et al* (1980a)
FINLAND	9.4	Huovinen *et al* (1983)
ITALY	20	Romero and Perduca (1977)
CANADA	3 (*Shigellae*)	Bannatyne *et al* (1980)
UNITED STATES	5 - 14	Mayer *et al* (1985)

TABLE 5.2: SPREAD OF PLASMID-MEDIATED, HIGH-LEVEL TRIMETHOPRIM RESISTANCE INTO VARIOUS BACTERIAL SPECIES

ORGANISM	COUNTRY	YEAR FIRST ISOLATED	REFERENCE
Escherichia coli	UK	1971	Fleming *et al*, 1972
Klebsiella species	UK	1971	Fleming *et al*, 1972
Proteus mirabilis	UK	1972	Datta and Hedges, 1972
Indole +ve *Proteus* species	UK	1973	Jobanputra and Datta,1974
Citrobacter species	UK	1973	Amyes and Smith,1978
Enterobacter sp.	UK	1973	Grey *et al*, 1979
Serratia sp	UK	1974	Coulanges, 1981
Salmonella typhi	FRANCE	1975	Goldstein *et al*, 1984
Salmonella sp	UK	1975	Threlfall *et al*, 1980
Shigella sp	CANADA	1978	Bannatyne *et al*, 1980
Vibrio cholerae	BANGALADESH	1979	Threlfall *et al*, 1980
Acinetobacter sp	FRANCE	1982	Goldstein *et al*, 1983
Pseudomonas aeruginosa	FRANCE	1983	Acar *et al*, 1973

TABLE 5.3 CHANGES IN THE LEVELS OF TRIMETHOPRIM RESISTANCE SINCE 1973

CITY [YEAR(S)]	REFERENCE(S)	NO OF STRAINS ISOLATED	% STRAINS RESISTANT TO Tp	% STRAINS WITH HIGH LEVEL RESISTANCE TO Tp	% HIGHLY RESISTANT STRAINS CAPABLE OF RESISTANCE TRANSFER	NO OF Tp RESISTANCE PLASMIDS
PARIS	Goldstein et al (1986)					
1974-75		10,105	17.5	44.9	31.2	92
1978-79		10,189	23.2	75.3	48.3	84
1980-81		10,246	38.6	83.6	62.6	71
1982-83		10,823	35.4	91.9	44.3	116
1984		5,494	24.3	95.4	50.0	31
PAVIA (ITALY)	Romero & Perduca (1977)					
1973-75		670	20.7	-	16.2	32
LONDON	Amyes et al (1978)					
1973-75	Datta et al (1980)	-	3.2	10.0	-	-
1975-77	Hamilton-Millar et al	1,651	9.4	-	11.5	18
1977	(1981)	1,618	13.0	27.4	23.3	18
1973-75	Brumfitt et al (1983)	3,129	4.3	20.3	-	-
1978-79		4,773	11.5	60.0	24.7	72
1981		-	12.2	78.0	-	-
1985		-	24.2	81.0	-	-
NOTTINGHAM	Towner (1979)					
1978	Towner et al (1979)	3,998	4.8	17.1	12.5	24
1979	Towner et al (1980)	4,069	2.9	57.6	34.7	41
	Towner & Wise (1983)					
EDINBURGH	Amyes et al (1981)					
1978-81	Amyes et al (1986)	359	21	5.0	5.0	-
1981		200	64	25.0	11.0	-
1982-84		-	16.5	12.0	5.0	-
TURKU, FINLAND	Huovinen & Toivanen (1980)					
1979	Toivanen (1980)	222	41.0	60.4	13.0	6
1980-81	Huovinen et al (1983)	560	35.4	68.7	20.0	27
1980-81	Huovinen et al (1986)	633	9.0	38.6	19.0	4
1983		-	32	-	-	-
1984		-	35	-	-	-

Levels of Tp resistance in Greece (Saroglou et al, 1980), Finland (Huovinen and Toivanen, 1980; Huovinen et al, 1982; Huovinen et al, 1983) and Italy (Romero and Perduca,1977) are reported to range from 20% to 40%. In contrast levels of 5 - 8% have been reported for similar periods in the UK (Towner et al, 1980; Chirnside et al, 1985), Denmark (Fruensgaard and Korner, 1974) and the United States (Mayer et al, 1985). Whilst the prevalence of Tp resistant E coli increased from 2% to 6% in Boston during 1978 -1981 (Mayer er al, 1985) and from 8% to 30% in Paris (Papadopoulou et al, 1986), the prevalance of Tp resistant P mirabilis during the same period was 1% in Boston (Mayer et al, 1985) and 20 - 25% in Paris (Goldstein et al, 1984), thus indicating differences in Tp levels with respect to both organism and location. Different trends in the development of Tp resistance have with out doubt emerged (Brumfitt et al, 1983; Towner and Wise, 1983; Amyes et al , 1986): in London between 1981 and 1983 there was a marked increase in the level of transferable Tp resistance (Chirnside et al, 1985) whereas in Edinburgh there was a decrease (Amyes et al, 1986). Between 1979 and 1981 there was an emergence of low level transferable Tp resistance (Anderson, 1980; Towner and Pinn, 1981) which was thought to be a possible precursor of the high level Tp resistance that had been observed. The incidence of Tp resistant bacteria is especially high in developing countries eg 44% of E coli in Chile and 40% in Thailand were observed to be Tp resistant (Murray et al, 1985) and 64% in India (Young et al, 1986). However, despite the variation in overall prevalence of Tp resistance between countries, an increase in the relative % of high level resistance, presumably encoded by R plasmids or transposons, is common to the different countries (Table 5.3). Increases were observed in London between 1973 and 1981 (Amyes et al, 1978; Datta et al, 1981; Brumfitt et al, 1983), in Nottingham from 1978 to 1979 (Towner

et al, 1979; Towner et al 1980) and in Paris an increase from 40.2% to 95.4% was observed between 1972 and 1984 (Goldstein et al, 1986). Similar increases have also been observed in Mexico, Chile and Thailand (Murray et al, 1982; Rudy and Murray, 1984; Murray et al, 1985).

The diversity of organisms now resistant to Tp and the changing levels of Tp resistance can be related to the spread of transposons and R plasmids. Since the first report in 1972 many epidemics of Tp resistant bacteria with plasmid mediated resistance have been described (Table 5.4). The first epidemic of Tp resistance encoded by an R plasmid occurred in London in 1971 (Fleming et al, 1972) and was due to a single plasmid of incompatability group W (Jobanputra and Datta, 1974). (During this time extensive studies in Bristol (Lacey et al, 1972) and in Dublin (Moorhouse and Farrell, 1973) failed to indicate any emergence of Tp resistant plasmids. By 1977, however, other hospitals in London and in Bristol were reporting the isolation of these plasmids (Brumfitt et al, 1977; Grey and Hamilton Millar, 1977; Marks et al, 1977).). This situation was mirrored by results from a hospital in Boston (Mayer et al, 1985) where Tp resistance amongst Enterobacteriaceae, was due to the dissemination of a single conjugative plasmid. The situation observed in Paris in 1974, however, was quite different (Goldstein et al, 1975; Acar et al, 1977); Tp R plasmids belonged to 6 different Inc groups. Subsequent reports from Italy and the UK (Table 5.5) have indicated that R plasmids from a wide range of Inc groups are now responsible for Tp resistance, although initially, in Italy, almost all plasmids belonged to the M Inc group (Romero and Perduca, 1977). The emergence of R plasmid Tp resistance seems to have been more rapid in France and Italy than in the UK. The spread of Tp resistance genes to plasmids of such a wide variety

TABLE 5.4 EPIDEMICS OF TRIMETHOPRIM RESISTANT BACTERIA DUE TO TRANSFERABLE Tp PLASMIDS

YEAR	COUNTRY	REFERENCE
1971	UK	Flemming *et al* (1977)
1974	France	Acar *et al* (1977)
1976	Italy	Romero and Perduca (1977)
	Japan	Terakado *et al* (1980)
1977	United States	O'Brien *et al* (1982)
	Canada	Bannatyne *et al* (1980)
	Greece	Saroglou *et al* (1980)
	New Zealand	Anderson (1980)
1979	Finland	Huovinen *et al* (1983)
	East Germany	Tietze *et al* (1982)
	Bangladesh	Threlfall *et al* (1980)
	Southeast Asia	Agarwal *et al* (1981); Butler *et al* (1982); Goldstein *et al* (1986)
1980	Madagascar	Coulanges (1981)
1981	Peru	Goldstein *et al* (1986)
	UK	Amyes *et al* (1981)
1982	Spain	Palenque *et al* (1983)
1983	Brazil	Goldstein *et al* (1983)
	Kenya	Gerboud *et al* (1983)
	Tanzania	Goldstein *et al* (1986)
	Algeria	
	Thailand	
	Indonesia	
1984	Ivory Coast	Goldstein *et al* (1985)
	Zaire	Goldstein *et al* (1985)

TABLE 5.5 INCOMPATIBILITY GROUPS OF PLASMIDS CONFERRING RESISTANCE TO TRIMETHOPRIM IN DIFFERENT COUNTRIES

CITY	REFERENCE	PERIOD	NO OF PLASMIDS	INCOMPATIBILITY GROUPS
Paris	Acar *et al* (1980)	1974-75	91	C, Fll, N, M, I, B
Pavia, Italy	Romero & Perduca (1977)	1973-75	32	Fll, N, M, S
London	Datta *et al* (1980)	1977	18	C, Fll, N, I , W, P, X, H_2
Nottingham, UK	Towner (1979)	1983	52	Fll, I , I , K, M, B, P, W, X
Lima, Peru	Goldstein *et al* (1986)	1981-82	34	H_1

of Inc groups is likely to be due to transposable elements and would suggest an analogy with the spread of Ap resistance (Richards et al, 1978). The ubiqiity of the TEM β lactamase gene (Hedges et al, 1974) has been shown to result from its ability to transpose from replicon to replicon (Heffron et al, 1975), and Hedges and Jacob (1974) later confirmed the presence of this gene on a transposon, thus accounting for its rapid transmission through populations of bacteria and between bacterial families eg to N gonorrhoeae (Elwell et al, 1977) and H influenzae (Laufs and Kaufers, 1977). As previously mentioned (Chapter 4) a number of Tp resistant transposons have been identified; Tn1527 (Goldstein et al, 1986), Tn1824 (Tietze et al, 1982), Tn71 and Tn72 (Barth and Datta 1977a,b), Tn79 and Tn80 (Richards and Nugent, 1979), Tn78 (Datta et al, 1979) and Tn4132 (Young and Amyes, 1983), but the most extensively studied have been Tn7 (Barth et al, 1976) and Tn402 (Shapiro and Sporn, 1977). Tn402 has been transposed from R751 but it does not appear to integrate into the chromosome or other plasmids (Amyes, 1979). (Preliminary evidence suggested that the resistance gene of R388 might also reside on a transposon (Amyes and Smith, 1977), but it has not been proven, nor has the suggestion that Rss28 and Rss42 might carry Tp transposons (Amyes and Smith, 1978).). Various reports have indicated the ease with which Tn7 transfers between replicons (Barth et al, 1978; Hassan and Brevet, 1983; Taylor, 1983; Ouartsi et al, 1985), eg Tn7 has been shown to insert at multiple sites of some plasmids, such as RP4 (Barth et al, 1978), occasionally inactivating existing resistance characters or inducing transfer defective mutants (Taylor, 1983).

A large proportion of the high level resistance to Tp can be attributable to Tn7; Tp resistant enterobacteria isolated since 1978 in the UK (Richards et al, 1978; Datta et al, 1979; Richards and Nugent,

1979; Datta et al, 1981; Richards and Datta, 1982; Towner et al, 1982; Towner and Wise, 1983), Sweden (Steen and Skold, 1985) and Finland (Pulkkinen et al, 1984) owed their Tp resistance to chromosone or plasmid located Tn7.

The observed increase in the incidence of high level non transferable resistance to Tp (Towner et al, 1980; Hamilton Millar et al, 1981; Towner et al, 1982; Amyes et al, 1986; Amyes, 1986) is disturbing. This could be due to the spread of plasmids incapable of transfering into standard E coli K12 recipient strains or due to the transpostion of Tp genes to the chromosome. Tn7 has been shown to transpose into the chromosome of E coli at a specific site (Barth et al, 1976; Lichtenstien and Brenner, 1981) and has also been found located in the chromosomes of other enteric bacteria (Goldstein et al, 1986), Vibrio species (Thomson et al, 1981; Goldstein et al, 1986), Agrobacterium (Hernalsteens et al, 1978) and Caulobacter (Ely, 1982), inducing mutagenesis in some cases. This integration of resistance genes is not uncommon, Richmond and Sykes (1972) found that the TEM B lactamase gene was capable of integration into the bacterial chromosome. This is a potentially disquieting trend, however, since this resistance is inherantly more stable than plasmid mediated resistance and not likely to be lost once the selective pressure is removed. An integrated transposon also retains the ability to spread amongst further plasmids which may enter the cell. Towner (1981) indicated an increase in non transferable high level Tp resistance from 4.7% in 1978 to 22.9% in 1979; results which suggested that transposable Tp resistance, in the absence of a conjugative Tp resistant plasmid, was probably fairly common in hospitals of the Nottingham area of the UK. Non transferable Tp resistance seemed to be particularly common in strains of Proteus species (Towner and Wise,

1983). This shift is also being observed in other areas (Kraft *et al*, 1984; Steen and Skold, 1985).

Clearly there have been changes in the distribution of Tp resistance genes in the environment over the last decade, brought about by the spread of trasnposons, such as Tn*7*. The emergence of Tp only transposons, such as Tn*4132* (Young and Amyes, 1983) and Tn*4135* (unpublished results) has lead not only to speculation about the evolution of the resistance genes but also the changes that are likely to occur over the next decade, with regards spread of Tp resistance. In order to understand the events that have occurred so far; the increase in high level resistance and the movement of transposons, and thus resistance genes, into the chromosome, there is a need to monitor the changes that occur at the molecular level. The purpose of this study was therefore to delineate the transfer potential , and the genetic aspects associated with transposition of the Tp only transposon - Tn*4135* - in comparison with Tn*7* transposition.

MATERIALS AND METHODS

The experimental procedures for plasmid transfer, preparation of selection plates and resistance testing have been described previously (chapter 3). The method adopted for transposon transfer was that utilised in chapter 4 and DNA was prepared by the method of Takahashi and Nagano (1984) as outlined in chapter 1.

REPLICA PLATING

Purified colonies were either spotted onto nutrient agar plates, in a grid like manner with sterile sticks, or alternatively, 0.1 ml of suitably diluted nutrient broth cultures were spread on nutrient agar plates. All plates were incubated at 37°C overnight. Replica plating (Lederberg and Lederberg, 1952) was achieved by inverting the incubated plates over a square of sterile velvet held on to a cylindrical rubber block (8 cm in diameter) by a perspex collar. The agar surface was pressed gently against the pile and removed. Replica plates, containing suitable antibiotics and marked for subsequent orientation, were inverted in turn over the fabric and the colonies transferred from pile to plate by gentle pressure on the back of the inverted plate. The plates were incubated at 37°C overnight.

PHAGE OVERLAYS

Agar overlays were prepared by dissolving 0.7 g of ion agar (Oxoid) in 100 ml of nutrient broth (oxoid no 2). Aliquots (2 ml) were dispensed into bijoux bottles, autoclaved and kept molten in a water bath at 45°C. Cultures to be tested were grown up overnight in nutrient broth and phage preparations were diluted in nutrient broth as appropriate. Three drops of bacterial culture were added to a warm overlay, followed by 100 ul of the appropriate phage suspension. The bijoux was rolled quickly in the hands to mix, being careful not to create any air bubbles, and poured onto a nutrient agar plate. Plates were incubated at 37°C overnight.

PHAGE SELECTION FOR LOSS OF PLASMID DNA

Phage PRR1 was used to test cultures for the presence or absence of the *Inc* P plasmid RP4. (This phage is specific for *Inc* P plasmids - Olsen and Thomas, 1973 - lysing any cells harbouring one of these plasmids). Bacterial cultures were grown up overnight in Isosensitest broth containing an appropriate drug and subcultured in the same medium. A phage overlay was set up with one drop of bacterial culture and 100 ul of either a neat, 10^{-1} or 10^{-2} diluted PRR1 phage. Colonies growing through the phage plaques (ie those lacking RP4 and therefore not lysed) were transferred by sterile stick onto appropriately supplemented DM plates, and DM plates containing one of the drugs to which the unwanted RP4 plasmid was resistant. All drug sensitive colonies were then tested for desirable resistance determinants.

RESULTS

TRANSFER OF Tn4135 FROM RP4 TO Sa-1 AND ANALYSIS OF THE RESULTING TRANSCONJUGANTS

When Tn4135 was transposed into the Inc P plasmid RP4, the plasmid was found to be unstable when the host strain was grown in medium containing Tp. Therefore, Tn4135 was transferred to the Inc W plasmid Sa - a naturally occurring plasmid of clinical and experimental relevance and commonly used as a standard in molecular biology.

i. Transfer

The plasmid Sa-1 was introduced into E coli J62-2(RP4::Tn4135) in a 5 hour standard mating and selection was made for Tp and Sm resistance (Table 5.6; mating 1). Transconjugants were purified and their resistance markers checked, before promoting transposon transfer by subculturing twice in nutrient broth. A single transconjugant colony carrying the resistance determinants for Tp, Ka, Tc, Tp, Sm and Su was used as a donor in a standard 5 hour mating with E coli J53 (Table 5.6; mating 2). Selection was carried out on DM plates containing supplements for strain J53 and Tp, Sm or TpSm together. Resistances of transconjugants were again checked and a single colony, conferring resistance to Ka, Su, Sm and Tp, was used as a donor to transfer Sa-1::Tn4135 back to E coli J62 (Table 5.6; mating 3). Selection was carried out on DM plates, supplemented for strain J62 and containing Tp, Sm or Tp and Sm.

TABLE 5.6: TRANSFER FREQUENCIES FOR THE TRANSFER OF Tn4135 FROM RP4 TO Sa-1

MATING	SELECTION MEDIA	TRANSFER FREQUENCY PER DONOR CELL
1	Tp Sm	1.73×10^{-1}
2	Tp	1.74
	Sm	1.14
	Tp Sm	9.80×10^{-1}
3	Tp	1.40×10^{-1}
	Sm	8.40×10^{-2}
	Tp Sm	1.73×10^{-2}

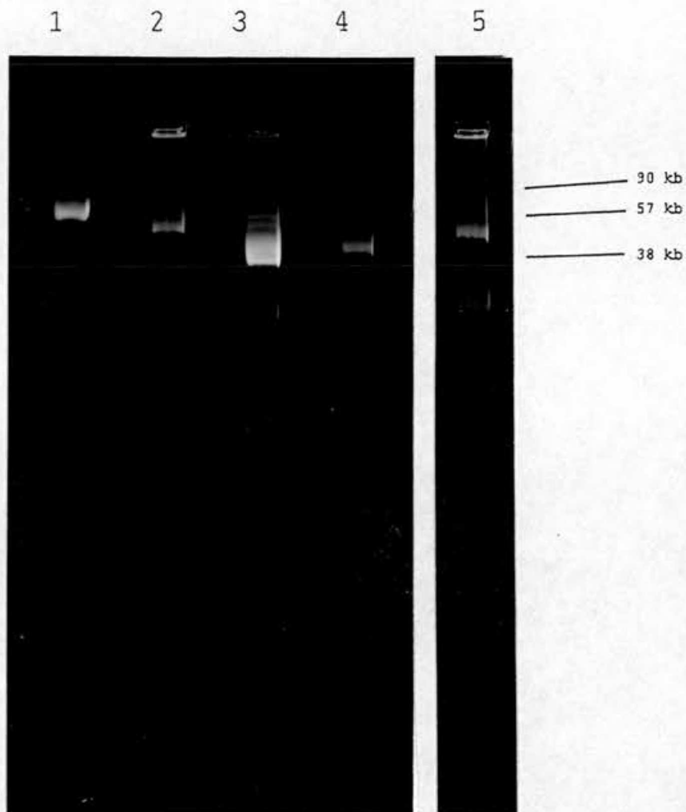
a

ii. Preliminary Analysis of E coli J62(Sa-1::Tn4135)

DNA from E coli[1] J62(Sa-1::Tn4135)[a] was prepared by the method of Takahashi and Nagano (1984) and analysed by agarose gel electrophoresis, in comparison with DNA isolated from standard plasmids. Figure 5.1 indicates the results of electophoresis on 0.5% gels run at 70 v for 16 hours. The plasmid Sa-1::Tn4135 was found to have a molecular size of approximately 60 kb, which does not correspond to single copies of Sa-1 (33 kb) and Tn4135 (3 - 6 kb). Repeated examination of the DNA of E coli J62(Sa-1::Tn4135)[a] confirmed this unusually large size. This transconjugant strain was tested for its resistance to a number of antibiotics and was found to be resistant to Tp, Sm and Sp only , suggesting that neither RP4 nor Sa-1 were present, although DNA analysis had indicated the presence of plasmid DNA.

1 To avoid confusion transconjugants from the initial transfer shall be designated E coli J62(Sa-1::Tn4135)[a] and those from the second transfer E coli J62(Sa-1::Tn4135)[b].

FIGURE 5.1: AGAROSE GEL ELECTROPHORESIS OF E COLI J62 (Sa-1::Tn4135)[a] DNA



TRACK 1. R1

2. RP4

3. R6K

4. Sa-1

5. (Sa-1::Tn4135)[a]

iii. Repeat transfer of Tn4135 to Sa-1 from RP4

In order to check the validity of the initial tranfer result, and confirm that the large molecular species of E coli J62(Sa-1::Tn4135)[a] was as a result of the transfer, and not due to contamination, the above transfer was repeated. Su was used instead of Sm to select for the plasmid Sa to circumnavigate the possibility that Tn4135 may also encode low-level Sm resistance. The transfer frequencies are shown in Table 5.7 and indicate that Tp and Su are not being co-transferred. Transconjugants were found to be resistant to both Ap and Tc, indicating that RP4 has not been lost from the bacterium.

Repeated subculturing, to induce transposon transfer, followed by 5 hour conjugal matings with E coli J53 and J62-2 failed to produce a transconjugant encoding Tp and Su on the same molecular species. After subculturing twice, strain E coli J62-2(Sa-1, RP4::Tn4135)[b] was therefore conjugated with E coli J53 for one hour, (Table 5.7), selecting on DM plates containing J53 supplements plus Tp, Su or TpSu. Analysis of these transconjugants indicated that, like E coli J62-2(Sa-1::Tn4135)[a], E coli J53(Sa-1::Tn4135)[b] was resistant to Tp, Sm and Sp, but was additionally resistant to Ap, suggesting the presence of the Ap transposon from RP4. Repeated transfers failed to eliminate Ap resistance. Therefore 100 purified colonies of E coli J53(Sa-1::Tn4135)[b] were transfered on to nutrient agar plates with sterile sticks and replica plated on to DM plates supplemented for J53 and containing Ap, to look for loss of Ap resistance. All colonies were found to be resistant to Ap. No conclusive results could be drawn from plasmid DNA analysis of E coli J53(Sa-1::Tn4135)[b] transconjugants, because of the presence of the Ap transposon, but the results would appear to indicate the presence of a plasmid of comparable size to that of E coli J62-2(Sa-1::Tn4135)[a], indicating the genuine finding of an unusually large molecular species.

TABLE 5.7a: TRANSFER FREQUENCIES FOR THE REPEAT TRANSFER OF Tn4135 FROM RP4 TO Sa-1

MATING	SELECTION MEDIA	TRANSFER FREQUENCY PER DONOR CELL
1	Tp Su	6.70×10^{-5}
2	Tp	1.85×10^{-1}
	Su	8.54×10^{-6}
	Tp Su	9.49×10^{-7}
3	Tp	2.10×10^{-1}
	Su	2.20×10^{-5}
	Tp Su	1.00×10^{-6}

TABLE 5.7b: TRANSFER FREQUENCIES FROM THE CONJUGATION OF E COLI J62-2 (Sa-1::Tn4135)[b] WITH E COLI J53

MEDIA	TRANSFER FREQUENCY
Tp	4.0×10^{-1}
Su	1.5
Tp Su	2.8×10^{-1}

TRANSFER OF Tn4135 FROM THE ORIGINAL PIG ISOLATE, P-20, TO Sa-1

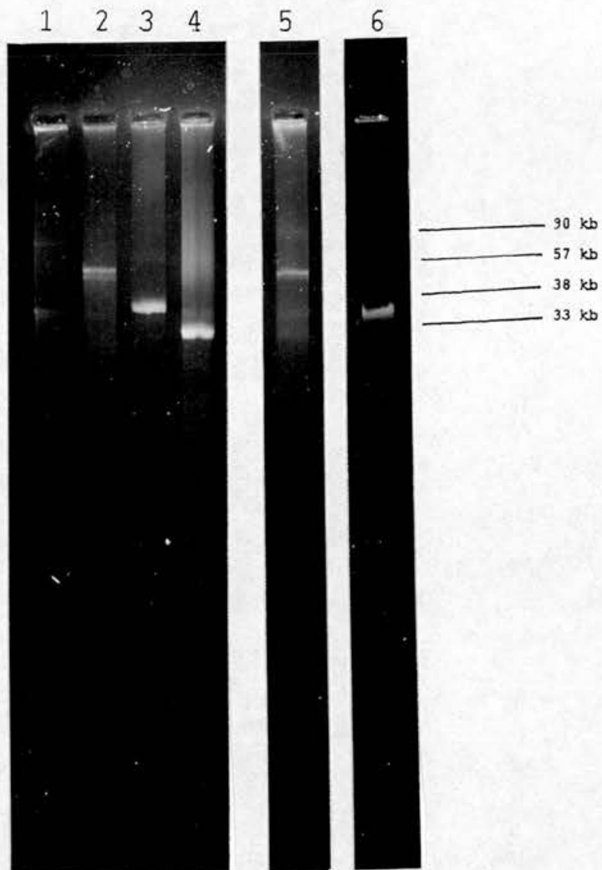
As a result of the unusually large size of the molecular species harboured by E coli J62(Sa-1::Tn4135)[a], and due to the possibility that the presence of RP4 may have contributed to this result, Tn4135 was mobilised directly from the original pig isolate, P-20, with Sa-1.

Sa-1 was transferred into P-20[F] in a 5 hour mating selecting on DSTA plates containing Ka (Table 5.8, mating 1). After purification and subculturing of transconjugants a single colony was used as a donor in a 5 hour mating with E coli J62-2, selecting on DM plates containing supplements for the J62 strain plus Rif and Tp, Ka or TpKa together (Table 5.8, mating 2). Resistances of transconjugants were checked and a single colony conferring resistance to Ka, Sm, Sp, Su, Tp and Rif was used as a donor to transfer Sa-1::Tn4135 to E coli J53 (Table 5.8, mating 3). (Sa-1::Tn4135 was also transferred back to E coli J62, transfer frequencies not shown.). E coli J62 transconjugants designated J62(Sa-1::Tn4135)[ORI], to distinguish them from similar transconjugants formed by the transfer of Tn4135 from RP4 to Sa-1 (E coli J62(Sa-1::Tn4135)[a]), were resistance tested and plasmid content analysed by agarose gel electrophoresis. E coli J62(Sa-1::Tn4135)[ORI] was found to be resistant to Ka, Sm, Sp, Su and Tp, and harbour a plasmid of molecular size 40 kb, characteristic of Sa-1 plus a copy of Tn4135 (Figure 5.2, track 6). This does not compare with the molecular size of the plasmid harboured by J62(Sa-1::Tn4135)[a] (Figure 5.2, track 5), suggesting that RP4 is in some way involved in the formation of the large molecular sized species of J62(Sa-1::Tn4135)[a].

TABLE 5.8: TRANSFER FREQUENCIES FOR THE TRANSFER OF Tn4135 DIRECT FROM THE ORIGINAL PIG ISOLATE, P-20^F, TO Sa-1

MATING	MEDIA	TRANSFER FREQUENCY
1	DSTA Ka	3.11×10^{-6}
2	J62 Rif Tp	4.20×10^{-6}
	Rif Ka	4.66×10^{-7}
	Rif Tp Ka	9.30×10^{-7}
3	J53 Tp	2.40×10^{-3}
	Ka	8.00×10^{-4}
	Tp Ka	1.04×10^{-4}

FIGURE 5.2: COMPARISON OF THE PLASMIDS HARBOURED BY E COLI J62(Sa-1::Tn4135)^{a} AND E COLI J62(Sa-1::Tn4135)^{ORI}



TRACK 1. R1
2. RP4
3. R6K
4. Sa-1
5. (Sa-1::Tn4135)^{a}
6. (Sa-1::Tn4135)^{ORI}

TRANSFER OF Tn4135 FROM RP4 TO Sa-2

As a result of the inherant variability of Sa-1 with regards molecular size and absence of the Cm resistance determinant normally associated with this plasmid, and in order to determine whether this contributed to the anomalies observed in J62(Sa-1::Tn4135)[a], Tn4135 was transferred from RP4 to Sa-2. The experimental procedure leading to the formation of E coli J62(Sa-2::Tn4135) was identical with the formation of E coli J62(Sa-1::Tn4135)[a] except Cm was used for selection instead of Sm. Initial transfers were met with problems with the persistance of the Ap transposon of RP4 (results not shown), as experienced with the latter transfers of Tn4135 from RP4 to Sa-1. Replica plating onto DSTA and DSTA plates containing Tp, Cm, and Ap failed to select any colonies that had lost Ap resistance. Transfers were therefore repeated. Table 5.9 indicates the transfer frequencies from a repeated experiment leading to the formation of E coli J62(Sa-2::Tn4135). Resulting transconjugants were found to be resistant to Ka, Sm, Sp, Cm, Su and Tp, characteristic of Sa-2 containing Tn4135, and DNA analysis revealed a plasmid of molecular size 48 kb, also characteristic of the above molecular species. The plasmid Sa-1, as opposed to Sa-2, would therefore appear to play a role in the formation of the large plasmid species harboured by E coli J62(Sa-1::Tn4135)[a].

TABLE 5.9: TRANSFER FREQUENCIES OF CONJUGATION EXPERIMENTS LEADING TO THE FORMATION OF E COLI J62(Sa-1::Tn4135)[a]

MATING	SELECTION MEDIA	TRANSFER FREQUENCY
INTRODUCTION OF Sa-2	Tp Sm	6.72×10^{-6}
TRANSFER TO E COLI J53	Tp	1.14×10^{-1}
	Cm	8.17×10^{-5}
	Tp Cm	4.54×10^{-7}
TRANSFER TO E COLI J62-2	Tp	1.44×10^{-2}
	Cm	2.62×10^{-2}
	Tp Cm	2.45×10^{-3}

TRANSFER OF Tn4135 FROM THE ORIGINAL PIG ISOLATE TO Sa-2

Due to difficulties in finding suitable selective markers for transfer studies with Sa-1 (Cm resistance is absent, Su is not a good selection marker, Sm may be expressed on Tn4135 and Ka may be expressed on some of the smaller plasmids of the original pig isolate, P-20 - Chapter 3), Sa-2 was used to mobliize Tn4135 from P-20^F in a similar manner to the mobilisation with Sa-1. No transconjugants were obtained, indicating the problems of introducing a 'standard' plasmid into a clinical isolate of unknown restriction and modification background.

TRANSFER OF Tn7 TO Sa-1 AND Sa-2 FROM R483 AND RP4

In order to ascertain whether Tn7 behaved in a similar manner to Tn4135, on transfer to Sa-1 and Sa-2 from RP4 and R483 - the plasmid from which Tn7 was originally isolated - the following transfers were set up. The resulting transconjugants were analysed for resistance markers and molecular size of plasmid content.

i. Transfer of Tn7 from RP4 to Sa-1

Table 5.10 indicates the transfer frequencies and the steps taken to form *E coli* J62(Sa-1::Tn7)^a. Plasmid exchange was initiated in 5 hour standard matings and transposon transfer induced by subculturing. *E coli* J62 and J53 transconjugants were tested for their resistance to unselected markers and were found to be resistant to Ap, Ka, Tc, Sm, Sp, Su and Tp.

TABLE 5.10: TRANSFER FREQUENCIES OF CONJUGATION EXPERIMENTS LEADING TO THE FORMATION OF J62(Sa-1::Tn7)

MATING	SELECTION MEDIA	TRANSFER FREQUENCY
INTRODUCTION OF Sa-1 INTO J62(RP4::Tn7)	DM J62 Tp Su	5.7×10^{-5}
TRANSFER TO E COLI J53	DM J53 Tp	1.20
	Su	4.97×10^{-1}
	Tp Su	2.48×10^{-2}
TRANSFER TO E COLI J62	DM J62 Tp	2.08×10^{-2}
	Su	< 10
	Tp Su	< 10

Due to the failure to eliminate RP4 by transfer, E coli J62(RP4::Tn7, Sa-1) was analysed for spontaneous loss of RP4, utilising phage PRR1 and by replica plating. E coli J62(RP4::Tn7, Sa-1) was subcultured twice in Isosensitest broth containing Tp at 10 ug/ml and challenged with PRR1 in a phage overlay. Resistant colonies were streaked out for single colonies on nutrient agar, before transfering by sterile stick to J62 supplemented DM plates, with and without Ap. All colonies were found to be resistant to Ap indicating the presence of the Ap transposon of RP4. Replica plating of 100 colonies of E coli J62(RP4::Tn7, Sa-1), purified from the mating plates, substantiated this finding. Colonies were replica plated on to DSTA, DSTA + Su and Tp together and DSTA + Tc. Those colonies that were Su and Tp resistant, but sensitive to Tc, were replica plated on to plates containing Ap and all were found to be Ap resistant, indicating the presence of the ampicillin resistance transposon TnA. Agarose gel electrophoresis of Ap, Tp and Su resistant colonies of E coli J62(Sa-1::Tn7), from the replica plates, indicated a plasmid species of molecular size 53 kb which is indicative of Sa-1 (33 kb), a single copy of Tn7 (14 kb) plus a single copy of TnA (6 kb), the Ap transposon of RP4. Resistance testing of these colonies for unselected markers indicated that Ka resistance had been lost, suggesting that Tn7 had inserted into the Ka resistance gene of Sa-1.

ii. Transfer of Tn7 from R483 to Sa-1

Experiments to transfer Tn7 to Sa-1, by introducing Sa-1 into E coli J53(R 483::Tn7) and utilising the Inc Iα plasmid R64 to eliminate R483, were unable to produce a transconjugant with a single plasmid band. Repeated tranfers failed to eliminate R64, and Tp and Ka resistance determinants were not found to be co-transferable.

iii. Transfer of Tn7 to Sa-2 via RP4

Sa-2 was introduced into E coli J62(RP4::Tn7) in a standard 4 hour mating, selecting on DM plates supplemented for J62 and containing Tp and Cm (Table 5.11, mating 1). After purification and subculture a single transconjugant was used as a donor to transfer Sa-2::Tn7 into E coli J53, selecting on plates containing Tp, Cm and TpCm together (Table 5.11, mating 2). Resistance testing of E coli J53(Sa-2::Tn7)[a] colonies indicated 3 different groups of transconjugants (Table 5.12) but the Ap transposon was present in all three classes. Replica plating failed to select any E coli J53(Sa-2::Tn7)[a] colonies that had lost Ap resistance. Agarose gel electrophoresis of those colonies thought to harbour Sa-2, Tn7 and TnA indicated two plasmid bands - one of 33 kb in size, characteristic of Sa, and one of 53 kb, indicative of Sa + Tn7 + TnA. Interestingly, examination of DNA from transconjugants possessing similar resistances to E coli J62(Sa-1::Tn4135)[a], namely Sm, Sp, Tp plus Ap, revealed a plasmid species of molecular size 74 kb. This size compares favourably with the unusually large size of 60 kb for E coli J62(Sa-1::Tn4135)[a], if the Ap transposon is assumed to have a size of approximately 6 kb (Hedges et al, 1974).

iv. Transfer of Tn7 form R483 direct to Sa-2

Tn7 was transfered to Sa-2 using the exclusion property of Proteus mirabilis for Inc Ia plasmids, such as R483. R483::Tn7 was introduced into E coli J53(Sa-2) in a 4 hour mating, selecting on DM plates supplemented for strain J53 and containing Ka and Tp (Table 5.13, mating

TABLE 5.11: TRANSFER FREQUENCIES FOR THE FORMATION OF J53(Sa-2::Tn7)[a]

MATING	MEDIA	FREQUENCY
1	Tp Cm	1.00×10^{-2}
2	Tp	3.24×10^{-4}
	Cm	1.63×10^{-5}
	Tp Cm	2.50×10^{-6}

TABLE 5.12: RESISTANCE MARKERS OF J53(Sa-2::Tn7)[a]

RESISTANCE MARKERS	SUGGESTED DNA CONTENT
Ap Sm/Sp Tp	TnA + Tn7
Ap Ka Tc Sm/Sp Cm Su Tp	RP4 + Sa-2 + Tn7
Ap Ka Sm/Sp Cm Su Tp	Sa-2 + Tn7 + TnA

TABLE 5.13: TRANSFER FREQUENCIES FOR THE FORMATION OF E COLI J53(Sa-2::Tn7)^{ORI}

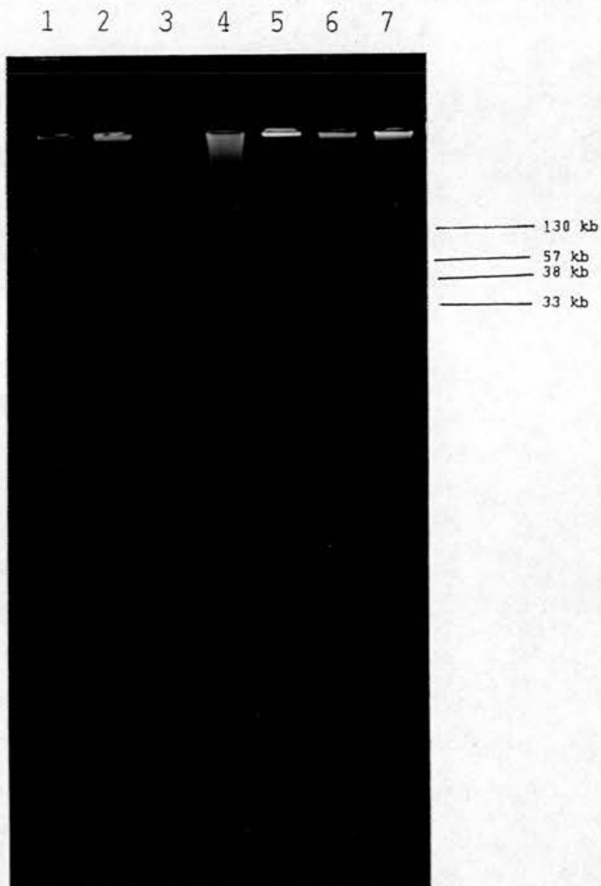
MATING	SELECTION MEDIA	TRANSFER FREQUENCY PER DONOR CELL
1	DM J53 Tp	5.13×10^{-3}
2	DSTA Ka Tp Rif	2.69×10^{-5}
3	DM J62 Tp	2.13×10^{-4}
	Ka	1.87×10^{-4}
	Ka Tp	7.47×10^{-6}
4	DM J53 Tp	7.60×10^{-4}
	Ka	9.13×10^{-5}
	Ka Tp	8.52×10^{-4}

1). After purification and subculture a single colony was used as a donor to transfer (Sa-2::Tn7, R483) to a Rif resistant mutant of P mirabilis in a 4 hour mating. Selection was made on DSTA plates containing Tp, Ka and Rif (Table 5.13, mating 2). Transconjugants were tested for their resistance to unselected markers and a single colony conferring resistance to Ka, Sm, Sp, Su, Tp and Rif was used as a donor to transfer Sa-2::Tn7 to E coli J62 (Table 5.13, mating 3) in a one hour mating. Transconjugants from this transfer were found to be resistant to Ka, Cm, Sp, Sm, Su and Tp, indicative of Sa-2 and Tn7. A single colony was used to transfer Sa-2::Tn7 back to E coli J53 (Table 5.13, mating 4). Transconjugants were found to be resistant to Ka, Sm, Sp, Su and Tp, suggesting that Tn7 had inserted into the Cm resistance gene of Sa-2, or that Sa-2 had been converted to Sa-1 by loss of the Cm resistance gene. Agarose gel electrophoresis of representative colonies proved difficult to interpret because of the presence of a number of bands (Figure 5.3), which probably corresponded to Sa alone and the Sa plasmid plus insertions of Tn7.

TRANSFER OF Tn4135 FROM J62(Sa-1::Tn4135)^{ORI} TO RP4

As a result of peculiarities arising from the transfer of Tn4135 from RP4 to Sa-1, the reverse transfer was set up to analyse suspicions that RP4 may be responsible for the anomalies. RP4 was introduced into E coli J62(Sa-1::Tn4135)^{ORI} in a 5 hour mating, selecting on DM plates supplemented for strain J62 and containing Tp and Tc. The transfer frequency was found to be 5.55×10^{-4} per donor cell. Transconjugants were tested for unselected markers and were found to be resistant to Tp and Tc only. This suggested that Sa-1 and probably RP4 had been lost

FIGURE 5.3: PLASMID DNA ANALYSIS OF E COLI J62(Sa-1::Tn4135)^{ORI} TRANSCONJUGANTS

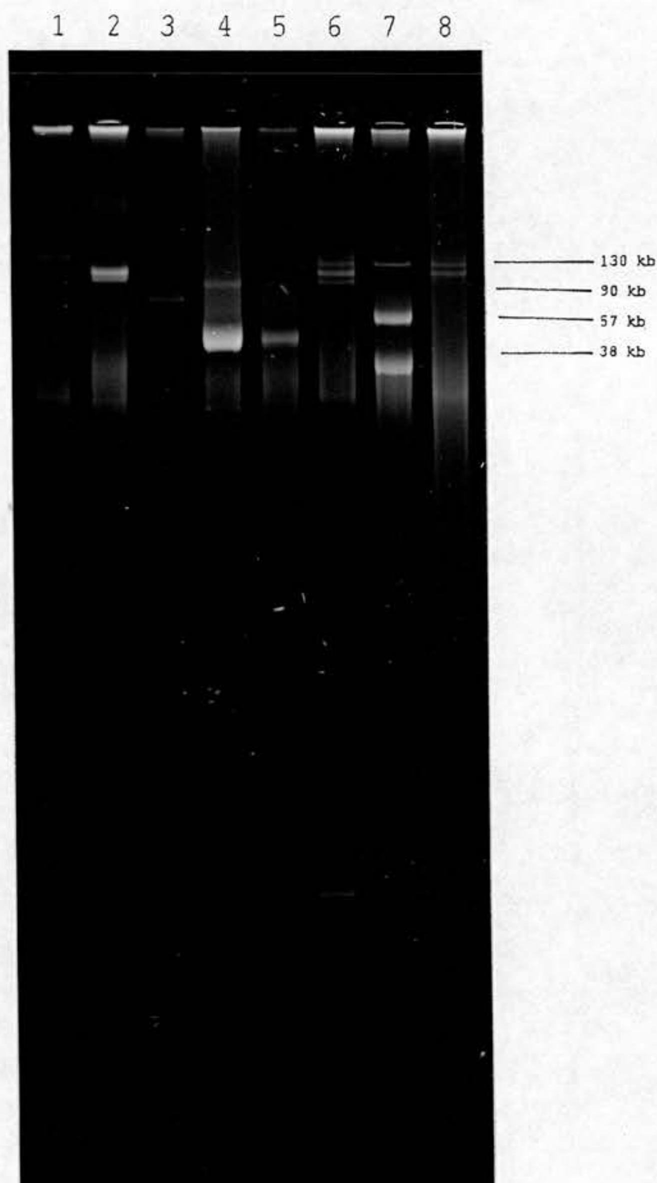


TRACK 1. R40a / Sa-2
2. Sa-1
3. RP4
4. R6K
5. J53(Sa-2::Tn7) transconjugant
6. J53(Sa-2::Tn7) transconjugant
7. J53(Sa-2::Tn7) transconjugant

from the cell, despite the expression ot Tc resistance. Agarose gel electrophoresis of these transconjugants, in comparison with the original pig strain, P-20^{F} and J62(Sa-1::Tn4135)^{ORI} (Figure 5.4), revealed further complications in transfer studies between P-20 and other replicons,. Whilst E coli J62 (Sa-1::Tn4135)^{ORI} was initially found to harbour one plasmid of molecular size 40 kb, its storage (before use in this experiment) resulted in the appearance of a large plasmid (90 kb) similar in size to the large plasmid of P-20^{F}. The introduction of RP4 into this strain would appear to have mobilised some of the smaller bands of P-20^{F}, whilst not being stably maintained itself.

Due to the appearance of multiple bands in E coli J62(Sa-1::Tn4135)^{ORI} after storage, rapid matings were carried out between this strain and E coli J53 and J62, to eliminate the plasmid bands of the original pig. This resulted in the production of a strain with one plasmid band, corresponding to Sa-1 and a copy of Tn4135. This strain, designated E coli J62(Sa-1::Tn4135)$^{ORI(New)}$, was utilised as a recipient for the introduction of RP4, as before, selecting on plates containing TpTc, TcSu and Tp (Table 5.14). Purified colonies from Tp plates were retested for their resistance to Tc, Tp and TpTc together. All colonies were found to be resistant to Tp but sensitive to Tc, indicating that RP4 was not present in transconjugants. This suggested that the previous finding of transconjugants resistant to Tp and Tc was due to the presence of Tc resistance on one of the original pig plasmids. These transconjugants additionally expressed Ka and Sm resistance, indicating

FIGURE 5.4: EXAMINATION OF TRANSCONJUGANTS FROM THE INTRODUCTION OF RP4 INTO J62(Sa-1::Tn4135)^{ORI}



TRACK			
1.	R40a	5.	Sa-2
2.	R1	6.	original pig P-20^{F}
3.	RP4	7.	(Sa-1::Tn4135)^{ORI}
4.	R6K	8.	(RP4, Sa-1::Tn4135)^{ORI} transconjugant

TABLE 5.14: TRANSFER FREQUENCIES FOR THE INTRODUCTION OF RP4 INTO J62(Sa-1::Tn4135)$^{ORI(NEW)}$

SELECTION MEDIA	TRANSFER FREQUENCY PER DONOR CELL
Tp Tc	$< 2.5 \times 10^{-3}$
Tc Su	1.04×10^{-2}
Tp	1.27×10^{-2}

the presence of Sa as well as Tn4135. Subsequent resistance testing of purified colonies from the TcSu transfer plates indicated these transconjugants to be resistant to Ap, Ka, Tc, Sm, Sp, Su but not Tp. These results would suggest an incompatibility between RP4 and the Tp encoding element: transconjugants harbour either RP4 and Sa, or Sa + the Tp encoding element Tn4135, but never RP4 and Tn4135.

DISCUSSION

The appearance of conjugally transferable resistance has been a recognised consequence of the use of antibiotics for some 25 - 30 years (Mitsuhashi, 1969). Resistance has been appearing in bacterial species and genera in which it was at first quite unexpected. The use of any new drug, to which resistance is intially unusual, provides an opportunity to study the evolution and epidemiology of bacterial resistance and Tp is no exception. In fact, the study of resistance plasmids conferring Tp resistance has given a rare opportunity to observe the emergence and development of a completely new plasmid borne resistance. The widespread use of Tp has lead to the spontaneous emergence of resistance plasmids in different areas, and lately Tp resistance determinants have been recognised on transposons. The spread of the Tp resistance determinant is uncharacteristic of conventional resistance plasmids, in that it can spread rapidly amongst resistance plasmids of different Inc groups and thus through bacterial populations of different genera . This promiscuity has been attributed to its residence on transposons, leading to a high degree of flexibility. Tn7 has accounted for much of the high level transferable and non transferable Tp resistance (Barth et al, 1976; Datta and Richards, 1981).

Whilst the transfer potential of Tn7 is well documented, that of the smaller Tp only transposons is uncertain. This study has revealed anomalies, so far undescribed in Tn7 transposition, in the transfer of the Tp only transposon - Tn4135 - to and from different replicons. Its transfer from the Inc P plasmid RP4 to the incompatability group W plasmid, Sa-1, resulted in a molecular species of size in excess of that

characteristic of Sa-1 and a single copy of Tn4135. Whilst resistance testing suggested that neither RP4 nor Sa-1 were present, and therefore Tn4135 may have integrated into the chromosome, agarose gel electrophoresis indicated that plasmid DNA of some kind was present.

There are a number of possible explanations for the appearance of a molecular species of this size, which relate to events taking place at the molecular level of both transposition, and plasmid transfer between replicons. It is possible that Tn4135 has multiply inserted into the Sa-1 plasmid resulting in insertional inactivation of Ka and Su, the resistance markers of Sa-1. Although possible this seems unlikely because of the large increase in size: approximately 38 kb to over 60 kb. Alternativly a natural amplification may have occurred resulting in an increase in plasmid size, or Tn4135 may have picked up some of the RP4 genome (lacking the RP4 resistance determinants), during aberrant excision, and transferred this to Sa-1. The involvement of RP4 in the formation of this large species, although not necessarily the cotransfer of RP4 DNA, was confirmed by transferring Tn4135 direct from P-20 to Sa-1 without an intermediary RP4 step. The plasmid species generated was that characteristic of Sa-1 and a copy of Tn4135. The apparent instablility of the Sa-1 plasmid could also help to explain the observations and, in fact, the transfer of Tn4135 from RP4 to Sa-2 did reveal that Sa-1 not only differed from Sa-2 in its expression of resistance markers, but also in its involvement with Tn4135 transposition: Sa-2::Tn4135 was of predicted size. This implication that Sa-1 instability was involved, however, was not substantiated by experiments with Tn7, although it is reasonable to assume that a combination of Sa-1 instability and the presence ot the small Tp only transposon may be required to produce the large molecular species. The transfer of Tn7 from RP4 to Sa-1 produced a

plasmid species indicative of Sa-1 with Tn7 inserted into the Ka resistance gene. Experiments with Sa-2, however, produced some transconjugants with similar resistances to E coli J62(Sa-1::Tn4135)[a] ie Sm/Sp Tp (plus Ap), and agarose gel electrophoresis indicated that the plasmids were larger than those expected for Sa-2::Tn7. This finding coupled with the results for E coli J62(Sa-1::Tn4135)[a] not only indicates that both Tn7 and Tn4135 are involved in the formation of aberrant plasmid species, but is further evidence for the molecular relatedness of these two transposons.

Other possibilities to explain the results are that the large plasmid species is a contaminant or that it is a fusion product of either or both RP4 and Sa-1 plasmids, mediated by Tn4135. Results of repeated transfers of Tn4135 to Sa-1 from RP4, to check the reproducibility of the experiment, despite interference with Ap resistance, would tend to rule out the former explanation, as the same large molecular species was created again. Replicon fusion, on the other hand, with loss of the resistance determinant regions of RP4 and Sa-1, could result in the observed species. Bennett et al (1986) have suggested that there are potential alternatives to 'conventional' transposition and reciprocal recombination as a means to effect plasmid DNA rearrangements. The existance in nature of dual replicons, constructed relatively easily in the laboratory, have been suggested by Guerry et al (1974), Hedges et al (1975), Jacoby et al (1976), Olsen and Wright (1976) and this is a potential threat to the spread of resistance. A gene that is not transposable and which resides on a non transmissable plasmid, could , by replicon fusion, become part of another plasmid with a much wider host range. The involvement of transposons ie Tn4135, in this replicon fusion would be analogous to cointegrate formation (Grindley and Reed, 1985;

Schmitt, 1986) or, alternatively, IS elements could mediate the fusion.

A comparison of transfer frequencies for Tn7 and Tn4135, while not conclusive, indicated that both these transposons transfer with a similar frequency between RP4 and Sa plasmids. In both cases transfer takes place 100 fold more readily between RP4 and Sa-1 than between RP4 and Sa-2, although why this should occur is uncertain. No firm conclusion can be drawn, however, because of the interference and persistance of Ap resistance. The presence of Ap resistance on a transposon - TnA - and its world wide spread amongst bacteria of many different genera is now well documented (Heffron et al, 1977). Ap is heavily used in the community and the increased use of Tp may result in analogous spread, although so far Tn7 is not as widely distributed.

Further evidence for the continuing diversity of drug resistance genes and their transfer, arose from studies of the back transfer of Tn4135 ie its transfer from $(Sa\text{-}1::Tn4135)^{ORI}$ to RP4, as opposed to the transfer from RP4 to Sa-1. The appearance of a large plasmid of 90 kb, on storage of a 40 kb plasmid containing strain, E coli J62 $(Sa\text{-}1::Tn4135)^{ORI}$, further reiterates the instability of the P-20 system described in chapter 3. While it was assumed that Tn4135 had been mobilised by the intoduction of Sa-1 into the original pig strain, it is possible that the smaller plasmids of P-20 were comobilised. Their integration into the bacterial chromosome, in a similar fashion to that in the transconjugant HI (Chapter 3), would explain the initial appearance of a 40 kb plasmid. Their subsequent excision from the chromosome after storage and their fusion with the 40 kb plasmid could then explain the observation of a 90 kb plasmid. Reappearance of the small P-20 plasmids, probably by reexcision from the 90 kb plasmid, occurrred on introduction of RP4, but RP4 itself was conspicuous by its absence from the resulting Tp resistant

transconjugants. This phenomenon of mobilisation of small plasmids by a conjugative plasmid that is itself not detectable, has been described by Flett et al (1981). The ability of transconjugants to harbour both Sa and RP4, or Sa and Tn4135 but not RP4 and Tn4135, as determined by further examination of colonies, suggests a transposon mediated incompatibility function. This result is unexpected, since initial studies of Tn4135 were performed with E coli J62(RP4::Tn4135).

These results may be further evidence for the existence of two different Tp resistance determinants in the original pig strain P-20 (chapter 3): one mobilised from P-20 by RP4 (E coli J62(RP4::Tn4135)) with similar transfer properties to Tn7 and resulting in a plasmid of unusually large molecular size on tranfer to Sa-1, and a second, mobilised directly by Sa-1 which carries an incompatibility P function, preventing its cohabitation in a strain with RP4. These genes are likely to reside on the large P-20 plasmid and one of the smaller plasmids (Chapter 3). Datta et al (1979) isolated an E coli strain with two unrelated Tp resistance plasmids, so this finding is not new. It is probable, therefore, that P-20 is an example of a strain possessing two systems for the transfer of Tp resistance: transposition (possibly form the large plasmid) and mobilisation of small non transferable plasmids. Datta and Barth (1976) indicated that two methods were available to R483, the plasmid from which Tn7 was first isolated, to manipulate the movement of Tp markers - transposition and integration of R483 into the chromosone, although this latter method was less flexible. (R751 has also been shown to integrate into the chromosome of Bacteroides species - Shoemaker et al, 1986). Other reports (Towner, 1981; Towner et al, 1982; Popadopoulou et al, 1986;) have also indicated the coexistance of two genetic systems

(transposition and self-transmissible plasmids) which may have accounted for the rapid dissemination of resistance to Tp in bacteria isolated from human and veterinary specimens.

With the possibility that isolates may contain more than one system for the transfer of Tp resistance, there is clearly a need to monitor the effects of changing selection pressures on these systems. The transfer systems are both complex and dynamic and are subject to the effect of many selective pressures from within and external to the cell. The joint existence of elements capable of transferring and transposing Tp resistance, the particular properties of these elements, and the widespread use of Tp in humans and animals help to explain the emergence and continued spread of Tp resistance in different recipients.

CHAPTER 6

DETERMINATION OF THE ORIGIN OF *E.COLI* J62(Sa-1::Tn*4135*)[a] DNA

INTRODUCTION

The evolution of resistance via plasmid DNA rearrangements is well established and molecular studies of related, but different R plasmids have clearly established the role of transposable elements (Chapter 4) in many of these events. Transposition, together with reciprocal recombination, using transposable elements (Schmitt, 1986) and other regions of DNA (Peterson and Rownd, 1985) as units of DNA homology provided an adequate explanation of the behaviour of plasmid complexes (Labigne-Roussel et al, 1981, Nies et al, 1986). However, it has become apparent that there are potential alternatives to conventional transposition and reciprocal recombination as a means to effect plasmid DNA arrangements and the generation of aberrant forms.

Rearrangements involving DNA sequences on two independent replicons can generate dual replicons; defined as a single DNA molecule with two independent origins of replication (Bennett et al, 1986). Such molecules are likely to express dual incompatibility properties and probably demonstrate unidirectional incompatibility towards plasmids similar to either of the constituents, since their survival will not be dependent on one specific origin of replication (Bennett et al, 1986). These replicons are relatively easy to construct in the laboratory, and their existence in nature has been attested by a few references to plasmids which have arisen, or thought to have arisen, as the result of illegitimate recombination between plasmids of different incompatibility groups (Guerry et al, 1974; Hedges et al, 1975; Jacoby et al, 1976; Olson and Wright, 1976) and plasmids carrying two origins of replication (Crosa et al, 1975; Crosa et al, 1976; Clerget et al, 1982). Although such

recombination events are expected to be rare, the potential of dual replicons can not be ignored. The fusion of a non-transmissible plasmid, carrying a non-transposable gene, with a plasmid of broader host range, would have far reaching consequences to clinical medicine. Both Insertion sequences (IS elements) (Kleckner, 1981; Grindley and Reed, 1985) and transposons (Heritage and Bennett, 1985) can mediate the formation of such hybrid replicons; that consist of both donor and target replicons with a copy of the transposable element at each of the junctions formed between the two plasmids. Such structures will inevitably show a degree of instability (Nies et al, 1986) depending on the size of the transposable element involved, (the larger the element, the greater the degree of homology, and thus the greater the potential for resolution of the hybrid replicon by reciprocal recombination) and the physical nearness of the two participating elements on the cointegrate. When IS elements themselves are involved, the cointegrates formed are relatively stable. R-plasmids such as R1, R6 and R100 have structures consistent with their formation via IS1 mediated contegrate formation and Clerget et al (1982) have demonstrated that both sections of these plasmids carry sequences capable of functioning as replicative origins. However, cointegrates mediated by transposons, such as Tn3, and formed as a normal consequence of the transposition process (Arthur and Sherratt, 1979; Shapiro, 1979) are normally only transient owing to the site specific recombination systems encoded by the transposon. However, it has been reported that derivatives of these elements lacking one of the short terminal inverted repeats, that are characteristic of all transposons (Kleckner, 1981; Grindley and Reed, 1985; Schmitt, 1986), although no longer able to mediate normal transposition, can mediate a transposition like recombination that generates replicon fusions (Heritage and Bennett, 1985). Although this

Tn3 like system is artificial a similar event has been shown to occur in nature with the Km transposon of the small 8.6 kb pUB2380 plasmid, isolated from a strain of E coli, obtained from farm sewage. The length of DNA transposed, however, is not uniform, but a proportion of the products are replicon fusions. There are also one or two reports of recombination between plasmids via site specific recombination utilising replication origins (Kilbane and Malany, 1980; Hirshel et al, 1982; Clerget, 1984; O'Connor and Malamy, 1984).

An alternative means of generating DNA species of increased size is afforded by DNA amplification: a mechanism whereby cells can alter their phenotypic expression when increasing amounts of specific proteins are required (eg during development) and, when faced by an environmental challenge, can over produce specific proteins, thus conferring resistance to otherwise lethal cytotoxic agents (Montogomery et al, 1983). The duplication and amplification of DNA regions has played a relevant role in evolution, but the significance of the phenomenon in gene regulation and development has yet to be fully recognised. If the natural transfer of genetic material is assumed to be a rare event, the advantages of transferring high levels of resistance in one step are obvious and this would have far reaching consequences on the administration of antibiotics; effective chemotherapy would become even more limited. The beginings of such an event may have been realised with the discovery of resistance genes capable of amplification within a transposon. Eg. the 10.7 kb transposon Tn1721 of the E coli plasmid pRSD1 was found to form multiple duplications of a 5.3 kb region specifying Tc resistance (Schmitt et al, 1979; Mattes et al, 1979; Wiebauer et al, 1981).

The amplification of specific DNA segments has been observed both in prokaryotes (Horiuchi et al, 1963; Rownd et al, 1975; Yagi and Clewell, 1976; Anderson and Roth, 1977; Meyer and Iida, 1979; Chandler et al, 1979; 1982; Fishman and Hershberger, 1983; Spies et al, 1983; Stark and Wahl, 1984; Tlsty et al, 1984) and Eukaryotes (Schimke, 1982; Stark and Wahl, 1984; Hamlin et al, 1984). It has been implicated in the development of drug resistance in bacteria (Foster, 1983) and in eukaryotic cells (Schimke, 1982; Scotto et al, 1986), eg. the amplification of the mammalian DHFR gene is well documented (Alt et al, 1978; Federspiel et al, 1984). Additionally amplification has been shown to be involved in normal cellular development (Long and Dawid, 1980; Spradling and Mahowald, 1980; Chisholme, 1982) as well as in oncogenic transformation (Della Favera et al, 1982; Collins and Groudine, 1982; George, 1984; Little et al, 1983) and in aging (Shmooklert Reis et al, 1983)

Some aspects of gene amplification in bacteria appear strikingly similar to facets of gene amplification in higher cells (Schimke, 1982). However, much of the work on DNA amplification has centred on bacterial drug resistnce genes because of the suitability of this system for investigating the genetic and molecular basis of the amplification process, and because of the significance of this process to the spread of bacterial drug resistance. The selective amplification of resistance genes carried by bacterial plasmids - a phenomenon often called R-factor transitioning (Peterson and Rownd, 1983; Rownd and Mickel, 1971) - and some phages, has been observed in several genera, including E coli (Mattes et al, 1979; Meyer and Iida, 1979), P mirabilis (Rownd and Mickel, 1971; Rownd et al, 1975) and Streptococcus faecalis (Clewell et al, 1974; Yagi and Clewell, 1976,1977). Gene amplification itself has been detected in

most regions of the bacterial genome (Anderson and Roth, 1977; Edlund et al, 1979; Young, 1984), although not at the same frequencey (Folk and Berg, 1971), hot spots for amplification have emerged (Stark and Wahl, 1984). The integration of plasmids into the chromosome has also been shown to lead to amplification (Gutterson and Koshland, 1983; Young, 1983), possibly by providing suitable homologous regions at which recombination can take place. A positive correlation between the level of drug resistance and gene dosage has often been observed (Rownd and Mickel, 1971; Uhlin and Nordstrom, 1977; Edlund et al, 1979; Meyer and Iida, 1979; Clewell, 1981; Schmitt et al, 1981; Scott et al, 1982), although an increase in gene dosage may also have occurred as a result of an increase in plasmid copy number as opposed to the selective amplification of specific regions of DNA carrying the drug resistance genes (Kontomichalou et al, 1970; Cabello et al, 1976; Futcher, 1986). It is, however, widely believed that antibiotics select but do not induce gene amplification (Hashimoto and Rownd, 1975; Iida et al, 1983); that is to say amplified forms already exist at low levels in the environment as a result of recombination or spontaneous amplification. Gene amplification in bacteria, phage and plasmids has been shown to occur spontaneously at a frequency ranging from 10^{-1} to 10^{-5} (Anderson and Roth, 1977). The finding of a return to non amplified forms in bacterial cultures after removal of drug stress, is further evidence for amplification merely being the selective outgrowth of plasmid or host determinants with enhanced amplification potential.

Investigations of the amplification process have involved studying the structure of the molecules (Rownd et al, 1979) and proposing models to explain the observed amplification (Clewell, 1981; Foster, 1983; Mahajan

et al, 1985). It is known that the amplifiable DNA sequences are flanked by directly repeated homologous sequences, often IS sequences (Ptashne and Cohen, 1975; Chandler et al. 1977), although Peterson and Rownd (1983) have shown that sequences other than IS elements can serve as recombination sites, and certain host recombination and replication functions are involved in the amplification process (Yagi and Clewell, 1980; Foster, 1983; Iida et al, 1983; Spies et al, 1983; Chandler and Galas, 1983). Work by Mahajan et al (1985) confirmed the involvement of the Rec A gene product in amplification and implied that the amplification process was dependent on a large number of host genes, some of which were not involved in recombination.

Between 1965 and 1970 there was considerable controversy as to the exact nature of the R factors involved in amplification and this appeared to vary with the host. Japanese coworkers supported the view that R-plasmids were single units of transfer and replication (Watanabe, 1963; Mitsuhashi, 1969) , whereas Anderson (1969) in Great Britain believed they were often dissociated independent units - a resistance transfer factor (RTF) carrying the genes for plasmid replication and transfer and the resistance determinant (R-det) carrying the resistance genes. It is now thought that both forms occur, the genetic nature being determined by the particular ecological situation. In E coli and Serratia marcescens RTF and R-dets appeared to be associated in the form of composite structures (Cohen and Millar, 1970; Nisioka et al, 1970; Cohen et al, 1971; Rownd et al, 1972; Clowes, 1972; Kontomichalou et al, 1970), whilst in P mirabilis there is considerable evidence that RTF and r-dets dissociate and reassociate in such a way as to regulate the number of copies of r-dets (and therfore drug resistance genes) per host cell (Rownd and Mickel, 1971) . It was Anderson in 1968 who first proposed that 'under suitable

conditions' many R-factors could dissociate physically into r-dets and RTF and this was confirmed by further genetic experiments (Anderson and Natkin, 1972) and by molecular studies (Milliken and Clowes, 1973). The bacterial R plasmid NR1 of incompatability group FII is one of the most extensively characterised systems of antibiotic resitance gene amplification (Hashimoto and Rownd, 1975; Peterson and Rownd, 1985). The drug resistance genes, apart from Tc resistance (Miki et al, 1978; Rownd et al, 1978) are separated from the rest of the plasmid by IS1 elements in direct orientation (Hu et al., 1975; Rownd et al, 1979). These elements serve as recombination sites for amplification of the r-det region in drug containing medium (Chandler et al, 1977; Silver et al, 1980; Peterson and Rownd, 1985), resulting in tandem r-det multimers. Although the r-det region is present as an independent supercoiled molecule, under certain conditions, it does not appear to be a replicon (Chandler et al, 1979; Perlman and Rownd, 1975; Rownd and Mickel, 1971; Silver et al., 1980; Chandler et al, 1982b) although Clowes (1972) suggested that this r-det could replicate under relaxed control. Regions other than transposable elements have also been shown to facilitate recombination and other genetic rearrangements eg Peterson and Rownd (1983) implicated a part of the chloramphenicol resistance gene of NR1.

The amplification process itself, has been described in a number of ways (Rownd et al, 1979; Clewell et al, 1979; Wiedemann, 1981; Peterson and Rownd, 1985): the unequal crossing over between the flanking direct repeats of two newly formed copies of a sequence in a partially replicative genome (Edlund and Normark, 1981); the homologous exchange between the flanking direct repeats of the same element, leading to the excision of a small circle which can then reintegrate next to a

preexisting homologous sequence (Yagi and Clewell, 1977). This type of dissociation and reassociation mechanism has been described for NR1 (Rownd et al, 1972). An alternative model involves recombination between two copies of a circular genome at the position of the flanking repeats of the amplifiable sequence, to generate a dimer in which the two copies of this sequence lie in tandem. Resolution of this dimer by crossing over between the direct repeats would generate monomers; one with both copies of the amplified sequence and one with only one copy of the direct repeat (Yagi and Clewell, 1977). Whilst all these models involve postreplicative redistribution of the amplified element among the progeny genomes, leading to clustering of several copies, Mahajan et al (1985) describe a model for Tn9 amplification that takes place under conditions where the normal genomic replication is inhibited. There are thus a number of ways in which extrachromosomal DNA can be amplified and rearranged.

To investigate the possibility that the enlarged molecular species of E coli J62(Sa-1::Tn4135)[a] may have resulted from a spontaneous amplifiction, from replicon fusion of the plasmids RP4 and Sa-1 or from multiple transposition, the nature of this species was examined further.

MATERIALS AND METHODS

BACTERIAL STRAINS

The strains, other than standard strains, utilised in this chapter, and their source, are given in table 6.1.

PHAGE SENSITIVITY BY SPOT TESTING

Nutrient agar plates were flood seeded with overnight broth cultures of the bacteria to be tested. Once dry, 20 ul quantities of the phage suspensions were spotted on to the plate. The plates were incubated overnight and the observation of any plaques was scored as sensitivity.

PHAGE PURIFICATION

Phage overlays were set up with neat, 10^{-2} and 10^{-4} diluted phage, as described in chapter 5. A single large plaque was removed from the agar and placed in 1 ml of nutrient broth. After vortexing, the mixture was centrifuged (2874 g for 1 minute) and the supernatant collected. This was diluted 10^{-2}, 10^{-4} and 10^{-6} in nutrient broth and titred on appropriate plasmid containing bacterial cells. One to two millilitres of nutrient broth was added to the plate displaying confluent lysis and allowed to soak in. The top agar layer was removed by gently scraping the surface of the plate with a spreader. This was pipetted into

TABLE 6.1: BACTERIAL STRAINS

STRAIN	PLASMID/TRANSPOSON	MOLECULAR SIZE	RESISTANCE MARKERS	REFERENCE
E coli J62	(Sa-1::Tn4135)[a]	70 kb	Sm/Sp Tp	Chapter 5
E coli J62	(Sa-1::Tn4135)[ORI]	40 kb	Sm/Sp Su Ka Tp	Chapter 5
E coli J62	HI	$>$ 90 kb	Tp	Chapter 3
E coli J62	(RP4::Tn7)	70 kb	Ap Ka Tc Tp Sm/Sp	Chapter 4

an eppendorf tube and spun in a microfuge (2874 g for 1 minute) to pellet the agar. After transferring the supernatant to a fresh eppendorf, the solution was spun again for 4 minutes to pellet the bacteria. The supernatant was filter sterilised and the phage suspension retitred on an appropriate plasmid containing strain, by overlaying neat, 10^{-2} and 10^{-4} diluted samples.

The methods used for the preparation of media, resistance testing and the standard conjugal mating are as indicated in Chapter 3. Agarose gel electrophoresis for molecular weight determination was performed as described in Chapter 1 and the techniques for phage overlays and restriction enzyme analysis can be found in chapters 5 and 2 respectively.

RESULTS

PRELIMINARY ANALYSIS OF E COLI J62(Sa-1::Tn4135)[a] BY PHAGE SENSITIVITY

The resistance profile of E coli J62 (Sa-1::Tn4135)[a] suggested that neither RP4 nor Sa-1 was present. Therefore, this strain, plus E coli J62(Sa-1), E coli J62 (RP4) and E coli J62 were tested for their sensitivity to phage PR4 (a phage specific for strains harbouring Inc P and Inc W plasmids) with simple spot tests. PR4 lysed Sa-1 and RP4 containing strains as expected and also lysed E coli J62 (Sa-1::Tn4135)[a] indicating that this latter strain contains an Inc W and/or an Inc P plasmid.

INCOMPATIBILITY TESTING OF THE PLASMID HARBOURED BY E COLI J62(Sa-1::Tn4135)[a]

In order to determine whether E coli J62 (Sa-1::Tn4135)[a] harboured an Inc W or an Inc P plasmid, the plasmids RP4 (Inc P) and R7K (Inc W) were individually introduced into E coli J62 (Sa-1::Tn4135)[a] and E coli J62 (Sa-1) in standard 5 hour matings, selecting for the incoming plasmids. Mating plates contained DM base supplemented for strains J62 or J53, plus Ap at 20 ug/ml. Table 6.2 indicates the transfer frequencies for the introduction of RP4 and R7K into the two strains. After subculturing colonies twice on nutrient agar plates with out selection, a single colony was sub-cultured in nutrient broth and incubated overnight, before diluting 10^{-6} and plating on nutrient agar. Twenty of these colonies from each mating were tested for cotransfer of the other resistance markers (Table 6.3)

TABLE 6.2: TRANSFER FREQUENCIES FOR THE INTRODUCTION OF RP4 AND R7K INTO E COLI J53(Sa-1) AND E COLI J62(Sa-1::Tn4135)[a]

DONOR PLASMID	RECIPIENT STRAIN	TRANSFER FREQUENCY PER DONOR CELL
RP4	Sa-1	5.759×10^{-5}
R7K	Sa-1	3.001×10^{-5}
RP4	(Sa-1::Tn4135)[a]	9.550×10^{-1}
R7K	(Sa-1::Tn4135)[a]	4.270×10^{-1}

TABLE 6.3: SENSITIVITY OF TRANSCONJUGANTS TO Ap,Tp AND Sp

TRANSCONJUGANT	Ap	Tp	Sp
E coli J53(RP4, Sa-1)	R	S	R
E coli J53(R7K, Sa-1)	R	S	S
E coli J62(RP4, Sa-1::Tn4135)[a]	R	S	S
E coli J62(R7K, Sa-1::Tn4135)[a]	R	R	R

As expected the resistances indicate that RP4 and Sa-1 coexist in the same cell, and the introduction of R7K results in the loss of Sa-1. The introduction of RP4 and R7K into E coli (Sa-1::Tn4135)[a], however, suggests that the large plasmid of this strain has an Inc P function, since the introduction of RP4 results in the loss of Sa-1::Tn4135 whereas R7K and this large plasmid species can exist stably in the same cell.

Agarose gel electrophoresis of plasmid DNA from representative colonies (results not shown) confirmed the antibiotic sensitivity results; two plasmid species were observed in strain E coli J62(R7K,Sa-1::Tn4135)[a], representative of R7K and the large plasmid species and only one, of a size commensurate with RP4, was observed in preparations of E coli J62 (RP4,Sa-1::Tn4135)[a].

SENSITIVITY OF E COLI J62 (Sa-1::Tn4135)[a] TO PHAGES PR4 AND PRR1

In order to check the hypothesis that E coli J62 (Sa-1::Tn4135)[a] might contain the plasmid RP4 (as suggested by the above incompatibility experiment), or at least the incompatibility region of this plasmid, cells were tested for lysis with phages PRR1 and PR4. E coli strains harbouring RP4, Sa-1, R7k, RP4::Tn4135 and RP4::Tn7 were used as controls to check the selectivity of the PR4 and PRR1 phage suspension. E coli J62(Sa-1::Tn4135)[ORI] was also tested since this strain, unlike E coli J62(Sa-1::Tn4135)[a], had been generated without an intermediary RP4 transfer step.

1. Lysis with stock PRR1 and PR4 phage

E coli J62 (RP4), *E coli* J53 (Sa-1), *E coli* J62 (R7K), *E coli* J62 (RP4::Tn7), *E coli* J62 (RP4::Tn4135), *E coli* J62 (Sa-1::Tn4135)[a] and *E coli* J62 (Sa-1::Tn4135)[ORI] were grown up overnight in nutrient broth (4.5 ml) and diluted as appropriate. The phages PRR1 (neat and 10^{-2} dilutions) and PR4 (10^{-6} dilution) were utilised in phage overlays of the above cultures and the plaque counts are shown in Table 6.4. Unexpectedly, PRR1 lysed *Inc* W plasmid-carrying strains, as well as *Inc* P, which raised doubts as to the specificity of this phage.

11. Determination of PRR1 phage Titre

Due to the unexpected results with phage PRR1, this phage was titred on the *Inc* P plasmids RP4, R751 and the *Inc* W plasmid Sa-1. Overnight cultures of *E coli* strains harbouring these plasmids were set up and PRR1 was diluted 10^{-2}, 10^{-4} and 10^{-6}. Cultures were overlayed and the number of plaque forming units per ml for each bacteria were calculated. Although phage PRR1 lysed Sa containing strains, it did so at a lower efficiency of plating (eop). The phage count on strain J53 (Sa-1) was 8.0 x 10^3 pfu/ml, where as the same phage preparation gave 1.8 x 10^7 pfu on J53 (RP4) and 1.8 x 10^6 pfu/ml on J62 (R751). Therefore, PRR1 genuinely does lyse cells containing the *Inc* W plasmid Sa, albeit at an eop which was 800 times lower than its ability on *Inc* P plasmid containing cells. This is in contrast to previously reported data on this phage.

TABLE 6.4: PLAQUE COUNTS FOR PRR1 AND PR4 LYSIS

STRAIN	PRR1 pfu/ml	PR4 pfu/ml
E coli J53(RP4)	confluent	confluent
E coli J53(Sa-1)	$> 10^8$	3×10^7
E coli J62(R7K)	$> 10^8$	$< 1 \times 10^7$
E coli J62(RP4::Tn*7*)	confluent	confluent
E coli J62(RP4::Tn*4134*)	confluent	confluent
E coli J62(Sa-1::Tn*4135*)[a]	< 10	$< 1 \times 10^7$
E coli J62(Sa-1::Tn*4135*)^{ORI}	4.72×10^5	2.2×10^8

111. Lysis with Purified PRR1

As phage PRR1 was unexpectedly lysing Inc W containing strains, a new phage preparation was purified on E coli cells. The initial titre was 2.6 x 10^7 pfu/ml rising to 3.7 x 10^9 pfu/ml after the final purification. The phage lysis experiment (see 1. above) was repeated with this new purified PRR1 phage diluted 10^{-2} and 10^{-6} and a similar experiment was run in parallel with RP4. (One drop of bacterial culture was used instead of three). As with previous results, PRR1 lysed Inc W plasmid containing strains (Table 6.5) but the eop was 100 fold lower than it was on strains containing the Inc P plasmid RP4. If a difference of 100 fold in eop is considered significant then, contrary to the incompatibility results, the phage lysis experiments indicate that E coli J62 (Sa-1::Tn4135)[a] genuinely does contain the plasmid Sa-1 rather than regions of RP4.

ANALYSIS OF THE E COLI J62 (Sa-1::Tn4135)[a] PLASMID BY CONJUGATION WITH P AERUGINOSA

However, the phage lysis experiments remain inconclusive and thus a conjugation experiment with P aeruginosa was used as an alternative means of determining the presence or absence of the inc P plasmid RP4. (Inc P plasmids are easily transferred and stably maintained within this bacterial species, whilst Inc W plasmids are either not transferred at all, or not as readily.). any differences in transfer frequency between E coli J62(Sa-1::Tn4135)[a] and E coli J62(Sa-1::Tn4135)[ORI] would therefore help to establish the origin of the DNA in the former strain.

TABLE 6.5: PLAQUE COUNTS FOR PURIFIED PRR1 AND PR4 LYSIS

PLASMID SPECIES	PRR1 pfu/ml	PR4 pfu/ml
RP4	9.92×10^9	9.0×10^8
Sa-1	1.00×10^7	4.0×10^7
Sa-1::Tn4135[a]	4.00×10^7	7.0×10^7
Sa-1::Tn4135^{ORI}	7.00×10^8	1.4×10^8

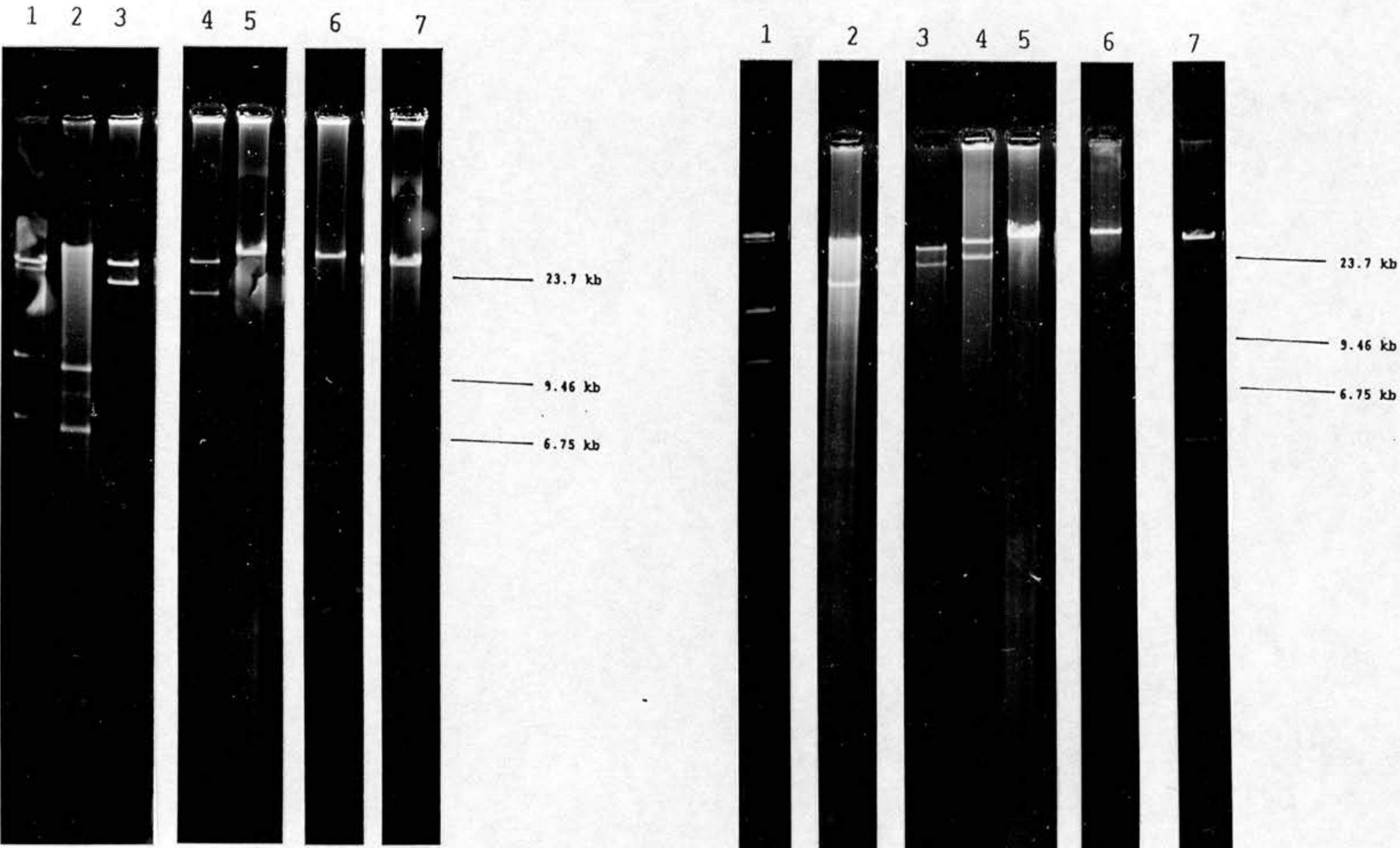
P aeruginosa Pa08, RP4 and Sa-1 controls were tested for their resistance to various selection agents (Ka, Su, Nal and plates containing methionine isoleucine and valine - met ilv - the auxotrophic requirements of P aeruginosa Pa08), to determine suitable markers for the transfer experiments. However, difficulties arose in finding suitable markers to select against E coli and differentiate between P aeruginosa cells devoid of any plasmids, and those harbouring RP4 or Sa-1 controls. Despite RP4 and Sa-1 having differing Ka MIC's, of 140 ug/ml and 40 ug/ml respectively, the intrinsic resistance of P aeruginosa Pa08 to Ka was too high (> 160 ug/ml) in the auxotrophic medium required to select against E coli. Attempts to select against E coli using Nalidixic acid at 10 ug/ml were unsuccessful. The use of sulphonamide resistance, although theoretically capable of distinguishing between individual strains, produced inconclusive results and it was not possible to ascertain the plasmid content of E coli J62(Sa-1::Tn4135)[a] by transfer into P aeruginosa.

RESTRICTION ANALYSIS OF E COLI J62 (Sa-1::Tn4135)[a] DNA

In order to characterise the large plasmid species of E coli J62 (Sa-1::Tn4135)[a] and determine its origin, restriction enzyme analysis was carried out. Figure 6.1 (a, b and c) indicate the results of restricting this strain, and E coli strains harbouring RP4, Sa-1, (Sa-1::Tn4135)[ORI], RP4::Tn4135 and transconjugant H1 with the restriction endonucleases Hind III, Bam H1 and Pst I respectively.

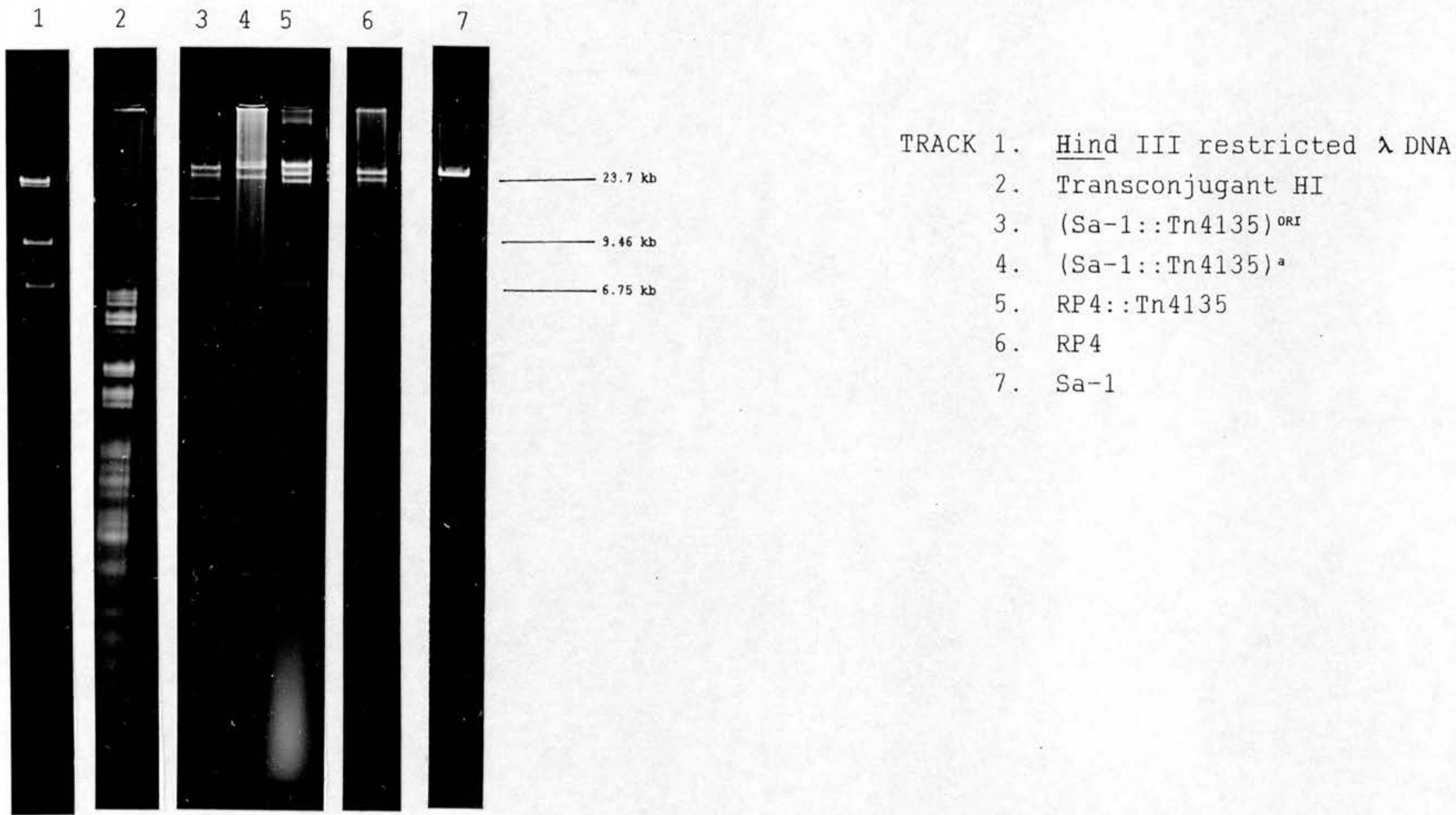
Restriction with all three enzymes indicated differences between E coli J62 (Sa-1::Tn4135)[a] and E coli J62 (Sa-1::Tn4135)[ORI]. Whilst Sa-1::Tn4135[a], like RP4::Tn4135, possessed the two characteristic

FIGURE 6.1: RESTRICTION OF E COLI J62(Sa-1::Tn4135)* IN COMPARISON WITH RP4, Sa-1 AND OTHER TRIMETHOPRIM RESISTANCE PLASMID CONTAINING STRAINS



(a) Hind III digested DNA

(b) Bam HI digested DNA



TRACK 1. <u>Hind</u> III restricted λ DNA
2. Transconjugant HI
3. (Sa-1::Tn4135)[ORI]
4. (Sa-1::Tn4135)[a]
5. RP4::Tn4135
6. RP4
7. Sa-1

(c) <u>Pst</u> I digested DNA

internal Hind III fragments of approximately 2.4 and 2.1 kb, Sa-1::Tn4135^{ORI}, and transconjugant H1, did not. These latter two strains also shared a small fragment (approximately 2 kb) not found in the former two strains. Bam H1 restriction of E coli J62 (Sa-1::Tn4135)^{ORI} indicated that the transposon possessed at least two Bam H1 sites whereas analysis of RP4::Tn4135 and Sa-1::Tn4135^{a} would suggest that Tn4135 lacked any Bam H1 sites. This result reiterates the view that the two transposons are different and therefore have different origins. Bam H1 restriction of Sa-1::Tn4135^{a} DNA, in comparison with Sa-1, indicated a common 21 kb fragment and a possible increase in size of the smaller Sa-1 fragment, inferring, not only that Sa-1 was present in this strain but that the transposon was located in the smaller Bam HI fragment of Sa-1. Pst I digestion of E coli J62 (Sa-1::Tn4135)^{ORI} in comparison with E coli J62 (Sa-1::Tn4135)^{a} also revealed additional bands in the former strain, confirming that the two strains harboured different transposons.

STABILITY OF (Sa-1::Tn4135)^{a} DNA DURING TRANSFER

In order to ascertain the stability of the plasmid harboured by E coli J62 (Sa-1::Tn4135)^{a}, this species was conjugated with E coli J53 in a standard one hour mating. Selection was made on DM plates supplemented for the J53 strain and containing Tp and Sp. (Sa-1::Tn4135)^{a} transferred with a frequency of 1.07×10^{-1} per donor cell and its resistance profile revealed that transconjugants were resistant to Tp Sp and Sm. This result, along with molecular weight analysis which revealed that transconjugants harboured a plasmid of approximately 70 kb, indicated that this molecular species was stable.

DISCUSSION

The evolution of bacterial drug resistance via plasmid DNA rearrangement is well established and many different mechanisms have been implicated in the generation of aberant forms (Nies et al, 1986; Bennett et al, 1986; Saunders et al, 1986; Grinsted, 1986). Genetic rearrangements via mutation, recombinational events and conjugal transfer are taking place all the time and those that prove to be beneficial are selected for and become established. The transfer of Tn4135 from the Inc P plasmid, RP4, to the Inc W plasmid, Sa-1, (Chapter 5) was shown to generate a molecular species of uncharacteristically large size (between 60 and 70 kb as opposed to the expected size of 36 - 40 kb) and it remains to be seen whether this is an evolutionary step that confers a selective advantage on the E coli host. Transfer studies have certainly indicated that this species is stable and can be readily exchanged between E coli J62 and J53 strains without a reduction in size. The mechanisms involved in the generation of E coli J62(Sa-1::Tn4135)[a] are unclear but transposition (Kleckner, 1981), replicon fusion (Bennett et al, 1986) and spontaneous amplification (Schimke, 1982) could all be inferred in the production of this large species.

The use of incompatibilty function to characterise plasmids is well established and the present investigation demonstrates that the molecular species harboured by E coli J62(Sa-1::Tn4135)[a] possesses an Inc P function. The introduction of RP4, an Inc P plasmid (Datta et al, 1971) into the above strain resulted in the loss of the (Sa-1::Tn4135)[a] species, whilst R7K, of Inc group W (Ward and Grinsted, 1982) and therefore of the same group as Sa-1, was able to stably coexist with

(Sa-1::Tn4135)^{a}. Whilst the resistance profile suggested that neither RP4 nor Sa-1 was present, this result confirms that RP4 , or at least the region of DNA encoding the incompatibility function, is present. The above finding, coupled with the large molecular size of the species could be explained by multiple transposition of Tn4135 into RP4. Insertional inactivation or transposition-associated deletion could account for the lack of resistance determinants: Barth and Grinter (1977) have shown that the insertion of Tn7 into RP4 caused deletions. Alternatively the above results could be accounted for by Tn4135-mediated transfer of some of the RP4 genome, after aberrant excision from the Inc P plasmid, or by a Tn4135 encoded incompatibility P function, that somehow switched off the plasmid encoded functions. Transposons are known to be capable of acting as biological switches either by direct insertion (Nies et al, 1986) or by causing polar mutations (Kleckner et al, 1975).

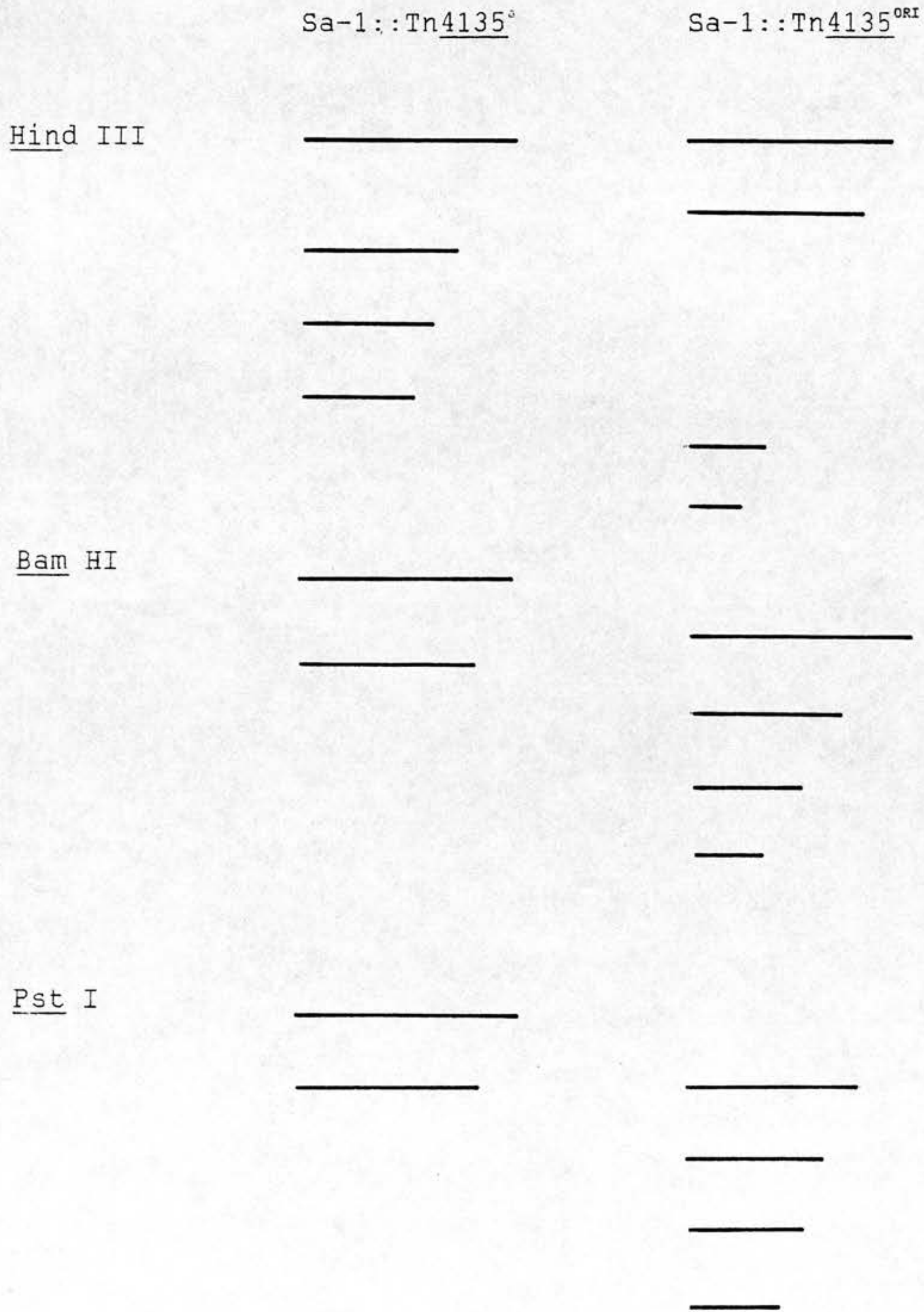
Contradictory results were obtained from phage lysis experiments. Whilst lysis with PR4 - a phage specific for P-, N- and W- group plasmids (Bradley and Rutherford, 1975) which attaches to the tips of the P-1 pili (Bradley and Cohen, 1977; Bradley, 1976) of host cells harbouring one of the above plasmids - indicated that either, or possibly both, an Inc P or an Inc W plasmid were present, Phage PRR1 lysis suggested that only Sa-1 was present. These latter experiments were not conclusive however, because of the poor specificity of the phage PRR1 preparations (Olsen and Thomas, 1973; Bradley, 1974) and they, therefore, did not rule out the possibility that RP4 was present also. If Tn4135-mediated transfer of RP4 DNA was reponsible for the observed increase in size, the PRR1 phage plating frequencies might be expected to be greater for E coli J62(Sa-1::Tn4135)^{a} than for E coli J62(Sa-1::Tn4135)^{ORI}. This, however, was not the case; there being little significant difference in

the eop for the above two strains. Whilst not ruling out this mechanism all together, the results indicate that if RP4 DNA is present, it does not encode the genes reponsible for conferring P-1 pilus production on the host cell.

The above paradox between the presence of RP4 DNA in some experiments and Sa-1 DNA in others, could be explained by replic on fusion (Bennett _et al_, 1986). Although dual replicons are likely to express dual incompatibility properties and demonstrate unidirectional incompatibility towards plasmids similar to either of the constituents, it is possible that one set of Inc functions are switched off or deleted by the fusion process. Just as amplification is thought to pose a biosynthetic burden on the cell, the fusion of two fairly large plasmids could result in a species too large to be accomodated or controlled by the host cell. By recombinational events certain genes, including those for drug resistance, could be lost. Transposon mediated fusions (cointegrates - Bennett _et al_, 1986) are known to be transient; recombination events resulting in their resolution. There is therefore the potential for aberrant resolution leading to the loss of some regions of DNA, but not the total separation into constituent replicons. This situation may be further complicated by the findings of Yusoff and Stannisch (1984). They indicated that RP1, which is physically and genetically indistinguishable from RP4, encodes two fertility inhibitory functions against _Inc_ W plasmids. Whilst not thought to affect surface exclusion properties, one of these functions affects the production of P-1 pili by the host cell and this inevitably effects phage attachment. This might account for phage results suggesting the presence of Sa-1 only, especially if Sa-1 encodes similar functions which affect RP4.

Restriction data, whilst not establishing which mechanism(s) was responsible for the generation of E coli J62(Sa-1::Tn4135)[a], revealed differences in the origin of the extrachromosomal DNA of this species and that of E coli J62(Sa-1::Tn4135)[ORI] (Figure 6.2). A comparison of Hind III fragments indicated that the E coli J62(Sa-1::Tn4135)[a] strain, like E coli J62(RP4::Tn4135), possessed the two characteristic 'Tn7 like' internal fragments (Barth and Grinter,1977), but E coli J62(Sa-1::Tn4135)[ORI], like transconjugant H1, did not. This result is further evidence for the view that the original pig isolate, P-20, contains more than one Tp resistance gene (Chapter 3). This theory is substantiated by the finding of a small (aproximately 2 kb) Hind III fragment common to E coli J62(Sa-1::Tn4135)[ORI] and transconjugant H1 (containing the large original pig plasmid) but absent from RP4::Tn4135 and (Sa-1::Tn4135)[a] containing strains. A comparison of Sa-1 and (Sa-1::Tn4135)[a] Hind III digested DNA confirmed the phage lysis results and indicated that Sa-1, or at least the large Hind III fragment, containing the genes for transfer and replication, was present. It was not possible to determine whether any RP4 DNA was present as well, since Hind III restricts RP4 only once (Barth and Grinter, 1977). Whilst the Hind III fragments of (Sa-1::Tn4135)[ORI] added up to the predicted molecular weight (38 kb) those of (Sa-1::Tn4135)[a] did not, suggesting that more than one copy of some of the fragments may be present. Amplification or multiple transposition would result in an increase in the number of copies of some fragments. Both Sa-1 and Sa-1::Tn4135[a] DNA would appear to be restricted twice by Bam HI, suggesting that Tn4135 has no Bam HI sites. Whilst this contrasts with results of Bam HI restriction of RP4::Tn4135 (Chapter 4), which inferred that Tn4135 had one Bam HI site, it is possible that the position of insertion of Tn4135 into Sa-1

FIGURE 6.2: A DIAGRAMATIC COMPARISON OF THE RESTRICTION FRAGMENTS OF THE TRANSPOSONS HARBOURED BY E COLI J62(Sa-1::Tn4135)^{a} AND E COLI J62(Sa-1::Tn4135)^{ORI}



could have lead to the generation of a fragment too small to be detected. This would result in the two strains appearing to have equal numbers of restriction sites. An increase in the size of the smaller fragment of Sa-1, indicates that the additional DNA may have inserted into the resistance gene portion of Sa-1. Again it is not possible to confirm the presence or absence of RP4 DNA because of the lack of restriction sites. (Sa-1::Tn4135)^{ORI} digestion with BamH1 again indicates that two different systems are being examined; this species has at least two BamH1 sites whilst (Sa-1::Tn4135)^{a} has none. Similar differences were observed after Pst 1 digestion although why (Sa-1::Tn4135)^{a} should possess two Pst 1 fragments when Sa-1 has only one Pst 1 site (Chapter 2) and Tn4135^{a} has no cuts sites; as determined by restriction of RP4::Tn4135 and RP4::Tn7 (Chapter 4), is unclear.

In conclusion, the generation of *E coli* J62(Sa-1::Tn4135)^{a} may have involved more than one of the previously mentioned mechanisms (Figure 6.2). RP4 DNA may have been integrated into the Sa-1 molecule , along with Tn4135, generating homologous sequences at which recombinational events can occur. Such events may have resulted in the deletion of unselected resistance genes as well as the spontaneous amplification of other regions. What is evident is that two different genetic elements are responsible for the formation of *E coli* J62(Sa-1::Tn4135)^{a} and *E coli* J62(Sa-1::Tn4135)^{ORI}.

CHAPTER 7

BIOCHEMICAL ANALYSIS OF THE DIHYDROFOLATE REDUCTASE ENCODED BY THE TRIMETHOPRIM RESISTANCE TRANSPOSON Tn4135

INTRODUCTION

Dihydrofolate reductase (DHFR) catalyses the NADPH-dependent reduction of dihydrofolate (DHF) to tetrahydrofolate, and is therefore usually an essential enzyme in bacterial metabolism. Its inhibition leads to impaired synthesis of protein, RNA, and DNA and is usually lethal for prokaryotic and eukaryotic cells (Blakely, 1969), although recently a bacterial mutant lacking DHFR has been reported (Singer et al, 1985). Due to the crucial role played by this enzyme in providing adequate levels of reduced cofactors, it has long been recognised as a susceptable site for chemotherapy of neoplastic disorders, malaria and certain other diseases (Blakely, 1969).

Over the last 25 years DHFR has attracted intense research interest for several different reasons. As a target for the actions of antineoplastic (Condit, 1971) and antimicrobial folate analogs, such as Tp (Burchall and Hitchings, 1965), the structure of the protein and of its binding sites has received considerable attention (Kisliuk and Brown, 1979). The small size of the protein and the ease of active site labelling have made DHFR one of the best systems for studying evolutionary aspects of protein structure as well as structure function relationships (Mathews, 1979; Stone et al, 1979). The importance of gene amplification as a genetic regulatory mechanism (Nunberg et al, 1978) has been spotlighted, as a result of studies on DHFR overproduction in drug resistant cells, and interest has centred around the possibility that the protein itself may be a genetic regulatory element (Sirotnak and McCuen, 1973; Sheldon and Brenner, 1976). In addition the finding that Tp drug resistance factors code for DHFR's of unusual properties has stirred

interest in the origins and roles of these plasmid encoded enzymes (Amyes and Smith, 1976; Pattishall et al, 1977; Smith et al, 1979). Information about the structure and biophysical parameters of DHFR from diverse sources has already helped in the understanding of its activity and in the design of species specific inhibitors. It may even have given some insight into the characteristics of rapidly proliferating cells such as those found in cancer and during development.

DHFR was first purified to homogeneity from T_4 phages (Erickson and Mathews, 1971) but it has been isolated and purified from many different sources including mouse leukemia cells (Bertino et al, 1965; Perkins et al 1967), mammalian cells (Greenberg et al, 1966; Perkins et al, 1967; Gauldie et al, 1973),trypanasomes such as Crithidia fasciulata (Iwai et al, 1981), protozoa (Diggens et al, 1970), avian sources (Kaufman and Gardiner, 1966), worms and schistosomes (Jaffe and McCormack, 1967), and many bacteria (Blakeley and McDougal, 1961; D'souza et al, 1972; Mandelbaum-shavit and Grossiwicz, 1974; Williams et al, 1977; Dann et al, 1976; Young et al, 1987). The bacterial and phage coded DHFR's have been intensively studied because of their relative abundance and ease of preparation. A number of DHFR genes have also been sequenced (Gleisner et al, 1974; Bennett, 1974; Stone et al, 1977; Bitar et al, 1977; Stone et al, 1979; Zolg and Hanggi, 1981; Swift et al, 1981; Simonson et al, 1983; Brisson and Hohn, 1984) and X-ray crystallography has been used to determine the structure of the DHFR molecules and their enzyme inhibitor complexes (Mathews et al, 1977,1978; Bolin et al, 1982; Volz et al, 1982). The aim of many of the studies has centred on understanding the interaction between the enzyme and tight binding inhibitors such as methotrexate, for the purpose of facilitating rational drug design (Montogomery et al, 1971; Gready, 1980; Cocco et al, 1981). However in

recent years interest has focused on the evolution and classification of DHFR's, and in particular those mediated by plasmids.

In 1972, Fleming et al reported the presence of bacterial R plasmids which conferred high levels of Tp resistance upon their hosts. Later reports (Amyes and Smith, 1974; Skold and Widh, 1974) demonstrated that the R plasmids encoded Tp insensitive DHFR's. These resistant enzymes were found to be much larger than the sensitive chromosomal DHFR, with molecular weights of about 35,000 as compared with 21,000 for the E coli chromosomal enzyme (Amyes and Smith, 1974; 1976; 1978; Pattishall et al, 1977). During the initial identification of the plasmid mediated DHFR's the properties of the enzymes from different plasmids appeared very similar (Amyes and Smith, 1978), the only difference being in the quantity of enzyme produced. However, it was clear from subsequent studies in a French Hospital that some R plasmids (R67, R67bis and R27) encoded DHFR's with very different properties (Pattishall et al, 1977). The enzymes were not only produced in lower quantities but were less susceptible to Tp. The plasmid encoded enzymes were therefore arbitrarily subdivided into two major groups, although it has recently become clear that there are at least four different classes (Amyes, 1986), distinguishable from each other on the basis of molecular size, inhibition profiles and heat lability (Pattishall et al, 1977; Broad and Smith, 1982; Joyner et al, 1984). The type I enzyme (exemplified by the DHFR from R483 - Barth et al 1976) was first identified in Britain on plasmids belonging to only one incompatibility group, but was later found to have spread to other groups (Fleming et al, 1972; Hedges et al, 1972; Datta et al, 1981). This diversity in plasmid host can be explained by the location of this gene on

a transposon, Tn7 (Barth et al, 1976; Elwell et al, 1979). The protein consists of two identical subunits of molecular weight 18,000 (Novak et al, 1983), is heat labile and the level of Tp needed to inhibit the enzyme by 50% is several 1000 fold higher than that required to inhibit the E coli chromosomal DHFR by the same amount. An enzyme with similar biochemical properties to the type I DHFR, but differing markedly in molecular weight - 24,500 (Young and Amyes, 1985a) - was extracted from a clinical plasmid isolated from a urinary pathogen in Edinburgh (Young and Amyes, 1983; 1985a). Like the prototype type I gene, this DHFR is encoded by a transposon, Tn4132, and due to it's strong enzymic similarity to the DHFR of Tn7, is thought to have evolved from the type I enzyme (Amyes, 1986). In contrast, the type II enzyme (exemplified by R67) consists of four subunits, all with a molecular weight of 9,000 (Smith et al, 1979; Fling and Elwell, 1980), is relatively heat stable and is about 100 fold less sensitive to Tp than the Type I, having a K_i value of about 0.15 mM (Amyes and Smith, 1976; Pattishall et al, 1977; Tennhammer Ekman and Skold, 1979), compared with 20 uM for the type I. The type II DHFR gene itself has been described less commonly than the type I gene, in association with the spread of Tp resistance (Hedges et al, 1972; Shapiro and Sporn, 1977; Patishall et al, 1977), but like the type I has been detected in plasmids of different incompatibility groups from Europe and North and South America. Surveillance however, has not been as comprehensive (Fling et al, 1982; Mayer et al, 1985).

Tennhammer Ekman and Skold (1979) reexamined the properties of the DHFR encoded by R751, R388 and R483 and concluded that these three enzymes were distinct from each other. Although R388 and R751 were found to be similar to the type II (Patishall et al, 1977), in terms of heat resistance and quantity of enzyme produced, they differed in their

relative sensitivities to Tp. Broad and Smith (1982) looked at isoelectric focusing as a means of classifying enzymes to type in a more exacting manner, and classified R388 to type II by virtue of having a pI of 5.5. R751 was found to exhibit a unique isoelectric point of pI 7.2 and was therefore thought to constitute a third type of DHFR. However, R751 and R388 were found to be serologically related (Fling and Elwell, 1980), suggesting R751 should be classified as a sub group of type II and not as a separate type.

A third type of plasmid Tp resistant DHFR (type III), encoded by plasmid pAZ1 (Anderson, 1980) has recently been identified and characterised (Fling et al 1982; Joyner et al, 1984). Unlike the type I and type II DHFR's, this enzyme was found to be monomeric (molecular weight 16,900), much more sensitive to Tp (K_i = 19 nM) and antiginically distinct from type I and II DHFR's. Additionally, strains harbouring pAZ1 were found to be only moderately resistant to Tp (MIC = 64 Mg/L) (Joyner et al, 1984), in contrast to the characteristic high level resistance expressed by strains harbouring Tp resistant plasmids encoding the type I or type II DHFR. This phenomenon has also been reported by Towner and Pinn (1981) who speculated that the plasmid pUN212 specified an intermediate type of DHFR, more sensitive to Tp than the 'normal' plasmid encoded reductases.

In 1985 Young and Amyes reported the detection of a new group of Tp R plasmids, derived from, bacteria isolated in Southern India (Young et al, 1985;1986), which also conferred a moderate level of resistance on their host. Characterisation of the DHFR specified by the plasmid pUK1123 (Young and Amyes, 1986a) indicated that the enzyme differed both physically and biochemically from all previous plasmid DHFR's and , in addition, was unique in it's capacity to be induced in the presence of

increasing concentrations of Tp. The molecular size of the pUk1123 encoded enzyme, designated a type IV, was found to be larger (46,700) than any other plasmid DHFR, although there has been a report that the type II enzyme of R67 may exist in various enzymatically active forms with molecular weights ranging up to 81,000 (Smith et al, 1979). So far, the only other DHFR's which have a molecular size of around 45,000 are from T_4 phages (Purohit et al, 1981). Although the type IV enzyme is less resistant to Tp than the T_4 enzyme (Purohit et al, 1981), Young and Amyes (1986a) postulated that the phage enzyme could have been a precursor to this plasmid DHFR. This view was based on the finding that a large proportion of the type IV DHFR activity was precipitated at 50% ammonium sulphate saturation; a property which was thought to be unique to the phage encoded DHFR (Purohit et al,1981; Erickson and Mathews, 1973).

Due to the increase in incidence of pathogenic bacteria with high level Tp resistance (Datta et al, 1981; Towner and Wise, 1983) it has been of considerable interest to determine the origins of plasmid encoded enzymes. However little progress has been made in this area and comparisons of enzyme properties have not proved useful. It seems unlikely, however, that these enzymes arose from an E coli chromosomal Tp-sensitive ancestral enzyme, due to the molecular weight differences between the chromosomal and plasmid mediated enzymes. A comparison of the amino acid sequences of various DHFR's does not support the view that the plasmid mediated Tp resistant enzymes are of chromosomal origin either (Simonson et al, 1983), although there is sufficient homology between the type I and other DHFR's to indicate relatedness (Doolittle, 1981). No obvious homology has been shown to exist between the type II DHFR and any other sequenced bacterial or vertebrate DHFR (Smith et al, 1979; Stone and Smith, 1979), indicating that this enzyme has an evolutionary origin

distinct from other DHFR's (Zolg and Hanggi, 1981; Swift et al, 1981; Fling and Richards, 1983). It has previously been suggested that the tetrameric type II enzyme may have arisen from an oxidoreductase unrelated to DHFR (Smith et al, 1979). The type III enzyme, on the other hand, has several properties similar to those of normal bacterial DHFR's (Joyner et al, 1984) so it is quite feasible that the type III enzyme is identical to the chromosomal DHFR of an (unidentified) bacterial species, that is intrinsically resistant to moderate levels of Tp. Alternatively, DHFR could have evolved from a reductase with another substrate (Foster, 1983), but this event would have to have occurred at least twice since there is no detectable homology between the genes encoding the type I and type II enzymes (Zolg and Hanggi, 1981). Just as the type IV enzyme appears to have a molecular size similar to that of the T_4 phage (Purohit et al, 1981; Young and Amyes, 1986a) so the type I and II enzymes would appear to have molecular weights similar to the T_6 bacteriophage emzyme - namely 31,000 (Mathews and Sutherland, 1965). Purohit et al (1981) postulated that the type I enzyme may be related to that of T_4 phages, due to their similarity in Tp resistance levels; although significant differences do exist (Mosher et al, 1977).

Further intrigue, with respect to the origins of DHFR genes, has recently arisen with the characterisation of a DHFR from a multi resistant *Staphylococcus aureus* (Young et al, 1987). The enzyme designated S1, differs both physically and biochemcally from all previous plasmid DHFR's. The molecular weight is similar to that of the *S aureus* chromosomal enzyme, although the other properties suggest it is not related to the bacterial enzyme. Hybridization studies (Archer et al, 1986) also showed no homology between an *S aureus* plasmid encoded Tp

resistance gene and the S1 enzyme. Structural studies have indicated that the S1 protein is monomeric, and in that respect similar to the type III plasmid enzyme. However, in physical properties the S1 enzyme is most similar to the type I gram negative plasmid DHFR, and more especially the smaller type Ib variant encoded by Tn4132 (Young and Amyes, 1985). S1 and the type Ib enzyme are unlikely to be related to each other, however, because of their very different responses to heat and the lack of subunit structure of the S1 DHFR.

The R plasmid DHFR's appear to be unique in both the variety of antifolate compounds to which they are resistant, and in the extreme nature of the resistance. However, there would appear to be considerable inter and intra evolutionary variation amongst DHFR's. The isolation of an additional small transposon, Tn4135, from an animal source, that appeared to encode Tp resistance only, has lead to further speculation about the evolution and spread of Tp resistance. In order to better understand the behaviour of this transposon, in relation to other Tp transposons such as Tn7 and Tn4132, a comprehensive biochemical analysis of Tn4135 harboured by different replicons was undertaken.

MATERIALS AND METHODS

BACTERIAL STRAINS

The strains used in this chapter, other than standard strains are indicated in Table 7.1.

PREPARATION OF 10 LITRE DAVIS-MINGIOLI CULTURES

Ten litre cultures of Davis-Mingioli (DM) medium were prepared by dissolving 70g of dipotassium hydrogen phosphate and 30g of potassium dihydrogen phosphate in 1 litre of distilled water. Litres of distilled water were used to dissolve each of 4.5g of trisodium citrate, 1g of magnesium sulphate heptahydrate and 10g of ammonium sulphate. These were added to the previous 1 litre and the volume was made up to 10 l with a further 6 litres of distilled water. After mixing, 1 litre was decanted and autoclaved. Glucose (40g in 78 mls of distilled water) and appropriate growth supplements were added to the two batches of medium in a ratio of 1:9. The 1 litre of medium was inoculated with the bacterial culture from which the enzyme was to be prepared, and incubated with shaking overnight. This was used to inoculate the 9 litres of medium prior to a further two and a half hour incubation, so that the cells were harvested in logarithmic phase.

TABLE 7.1: BACTERIAL STRAINS

STRAIN	PLASMID/TRANSPOSON	RESISTANCE MARKERS	MOLECULAR WEIGHT kb	SOURCE
E coli J62	RP4::Tn*7*	Ka Tc Ap Tp Sm/Sp	66	Chapter 4
E coli J62	(Sa-1::Tn*4135*)[a]	Ka Sm/Sp	60 - 70	Chapter 5
E coli J62	(Sa-1::Tn*4135*)[ORI]	Ka Su Tp Sm/Sp	40	Chapter 5
E coli J62	(Sa-2::Tn*4135*)[a]	Ka Su Cm TP SM/Sp	48	Chapter 5
E coli J62	(Sa-2::Tn*7*)[ORI]	Ka Su Cm Tp Sm/Sp		Chapter 5
E coli J62	(HI)	Tp	90 - 100	Chapter 3

PREPARATION OF DIHYDROFOLATE REDUCTASE

Overnight nutrient broth cultures were harvested by centrifugation (Sorvall RC-5B Dupont superspeed: 12,200 g for 20 minutes). Cells were washed in DM base and repelleted by centrifugation (12,200 g for 20 minutes), before resuspending in a minimum volume of buffer A (50 mM sodium phosphate, pH 7.4: 10 mM B-mercaptoethanol and 1 mM EDTA) (Amyes and Smith, 1974). The bacteria were disrupted ultrasonically with constant cooling (MSE Soniprep 150: 8 um for 3 x 1 minute) and the lysate cleared by centrifugation (40,000 g for 1 hour), as described by Amyes and Smith (1976). The volume of the supernatant was measured.

PURIFICATION OF DIHYDROFOLATE REDUCTASE

The DHFR was purified by a modification of the Amyes and Smith (1974) method. Nucleic acids were precipitated from the supernatant by the gradual addition of a 0.1 volume of 10% streptomycin sulphate, and the solution stirred constantly for 30 minutes at 4°C. After centrifugation (12,000 g for 30 minutes) the supernatant was dialysed overnight against 50% saturation ammonium sulphate (made up with buffer A). After further centrifugation (12,000 g for 30 minutes) the supernatant was dialysed for 4 hours against 80% saturation ammonium sulphate and the pellet collected by a final centrifugation step (12,000 g for 30 minutes). The pellet was resuspended in buffer A and applied to a sephadex G-75 column (90 cm x 2cm^2). (A small amount of sample was removed at each purification stage, for analysis of DHFR activity and protein content, and stored at -20°C.).

SEPHADEX EXCLUSION CHROMOTOGRAPHY

Solid Sephadex (1 g per 15 ml) was added to 250 ml of buffer A and kept at 100°C for 3 hours. The flask was shaken and its contents allowed to settle overnight. The fines were decanted, more buffer A added and the process repeated. The sephadex slurry was poured into the column, maintained at 4°C. When full the tap was connected and the flow changed to an upward direction with an LKB peristaltic pump. The flow rate was adjusted to between 6 and 8 ml/hour and the column washed continually with buffer A for 48 hours. The void volume was measured with Dextran Blue 2000. Samples (2-3 ml) were applied slowly at the bottom of the column and eluted with buffer A. Two millilitre fractions were collected and maintained at 4°C with an LKB ultro rac fraction çollector. The column was washed between each run with buffer A for 12 hours. The void volume of the column was 60 ml.

MOLECULAR WEIGHT DETERMINATION

Molecular weights were determined by sephadex exclusion chromatography (Amyes and Smith,1974). Marker proteins (50 mg each of chymotrypsinogen, ovalbumin and cytochrome C) were either dissolved in the sample or in 2 ml of buffer A before loading on the column. The positions of the marker proteins were found by measuring absorbance of each fraction at 280 nm and the cytochrome C peak was confirmed by measuring absorbance at 410 nm. All absorbance measurements were determined with a PYE Unicam SP 1800 UV/VIS spectrophotometer. The molecular weight of the sample DHFR was determined from a standard curve produced by plotting the logarithm of the molecular weight of the marker proteins against their elution volumes.

PROTEIN ESTIMATION

All protein concentrations were estimated by the method of Waddell (1956). The absorbance of each sample was read at 215 and 225 nm after diluting 1:1000 in distilled water and the amount of protein calculated.

PREPARATION OF DIHYDROFOLATE

A 1mM solution of Dihydrofolate (DHF) was prepared by dissolving 25 mg in 0.05 M sodium phosphate buffer, pH 7.4, containing 0.05 M B mercaptoethanol. Aliquots were dispensed into bijoux bottles and stored in total darkness at -20°C.

DIHYDROFOLATE REDUCTASE ASSAY

DHFR activity was assayed by the method of Osborn and Huennekens (1958) as modified by Amyes and Smith (1976), using a Pye Unicam SP 1800 spectrophotometer. This spectrophotometer had a constant temperature cuvette block which was continuously maintained at 37°C. The reading wavelength was set at 340 nm and the output continuously monitored by a chart recorder. The test cuvette was filled with 40 mM sodium phosphate buffer, pH 6.0; 10 mM B-mercaptoethanol; 0.1 mM NADPH; enzyme (10 ul of crude preparation or 100 ul of sample from the column) and the volume made up to 0.95 ml with distilled water. Similarly, the blank cuvette was filled as above, except the NADPH was omitted. The cuvettes were allowed to equilibrate in the spectrophotometer for four minutes, and any decrease

in absorbancy was noted before 50 ul of 1 mM DHF was added to each cuvette. The cuvettes were mixed and the decrease in absorbancy was again followed. The level of DHFR activity was calculated by subtracting the decrease in absorbancy before adding the DHF, (ie. the independent NADPH oxidase activity), from the decrease in absorbancey after adding DHF. Enzyme activity was expressed in enzyme units - one enzyme unit was defined as the amount of enzyme required to reduce 1 u mol of DHF/ minute, based on a molar extinction coefficient of 12.3×10^3 (Hillcoat et al, 1967). Trimethoprim sensitive and resistant DHFR's were distinguished by assaying in the presence and absence of 4 uM trimethoprim (Amyes and Smith, 1974).

DETERMINATION OF INHIBITION (ID_{50}) BY VARIOUS ANTIFOLATE COMPOUNDS

For each enzyme purified, an inhibitor profile was determined by examining the effects of trimethoprim (2,4-diamino-5-(3',4',5'-trimethoxybenzyl)pyrimidine) and methotrexate (amethopterin). In each case, the 50% inhibitory concentration (ID_{50}) was determined, for each inhibitor, by assaying the enzyme in the presence of different concentrations of the inhibitor, maintaining a constant level of DHF. The 50% inhibitory concentrations were determined from plots of log of inhibitor concentration versus the percentage of the uninhibited activity. ID_{50}'s were then obtained by interpolation at percentage activity = 50.

HEAT SENSITIVITY

Aliquots (200 ul) of purified enzyme preparation were maintained at 45°C for 20 s, 40 s, 60 s, 120 s, 180 s and 240 s. After the requisite time, the samples were cooled on ice and the enzyme activity assayed in the usual manner (Amyes and Smith, 1976).

PH PROFILES

pH profiles were determined by assaying the enzyme in the presence of buffers at varying pH's. For pH's between 4.0 and 5.5 sodium acetate buffer was used and for the pH ranges 6.0 to 7.5 and 8.0 to 9.0 sodium phosphate and Tris HCl buffers were used respectively.

DETERMINATION OF Km AND Ki VALUES

The decay of DHF was assayed spectrophotometrically with the aid of an IBM computer connected to the Pye Unicam SP1800 spectrophotometer. The test cuvette contained 40 mm sodium phosphate buffer (pH 6.0), 10 mm B mercaptoethanol, 0.05 mM DHF and 0.1 ml of purified enzyme. This was blanked against a similar cuvette containing the above solutions except the DHF. After allowing the cuvettes to equilibrate in the spectrophotometer for 4 minutes, 100ul of NADPH was added to the test cuvette and 100 ul of distilled water to the blank (control) cuvette. DHF decay was monitored for 30 minutes. The above experiment was repeated in the presence of 0.2 ml of 10^{-3} M Tp. The Km values were generated

automatically by the computer software[1], which plotted the reciprocal of the reaction velocity against the reciprocal of the substrate concentration by the method originated by Lineweaver and Burk (1934). Ki values were determined from the equation

$$K_i = \frac{I}{\frac{Kp}{Km} - 1}$$

where I is the inhibitor concentration, Kp is the apparent Km with inhibitor and Km is the Km in the absence of any inhibitor (Dixon and Webb, 1958).

[1] This computer program was compiled by Dr SGB Amyes and Dr CJ Adie of the Edinburgh Regional Computing Centre to collect enzyme kinetic data and automatically generate suitable plots to obtain the enzyme kinetic constants.

RESULTS

CHARACTERISATION OF THE DHFR OF Tn4135 AND IT'S COMPARISON WITH THE DHFR OF Tn7

In order to determine whether the DHFR's of Tn4135 and Tn7 were related, the specific activity and properties of the E coli J62(RP4::Tn4135), E coli J62(R483::Tn7) and E coli J62(RP4::Tn7) encoded enzymes were determined from 5 l nutrient broth preparations.

Specific DHFR activities were measured at pH 6.0 in phosphate buffer at 30°C before any separation of Tp sensitive and Tp resistant enzymes was attempted. The results in Table 7.2 indicate that the Tn4135 encoded enzyme is synthesised in amounts similar to the Tn7 enzyme and several fold higher than the host chromosomal enzyme.

Crude samples of each DHFR were purified by sephadex exclusion chcromatography (for purification Tables see Appendix 7.1) and the resultant 2 ml fractions analysed for DHFR activity, in the presence and absence of 4×10^{-6} M Tp. Separation of Tp resistant and Tp sensitive enzymes was achieved in all cases and the elution patterns, determined by plotting elution volume against DHFR activity (Figure 7.1), for E coli J62(R483::Tn7), E coli J62(RP4::Tn7) and E coli J62(RP4::Tn4135), were in most respects identical.

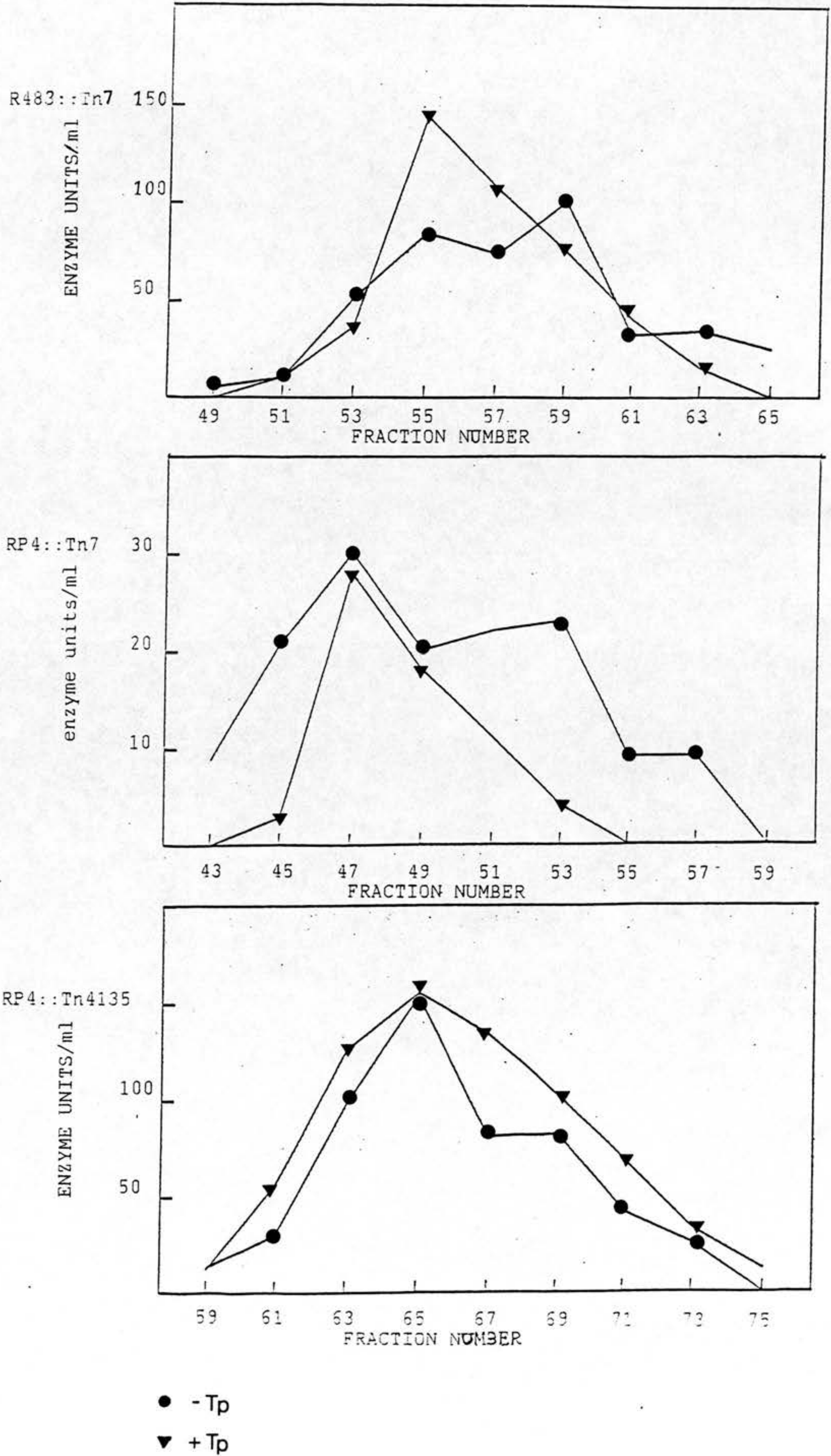
Molecular weight estimations from the gel filtration elution volumes of marker proteins (see Appendix 7.2) indicated the expected molecular weight (Amyes and Smith, 1974) of approximately 21000 for each chromosomal, Tp-sensitive enzyme. A molecular weight of between 30000 and

TABLE 7.2: A COMPARISON OF THE SPECIFIC DIHYDROFOLATE REDUCTASE ACTIVITIES OF CELL EXTRACTS OF E COLI HARBOURING Tn7 AND Tn4135

ORGANISM	SPECIFIC ACTIVITY
E coli J53(RP4)	< 1.5
E coli J62(R483::Tn7)	27.33
E coli J62(RP4::Tn7)	16.65
E coli J62(RP4::Tn4135)	21.75

Specific activities are expressed as nmol dihydrofolate reduced min^{-1} (mg protein)$^{-1}$

FIGURE 7.1 ELUTION OF DIHYDROFOLATE REDUCTASES FROM SEPHADEX G-75



33000 (Table 7.3) was determined for each Tp resistant enzyme present, suggesting that Tn7 and Tn4135 encoded enzymes were similar.

The pH profiles of the Tn4135 and Tn7 encoded DHFR were compared. Both enzymes exhibited a sharp peak at pH 6.0 in phosphate buffer (Figure 7.2).

Inhibitor profiles verified the similarity between the Tn7 and the Tn4135 encoded enzymes. (For example-plots of inhibitor concentrations against the percentage of uninhibited activity see Appendix 7.3). Purified samples of each enzyme were assayed in increasing concentrations of trimethoprim and methotrexate (Mtx) at pH 6.0 (see Appendix 7.4) and the concentration required to give 50% inhibition (ID_{50}) of each enzyme were determined (Table 7.4). Both enzymes were resistant to Tp and Mtx with little variation between them.

The heat sensitivity of each purified enzyme preparation was measured and the results (Table 7.5) indicate that both the Tn4135 and the Tn7 encoded DHFR's lose 50% of their activity in under a minute.

Table 7.6 indicates the results of Michaelis-Menten kinetics for the transposon encoded enzyme preparations after separation by exclusion chromatography. Each enzyme was continuously assayed in the presence of a decreasing concentration of dihydrofolate and Km values determined by computerised generation of double reciprocal plots of activity versus substrate concentration (Appendix 7.5). There was some variation between Km values for the enzymes concerned, but these differences are probably not significant. Assays in the presence of a decreasing concentration of DHF were repeated in the presence of Tp. The inhibitor constants (K_i) are seen in Table 7.6 and again, the differences observed are probably not significant. Tn7 and Tn4135 encoded enzymes would appear to have similar kinetic properties and are, therefore, very likely to be related.

TABLE 7.3: MOLECULAR WEIGHTS OF DIHYDROFOLATE REDUCTASE ENZYMES

SOURCE OF ENZYME	MOLECULAR WEIGHT
RP4	23000
R483::Tn7	30500
RP4::Tn7	33500
RP4::Tn4135	31000

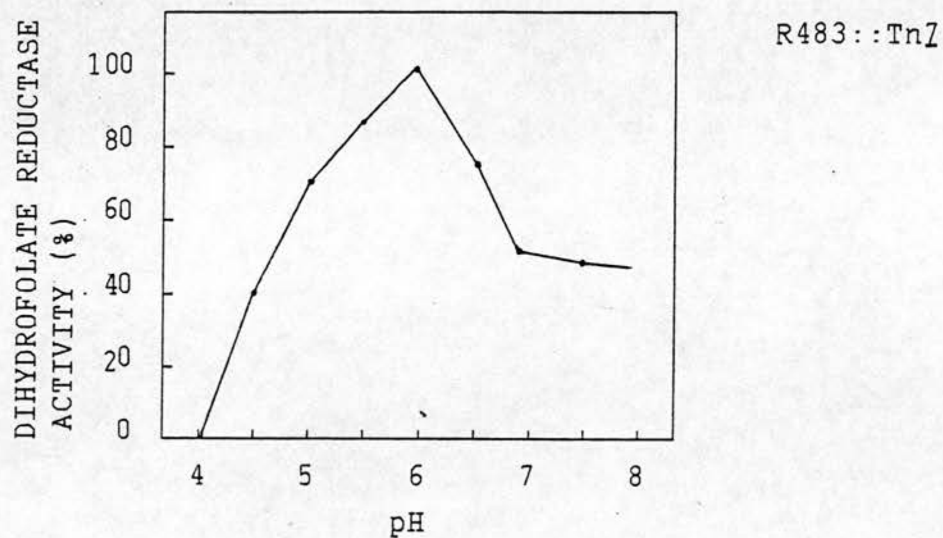
TABLE 7.4: TRIMETHOPRIM AND METHOTREXATE CONCENTRATIONS WHICH CAUSE 50% INHIBITION OF DIHYDROFOLATE REDUCTASE ACTIVITIES

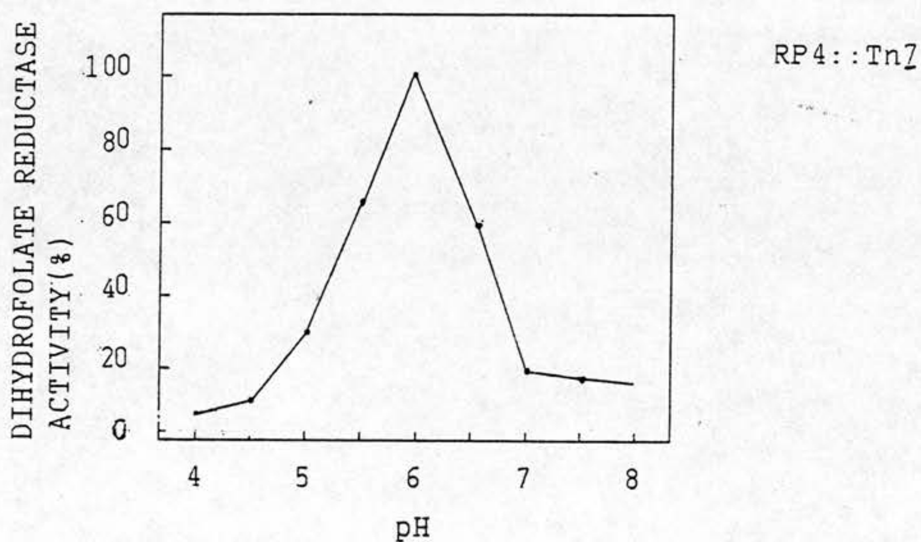
SOURCE OF ENZYME	TRIMETHOPRIM (M)	METHOTREXATE (M)
R483::Tn7	6.60×10^{-5}	8.90×10^{-6}
RP4::Tn7	8.30×10^{-5}	6.60×10^{-6}
RP4::Tn4135	3.98×10^{-5}	7.59×10^{-6}

TABLE 7.5: TIME TAKEN TO LOSE 50% DIHYDROFOLATE REDUCTASE ACTIVITY AT 45°C

SOURCE OF ENZYME	TIME (SECONDS)
R483::Tn7	49
RP4::Tn7	44
RP4::Tn4135	23

FIGURE 7.2: PERCENTAGE OF MAXIMUM DIHYDROFOLATE REDUCTASE ACTIVITIES AT DIFFERENT pH VALUES FOR THE TRIMETHOPRIM RESISTANT ENZYMES FROM E COLI STRAINS HARBOURING R483::Tn7, RP4::Tn7 AND RP4::Tn4135





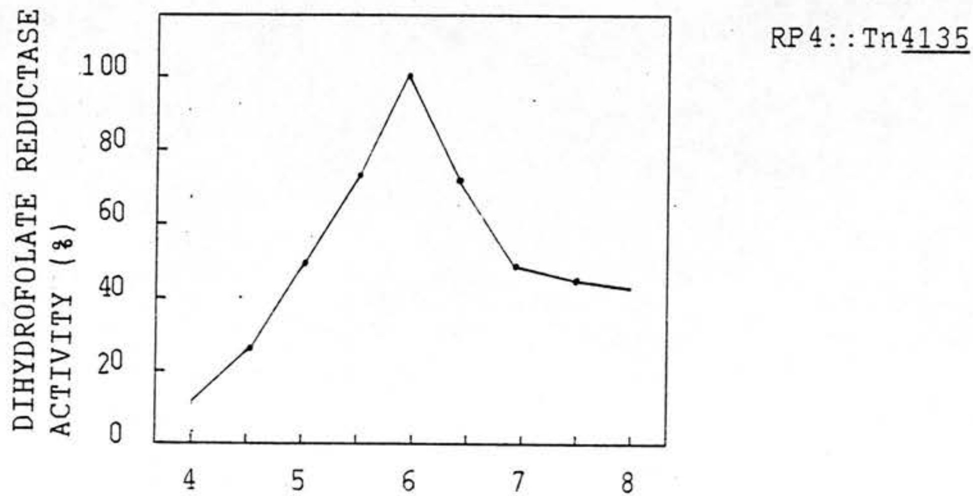


TABLE 7.6: MICHAELIS-MENTEN KINETICS OF DIHYDROFOLATE REDUCTASES, WITH DIHYDROFOLATE AS SUBSTRATE AND TRIMETHIPRIM AS INHIBITOR

SOURCE OF ENZYME	Km FOR DHF (M)	Ki FOR Tp (M)
R483::*Tn7*	2.63×10^{-5}	2.635×10^{-4}
RP4::*Tn7*	2.15×10^{-5}	2.714×10^{-4}
RP4::*Tn4135*	9.68×10^{-5}	4.800×10^{-4}

PRELIMINARY ANALYSIS OF THE DHFR OF E COLI J62(Sa-1::Tn4135)^{a}

Due to the possibility that multiple transposition or a spontaneous amplification might be responsible for the large size of the (Sa-1::Tn4135)^{a} molecule, the specific activity of the DHFR was determined and compared with that of RP4::Tn4135. Enzymes were prepared from 10 litres of DM cultures grown up for 2.5 hours with aeration . Whilst the E coli J62(RP4::Tn4135) enzyme was found to have a specific activity of 12.4, that of E coli J62(Sa-12::Tn4135)^{a} was found to be 50, suggesting that an increased number of copies of the DHFR gene might be present in the (Sa-1::Tn4135)^{a} molecule.

PRELIMINARY ANALYSIS OF THE DHFR OF E COLI J62 (Sa-1::Tn4135)^{ORI}

The high specific activity of the E coli J62(Sa-1::Tn4135)^{a} enzyme, and the large molecular size of the plasmid species harboured by this strain demanded that a more accurate characterisation of the Tn4135 encoded enzyme, harboured by Sa-1, was needed and this was achieved by analysing the DHFR of E coli J62(Sa-1::Tn4135)^{ORI}. The enzyme was prepared from 5 1 nutrient broth cultures of the two strains and the specific activity analysed prior to purification. E coli J62(Sa-1::Tn4135)^{ORI} was found to encode a DHFR of specific activity 1.336, indicating that this enzyme was not similar to the DHFR of Tn7 (specific activity 16.65). The DHFR encoded by (Sa-1::Tn4135)^{a}, whilst not as high as initial experiments, was found to have a specific axtivity of 9.24, approximately 10 fold greater than that of (Sa-1::Tn4135)^{ORI}. These results not only suggest that an amplification may be responsible

for the increased size and activity of the (Sa-1::Tn4135)[a] encoded enzyme, as compared with the (Sa-1::Tn4135)[ORI] encoded enzyme, but also indicate that RP4 may be involved in the amplification process, since the RP4::Tn4135 enzyme has a high specific activity as well (21.75).

THE EFFECT OF PASSAGE THROUGH RP4 ON THE SPECIFIC ACTIVITY OF THE Tn4135 ENCODED DHFR

It was possibile that RP4 was, in some way, responsible for the increase both in the size of the (Sa-1::Tn4135)[a] plasmid species, and the specific activity of the DHFR derived from it, compared with that of *E coli* J62(Sa-1::Tn4135)[ORI]. Therefore, DHFR was prepared from 1 litre overnight nutrient broth cultures of the original Pig strain (P-20), *E coli* J62(RP4::Tn4135), *E coli* J62(Sa-1::Tn4135)[ORI] and *E coli* J62(H1). The results (Table 7.7), in conjunction with the previous finding that *E coli* J62(Sa-1::Tn4135)[a] has a specific activity of approximately 10 in nutrient broth, suggest that passage through RP4 has a 'booster' affect on Tn4135 encoded DHFR activity.

However, preliminary experimentation with Tn7, to test the above hypothesis, indicated that this transposon did not behave in the same way. The specific activity of the DHFR encoded by (Sa-2::Tn7)[ORI] (23) was not found to be significantly different from that of R483::Tn7 (27) or RP4::Tn7 (17).

TABLE 7.7: SPECIFIC ACTIVITIES OF Tn4135 - ENCODED DHFR IN DIFFERENT PLASMID BACKGROUNDS

ENZYME SOURCE	PASSAGE THROUGH RP4	SPECIFIC ACTIVITY
RP4::Tn4135	+	18.72
[(Sa-1::Tn4135)[a]	+	10]
(Sa-2::Tn4135)[a]	+	25.22
original pig - P-20	-	5.8
(Sa-2::Tn4135)[ORI]	-	1.86
HI	-	2.02

CHARACTERISTAION OF THE DHFR ENCODED BY E COLI J62(Sa-1::Tn4135)^{a} AND E COLI J62(Sa-1::Tn4135)^{ORI}

In order to further characterise the (Sa-1::Tn4135)^{a} plasmid and determine whether an amplification step had occurred, a detailed analysis of specific activity and enzyme properties were carried out on the DHFR's of E coli J62(Sa-1::Tn4135)^{a} and E coli J62(Sa-1::Tn4135)^{ORI}. E coli J53(Sa-1) was used as a control.

Enzymes were prepared from 5 litres of nutrient broth and purified by sephadex exclusion chromatography (for purification tables see Appendix 7.1), but due to the low specific activity (1.336) and therefore the small amount of enzyme produced from (Sa-1::Tn4135)^{ORI}, this enzyme was prepared from 10 l of nutrient broth. Activity levels were still low, hampering any accurate determination of enzymic properties. A comparison of the plasmid encoded DHFR's from the two strains is given in Table 7.8 and Figure 7.3 indicates their gel filtration elution profiles.

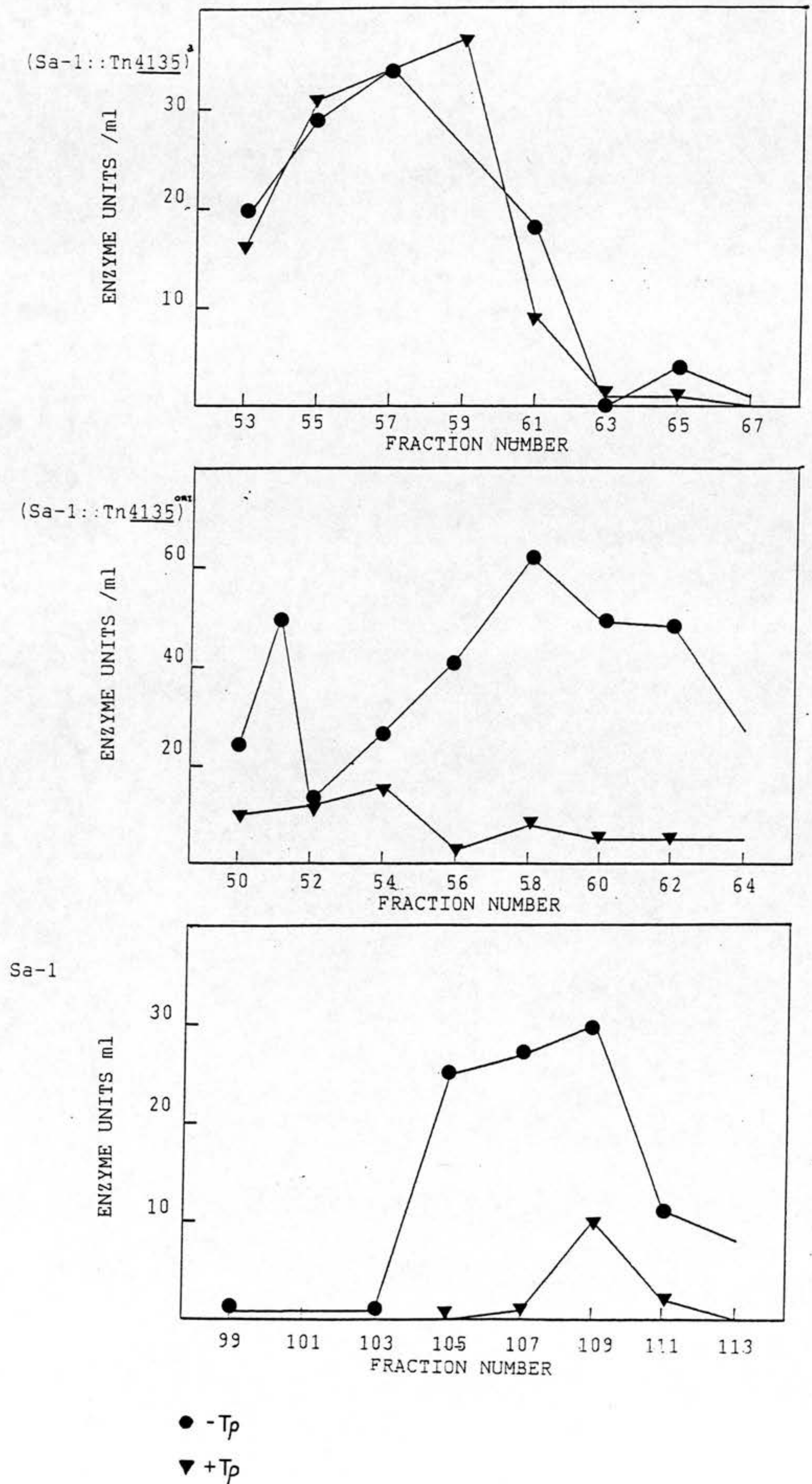
An analysis of enzyme properties indicates differences between the two strains, not only in specific activity but also in Tp ID_{50} and temperature sensitivity, but these differences are not consistent with an amplification step. Since initial temperature sensitivity results indicated an apparent increase in activity with exposure-time to a temperature of 45°C, the assay of DHFR activity was repeated in the presence of 2×10^{-6} M Tp, with exposure to heat for 0, 2 and 6 minutes. The results indicated that the E coli J62(Sa-1::Tn4135)^{ORI} enzyme had a TD_{50} of approximately 10 minutes, compared with less than 1 minute for the (Sa-1::Tn4135)^{a} enzyme. These results are suggestive of different origins for the two DHFR's rather than one being an amplified product of the other.

TABLE 7.8: A COMPARISON OF THE PROPERTIES OF (Sa-1::Tn4135)[a] AND (Sa-1::Tn4135)[ORI] MEDIATED DHFR

SOURCE	SPECIFIC ACTIVITY	Tp ID_{50} (M)	Mtx ID_{50} (M)	Km DHF (M)	Ki Tp (M)	TD_{50} (Sec)	MOLECULAR WEIGHT
E coli J62(Sa-1::Tn4135)[a]	9.24	1.78×10^{-4}	9.12×10^{-6}	1.089×10^{-5}	2.87×10^{-5}	38	36728
E coli J62(Sa-1::Tn4135)[ORI]	<1.0	$<1.58 \times 10^{-8}$*				>600*	34673
E coli J53(Sa-1)	0.0	1.58×10^{-9}					21000

* These properties were determined with the 80% $(NH_4)_2SO_4$ partially purified sample due to lack of activity of samples after sephadex exclusion chromatography.

FIGURE 7.3 ELUTION OF DIHYDROFOLATE REDUCTASES FROM SEPHADEX G-75



EFFECT OF MEDIA ON THE ENZYMIC PROPERTIES OF THE Tn4135 DHFR

The previous identification and characterisation of DHFR's has been carried out from cultures grown in different media, and therefore results may not be directly comparable. DHFR's were, therefore, purified from E coli J62(Sa-1::Tn4135)[a] and E coli J62(RP4::Tn4135) strains cultured in 10 litres DM, 5 litres nutrient broth and 15 litres Isosensitest broth. A comparison of the properties of the transposon encoded DHFR's is given in Table 7.9.

The results indicate that growth media not only affects the expression of DHFR but also the sensitivity of the enzyme to heat. Growth in Isosensitest broth greatly reduces the DHFR activity levels in both strains, whilst the expression of the two enzymes in DM and nutrient broth appears to be variable, but higher. Whilst not significantly affecting Tp ID_{50} or Mtx ID_{50} levels, growth in DM medium reduced the heat sensitivity of both DHFR's as compared with growth in the other two media.

TABLE 7.9: THE EFFECT OF GROWTH MEDIA ON THE ENZYMIC PROPERTIES OF THE Tn4135 ENCODED DHFR

SOURCE	MEDIA	SPECIFIC ACTIVITY PRIOR TO PURIFICATION	Tp ID_{50}	Mtx ID_{50}	TEMPERATURE SENSITIVITY (SECONDS)
(Sa-1::Tn4135)[a]	DM	24.25	5.01×10^{-5}	7.90×10^{-6}	142
(Sa-1::Tn4135)[a]	NB	9.24	1.78×10^{-4}	9.12×10^{-6}	38
(Sa-1::Tn4135)[a]	ISO	5.13	1.66×10^{-5}	1.74×10^{-6}	26
RP4::Tn4135	DM	12.40	4.80×10^{-5}	8.32×10^{-6}	154
RP4::Tn4135	NB	21.75	3.98×10^{-5}	7.59×10^{-6}	23
RP4::Tn4135	ISO	6.24	5.01×10^{-5}	6.61×10^{-6}	53

DISCUSSION

With the continuing isolation and characterisation of DHFR's, it has become clear that there is considerable variation in both their physical and biochemical properties. Bacterial and mammalian enzymes can be distinguished from each other on the basis of their relative abilities to bind a series of small molecule analogues of DHF (Baccanari et al, 1975). These differences in binding explain how drugs can function as potent and non toxic antibacterials even though the target is common to both host and parasite (Bushby and Hitchings, 1968). Whilst initial studies (Amyes and Smith, 1978) indicated a remarkable resemblance between the DHFR's encoded by R-factors of gram negative bacteria, further analysis has resulted in the subdivision of these enzymes into four classes: Types I to IV. Up until 1983, high level plasmid-mediated Tp resistance had only been found in Gram negative bacteria, but recently some multi-resistanct Staphylococcus aureus have been found to be Tp resistant (Lyon et al, 1983, 1984), and encode a new type of DHFR (Young et al, 1987). The mechanisms behind the generation of such diversity is unclear and the evolutionary origins of the different enzyme types would appear to be more complex than at first envisaged.

The properties of the RP4::Tn4135 encoded DHFR, characterised in this study, show marked similarities with the Tn7 encoded enzyme, despite the discrepancies between these and published results. Mtx and Tp ID_{50} results are comparable, indicating that the RP4::Tn4135 encoded enzyme, like that of Tn7, is a 100 - 1000 times less sensitive to inhibitors than the E coli chromosomal DHFR. Although the Tn7 and Tn4135 transposons differ in size (14 kb and 4 - 6 kb respectively), the molecular weights of

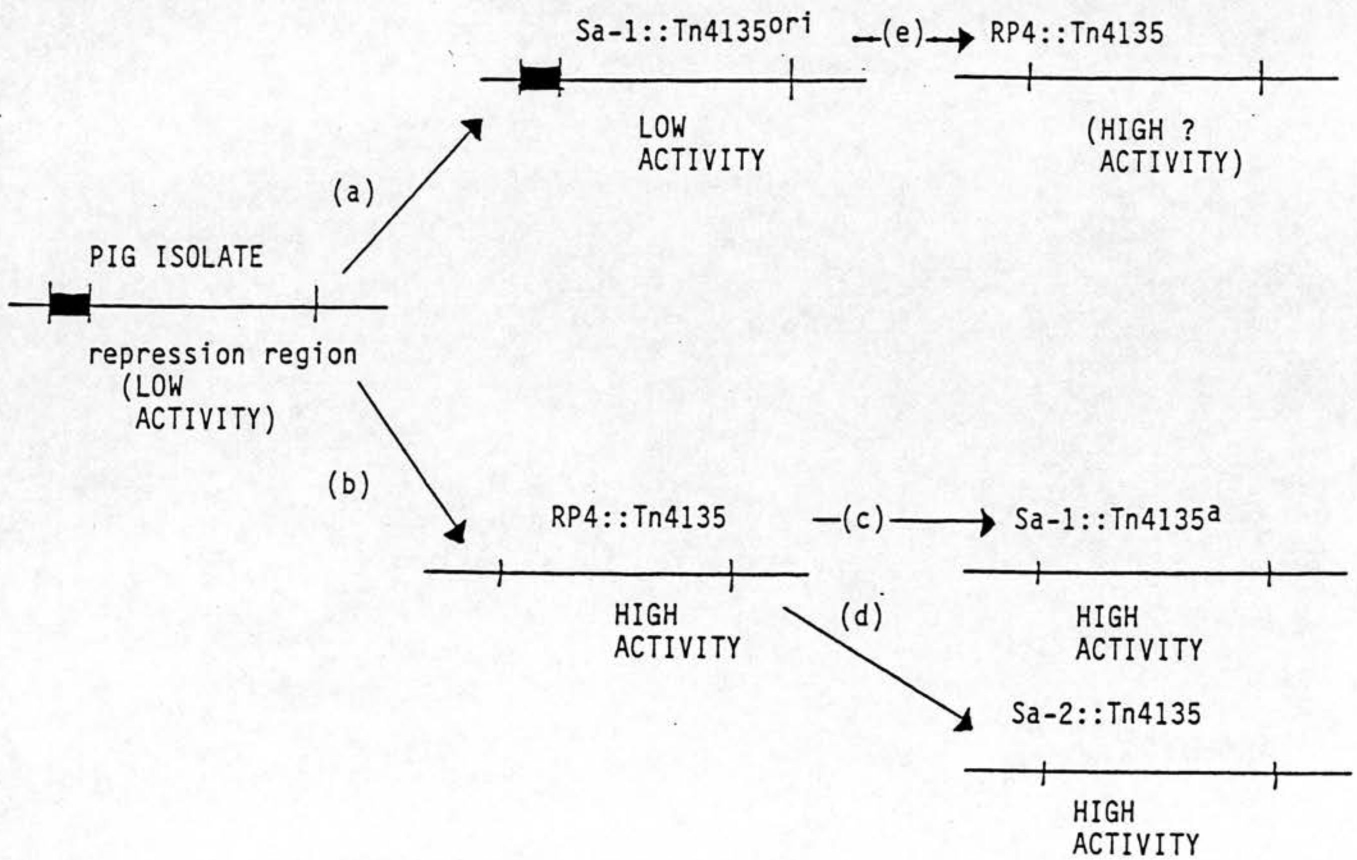
the encoded DHFR's are similar. This contrasts with reports on another small Tp resistance transposon, Tn4132 (Young and Amyes, 1983), which was found to have a reduced molecular weight of 24,500: a finding which suggested Tn4132 had evolved from Tn7 by deletion of part of the DNA encoding the structural gene for DHFR. Whilst Tn4135 would not appear to have been generated by such a deletion, the results do not rule out the possibility of deletions in other regions of the Tn7 genome.

The activity of the DHFR encoded by E coli J62(Sa-1::Tn4135)^{a} was initially found to be 50; a result that substantiated the view that the large molecular size of Sa-1::Tn4135^{a} was due to gene amplification. Extra gene copies have been responsible for the increase in chromosomal enzyme levels observed in mammalian cells (Schimke et al, 1977) and for the increase in B lactamase production in E coli (Edlund et al, 1979). The variations in specific activity observed for the Sa-1::Tn4135^{a} DHFR (10 - 50) could also be accounted for by differences in the degree of amplification , when it is considered that the Sa-1::Tn4135^{ORI} enzyme was found to have a specific activity of < 1. Examination of the Sa-1::Tn4135^{ORI} DHFR was expected to reveal the true classification of the Tn4135 enzyme, since this species was of the predicted size. However the specific activity appeared to resemble a type II enzyme, in contrast to that of the RP4::Tn4135 encoded DHFR which was indicative of a type I, suggesting that the results may not be accounted for by a simple amplification. The stability of the Sa-1::Tn4135^{a} species in the absence of drug selection would also tend to go against multiple gene copies as an explanation of increased enzyme synthesis. However the apparent overproduction of the DHFR of this strain may also be due to other phenomena. Kane et al (1979) suggested that the three fold increase in DHFR activity observed in rifampicin-resistant mutants of B subtilis was

due to a pleiotropic effect of the Rif mutation; leading to an altered recognition of the gene specifying DHFR, by RNA polymerase. Mutations in the structural gene for DHFR can also lead to hyperproduction of this enzyme (McCuen and Sirotnak, 1974), although such mutational events have usually been described for chromosomal DHFR's (Sheldon and Brenner, 1976; Flensburg and Skold, 1984), and would not explain the 'variations' in DHFR activity that have been observed. It is possible that a change in transcriptional regulation, brought about by insertion of Tn4135 into Sa-1, could be reponsible for the increased levels of enzyme activity, although this would not account for the increased plasmid size observed. Tennhammer-Ekman and Skold (1979) suggested that overproduction of DHFR in strain 1810 could be due to variations in transcription and Amyes and Smith (1978) implied that both the site and direction of insertion of transposons into other replicons could be the factor responsible for different levels of gene transcription. The controlling elements in the regulation of DHFR synthesis would appear to be closely associated with the structural gene (Breese et al, 1975; Spandidos and Smiminovitch, 1977), therefore a deletion in Tn7, could lead both to a smaller transposon and a deregulation of DHFR synthesis.

An attempt to explain the anomaly in enzyme activity levels, and account for the observed differences in plasmid size, lead to the hypothesis (Figure 7.4) that the presence of RP4, and possibly other Inc P plasmids could affect enzyme production. The activity of the DHFR encoded by the original Pig isolate is low and when Tn4135 is transferred direct to Sa-1 (a) the expression of Tn4135 DHFR activity is still low possibly due to some form of repression mechanism. On transfer to RP4 (b), however, this mechanism is derepressed, possibly by loss of a small region of DNA, and the expression of DHFR activity is high. Once this

FIGURE 7.4: HYPOTHESIS TO EXPLAIN THE VARIABLE SPECIFIC ACTIVITY OF Tn4135



repression mechanism has been lost, by passage through RP4, it can not be re-established and therefore transfer on to Sa-1 (c) again would result in a strain with high DHFR activity. The transfer of Tn4135 via RP4 to Sa-2 (d) backs up this hypothesis, as the activity is again high. Alternatively, because of a deletion, Tn4135 may lack its own regulatory function (promotor) which results in low expression of DHFR activity. Insertion of Tn4135 close to a regulatory region of RP4, could result in a complementation of regulatory functions and a subsequent increase in DHFR production. The transfer of a region of RP4, encoding this ' regulatory function', to Sa-1 with Tn4135, could account for both the size increase and the increase in DHFR activity. This latter hypothesis would not account for the high activity of the Sa-2::Tn4135 DHFR as there is no size increase.

It was hoped to prove the hypothesis of RP4 involvement in Tn4135 DHFR gene expression, by examining the DHFR from a strain generated by the transfer of Tn4135 from Sa-1::Tn4135^{ORI} to RP4. However, the formation of such a strain proved difficult, possibly due to incompatibility phenominum, and therefore the activity could not be ascertained.

The above hypotheses are based on the fact that Sa-1::Tn4135^{a} and Sa-1::Tn4135^{ORI} encode a similar DHFR, differing only in specific activity. However examination of the properties of the DHFR from E-coli J62(Sa-1::Tn4135)^{ORI}, in comparison with those of E coli Sa-1::Tn4135^{a}, would tend to indicate that the two enzymes were different. Some problems arose during the purification of DHFR from the former strain as a result of the very low specific activities, (and therefore the small amounts of enzyme produced), and the amount of NADPH oxidase present, which interferes with the optical assay of DHFR. Then and Angehrn (1979) were faced with similar problems in attempting to

isolate DHFR's from anaerobes. Insufficient enzyme was produced from Sa-1::Tn4135[ORI] to determine enzyme properties accurately, despite attempts to isolate the DHFR from larger volumes of culture. However the results do suggest that E coli (Sa-1::Tn4135)[ORI] encodes an enzyme of widely different properties from that of E coli J62(Sa-1::Tn4135)[a]. Despite the fact that both strains express Tp MIC's of greater than 1000 ug/ml, the Tp ID_{50} of the Sa-1::Tn4135[a] encoded enzyme is comparable to a type I enzyme whilst that of the Sa-1::Tn4135[ORI] DHFR more closely resembles that of the chromosomal enzyme, or possibly even the type IV. The TD_{50}'s are in agreement with this proposed classification and therefore imply that P-20, the original pig isolate, contains two different DHFR genes. The suggestion of two different DHFR genes residing in one R plasmid was implicated by the work of Joyner et al , (1984) although it is likely that, in the case of the P-20 isolate, the genes reside on different plasmids. The introducton of RP4 and Sa-1 into P-20 would appear to have resulted in the ' picking-up' of different DHFR genes. This however does not explain why E coli J62(Sa-1::Tn4135)[a] should harbour a plasmid of such uppredicted size. More than one mechansism may be involved in generating a large species with high DHFR activity.

The determination of kinetic parameters has also been dependent on isolating sufficient pure enzyme. Various graphical methods have been widely employed to determine the parameters involved in the Michaelis Menten equation

$$V = \frac{Vm.S}{Km + S}$$

where V = velocity and S = substrate concentration, but unless the data fit the relationship represented by the equation reasonably well, a considerable bias may be introduced in the

graphical methods. Hence the analysis of a progressive curve in a single experiment, by computer, has been regarded as being useful (Yoshimoto et al, 1984). It was not possible, however, to determine the kinetic parameters of the Sa-1::Tn4135^{ORI} encoded enzyme by computer either, as well as analysing Tp and TD_{50}'s, because of the very small quantities of DHFR isolated. Gilli et al (1986) have described an alternative method to spectrophotometric analysis for the determination of DHFR activity, which is thought to permit the detection of very low DHFR activities corresponding to 100 pmol of substrate reduced per minute. This method may prove useful in confirming the results of this preliminary investigation of the enzymic properties of the Sa-1::Tn4135^{ORI} encoded enzyme, and for analysing the 'low-activity' enzyme of the P-20 isolate.

When tested in vitro, the ability of Tp to inhibit various organisms is dependent upon the medium in which the test is performed (Bushby and Hitchings, 1968; Darrell et al, 1968). The medium acts as an exogenous source of metabolites whose normal de novo synthesis is blocked by the action of Tp, but once assimilated by the cell, these compounds would be expected to by pass, in a non competitive manner, the inhibition caused by Tp. The effect of various substances on bacterial sensitivity to Tp has been studied (Amyes and Smith, 1974; 1978a; 1978b; Then and Angehrn, 1974), but these results have not been applied to DHFR studies. This enzyme has been isolated from cells grown in different media, yet the results have been compared (Amyes, 1986). Young et al (1986) have shown that the presence of certain metabolites in the media influences the expression of Tp resistance, by affecting MIC's, and it is therefore possible that DHFR levels might be affected also. Whilst Young et al (1986) suggested that there was poorer expression of the type IV Tp resistance gene in complex media, the specific activity results obtained

in this study suggest that DHFR expression is poorest in Isosensitest broth. This latter broth, like Difco Mueller Hinton broth, is low in thymidine (Amyes and Smith, 1978), a potent antagonist of the action of both Tp and Su (Koch and Burchall, 1971; Amyes and Smith, 1976), and is therefore not expected to affect the action of Tp. The specific activity results from the comparison of E coli J62(Sa-1::Tn4135)[a] cells grown in DM and nutrient broth are in agreement with the view that the expression of Tp inhibition is greatest in nutrient broth, whilst cells grown in minimal media are four times as resistant as those grown in nutrient broth (Breeze et al, 1975). However, this may be coincidental, as analysis of the results of E coli J62(RP4::Tn4135) grown in similar media do not follow this trend. Whilst it is possible to explain the observed differences in DHFR expression by media effects, it is unclear why there should be such a discrepancy in temperature sensitivity results. Tp ID_{50} and Mtx ID_{50} figures are unaffected by growth in different media.

It is clear from the above findings that E coli J62(Sa-1::Tn4135)[a] and E coli J62(Sa-1::Tn4135)^{ORI} encode different DHFR; the former corresponding to a type I. The origin of the latter enzyme is unclear since its molecular weight and TD_{50} are characteristic of a type II but it's Tp ID_{50} more closely resembles that of the chromosome (Table 7.10). Just as Towner and Pinn (1981) speculated that the plasmid pUN212 specified an intermediate type of DHFR, more sensitive to Tp than the normal plasmid encoded reductase, so the Sa-1::Tn4135^{ORI} encoded enzyme may be an intermediate between chromosomal and type II DHFR's.

TABLE 7.10: PROPERTIES OF PLASMID AND TRANSPOSON DIHYDROFOLATE REDUCTASES

DHFR	PLASMID	TRANSPOSON	SPECIFIC ACTIVITY	Tp ID_{50} (uM)	Mtx ID_{50} (uM)	Km DHF (uM)	Ki Tp (uM)	TD_{50} (Min)	PI	MOLECULAR WEIGHT
Ia	R483	Tn7	13.6	57	5.6	5.6	7.4	0.5	6.4	35000
Ib	pUK163	Tn4132	4.5	32	2.8	11.0	41.0	1.2		24500
IIa	R67bis		0.85	70000	1100	4.6	6100	>12.0	5.5	35000
IIb	R751	Tn402	0.07	20000	1000	4.2	400	>12.0	7.2	34000
III	pAZI		2.0	2.1		0.4	0.019			16900
IV	pUK1123		600.0	0.2	0.02	37.0	0.063	>12.0		46700
CHROMOSOME				0.02	0.01	1.2	0.006	>12.0	4.2	21000
SI	pSKI		129.6	50.0	0.002	10.8	11.6	>12.0		19700
Ia	RP4	Tn7	16.65	83.0	6.6	21.0	271	<1.0		33500
Ia	RP4	Tn4135	21.75	39.8	7.6	96.0	480	<0.5		31000
	Sa-1	Tn4135[a]	10.0	178	9.1	10.9	28	0.5		36728
	Sa-1	Tn4135[ORI]	<1.0	0.02				>10.0		34673

REFERENCES: Amyes (1986); Young *et al* (1987)

a - transposon transferred from RP4 to Sa-1
ORI - Transposon transferred from original pig isolate, P-20, to Sa-1

CHAPTER 8

EXAMINATION OF Tn4135 DNA BY DNA-HYBRIDISATION

INTRODUCTION

During the last decade much interest has been generated in the handling of DNA, following the introduction of gene cloning or genetic engineering techniques. One technique that has become a very powerful tool for the detection and quantification of specific nucleic acid sequences, is DNA Hybridisation. This technique is based on the property of DNA to denature and separate into single strands, on heating or treatment with alkaline, and the ability of this single stranded DNA to reanneal with complementary single stranded DNA, to form a stable, double stranded molecule (Marmur and Lane, 1960; Doty et al, 1960).

In most hybridisation experiments, a sample of denatured DNA is immobilised by binding the DNA strands to a solid surface, frequently a nitrocellulose filter. This may be achieved by direct growth of cells on filters - colony hybridisation (Grunstein and Hogness, 1975; Hanaham and Messelson, 1980) or by transfer of DNA to filters by Southern blotting (Southern, 1975). (Recently a technique has been developed for the direct hybridisation of labelled DNA to DNA in agarose gels (Purrello and Balazs, 1983), which has the advantage that the gel can be reutilised several times). DNA single strands bound in this way to filters are unable to migrate and hence are unable to re-annneal with DNA from within the sample. Filters are challenged with radioactive probes, and after washing, subjected to autoradiography for detection of hybridised sequences. The probes themselves are usually generated by means of a Nick Translation procedure (Maniatis et al, 1975; Rigby et al, 1977), where by E coli polymerase I catalyses the replacement of existing unlabelled

nucleotides in DNA, with radioactive ones (Walker and Gaastra, 1983). By means of this 'hot for cold swop' of nucleotides, about 50% of the residues in the DNA can be labelled.

The most important property of the probe is its specific activity, as this will determine the sensitivity and accuracy of the detection and quantification of specific sequences. Adequate detection of unique sequences by Southern blotting, for example, requires a probe with an activity of 10^8 cpm/ug DNA. For this reason a probe often consists of a precise restriction fragment suitably recovered from an agarose gel (Blin et al, 1975; Smith, 1980; Burns and Beacham, 1983) and purified. The probe must also be labelled throughout at a uniform specific activity - as achieved by nick translation (Rigby et al, 1977), since a restriction fragment corresponding to a poorly labelled section of probe might not be detected in a southern blotting experiment.

The development of DNA-hybridisation has resulted in the ability to detect specific sequences in southern blots; to analyse quantitatively specific sequences, eg Rigby et al (1977) used nick translated ^{32}P SV40 DNA to detect one SV40 DNA molecule per haploid mouse genome, and Young (1984) used this same technique to determine the number of copies of amplified DNA in Bacillus subtilis; to screen for recombinant DNA molecules; as well as locate chromosomal sequences (Ruddle, 1981), study gene expression (Levitt et al, 1979; Stalder et al, 1980) and structure (Breathnach et al, 1977), visualise restriction fragments and analyse malfunctioning genes in various inherited cell lines (Kan and Dozy, 1978; Maniatis et al, 1980; Geever et al, 1981).

More recently DNA hybridisation has been used to characterise incompatibility group plasmids (Taylor and Brose, 1983) and study the epidemiology and evolution of their drug resistance genes; in particular

Tp resistance genes (Mayer et al, 1985). The rapid detection and classification of these latter genes is of primary importance for clinical and epidemiological studies of Tp resistance. Flensburg and Skold (1984) utilised DNA-DNA hybridisation to study regulatory changes in the formation of a chromosomal DHFR causing resistance to Tp, whilst Fling et al (1982) were able to detect a new resistant enzyme by monitoring plasmid-encoded Tp resistance genes with gene specific radiolabelled probe DNA. Such probes were not only able to detect the type of DHFR gene, but resulted in information as to the location of the type I and II genes and also their prevalence. Steen and Skold (1985) reported that in Sweden plasmid born or chromosomally mediated resistance by Tn7 was the most common responce to the ubiquitous use of Tp, a view substantiated by the work of Pulkkinnen et al (1984) in Finland. Campbell et al (1986) looked at the spread of Tp resistant Inc FIV plasmids via DNA hybridisation techniques, and identified three evolutionary lines amongst human and pig isolates. A remarkable degree of similarity was found amongst plasmids of the third line (containing examples from both human and animal sources), providing clear evidence of exchange of plasmid bearing E coli between humans and pigs.

Due to the potential of DNA hybridisation to not only distinguish between different DHFR genes (Fling et al, 1982), but also to detect novel DNA rearrangements associated with DHFR gene amplification (Federspiel et al, 1984), this technique was used to ascertain the origin of the Tn4135 encoded gene(s) and the nature of the plasmid spieces generated by Tn4135 transposition.

MATERIALS AND METHODS

BACTERIAL STRAINS AND PLASMIDS

The plasmids used in this study and their relevant properties are shown in table 8.1. All plasmids were harboured in E coli strains unless otherwise stated. Plasmids used for DNA probes were RP4 (Datta et al, 1971), Sa-1 (Ward and Grinsted, 1982), P872 (pBR322: pFE872 1618 bp Taq I partial fragment) which contains the type I DHFR structural gene (Fling and Richards, 1983), and p700 (PUC4: PFE364 800 bp EcoRl fragment), which will detect the type II DHFR (Elwell, personal communication). (pFE364 was constructed by inserting a 2.5 kb Bam HI - EcoRI fragment of plasmid R67 into pBR322 - Burchall et al, 1982)

RESTRICTION OF PLASMID DNA

Restriction enzymes Pst I, Hind III, EcoRI, Hpa I and Bgl II were purchased from Boehringer Mannheim Corporation. DNA was digested in the presence of 1 ul of BSA (5 mg/ml) (Boehringer Mannheim Corporation), as described in Chapter 1, unless otherwise stated. Conditions for digestion were as directed by the manufacturers.

TABLE 8.1: PLASMIDS USED IN HYBRIDISATION STUDY

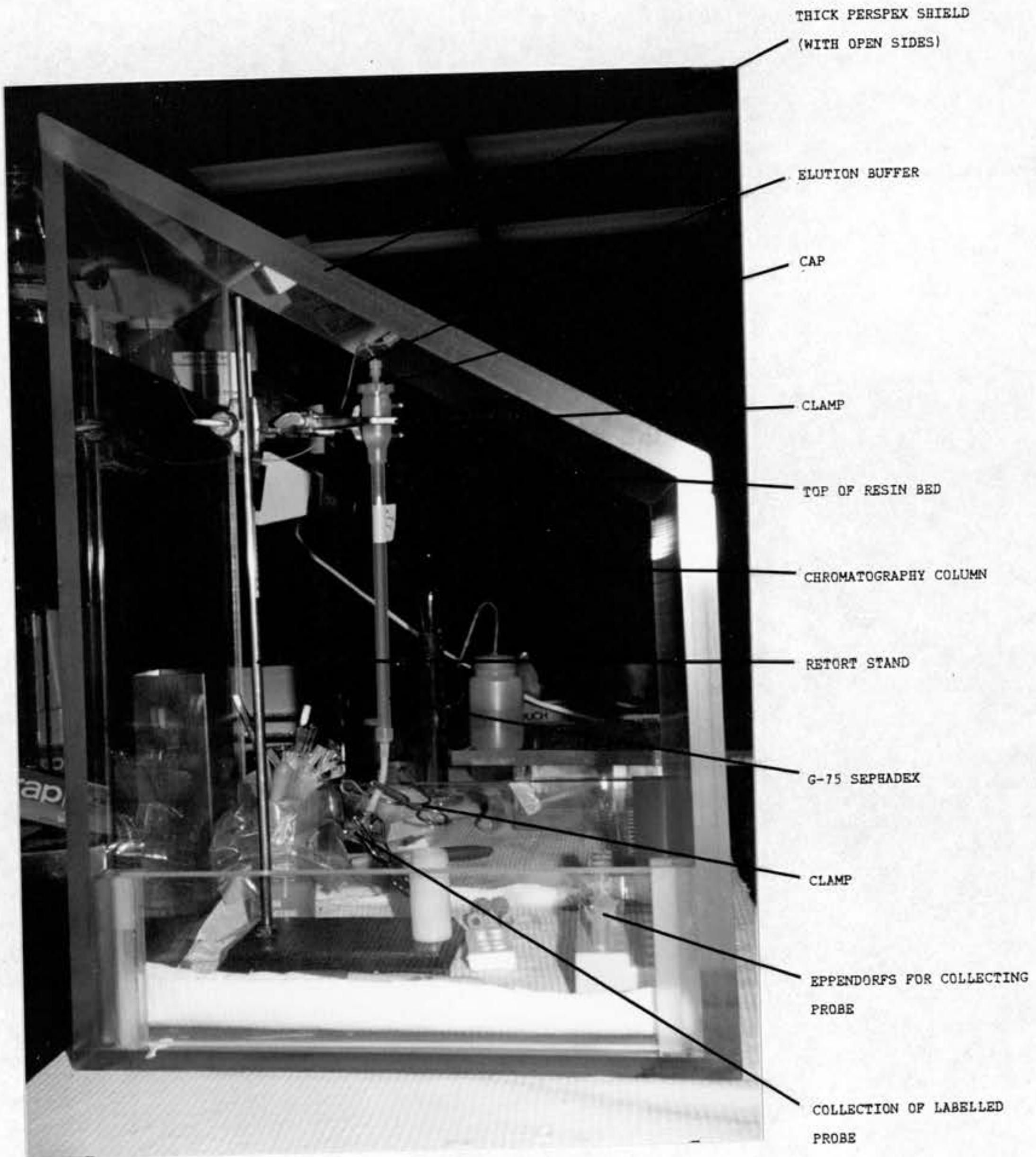
PLASMID	TRANSPOSON	RESISTANCE PATTERN	MOLECULAR SIZE kb	REFERENCE
Sa-1	Tn4135[a]	Su Sm/Sp Tp	70	Chapter 5
Sa-1	Tn4135[ORI]	Ka Su Sm/Sp Tp	40	Chapter 5
Sa-2	Tn4135[a]	Ka Su Sm/Sp Cm Tp	40	Chapter 5
RP4	Tn7	Ap Ka Tc Sm/Sp Tp	64	Chapter 4

a - Transposon transferred from RP4
ORI - Transposon transferred from original pig isolate, p-20

NICK TRANSLATION PROCEDURE

Probes were labelled with ^{32}P dATP by a modification of the Maniatis et al (1975) method. Each unlabelled nucleotide dCTP, dTTP and dGTP was made up to a 1 mM stock solution in NTP buffer (5 mM Tris pH 7.5, 2 mm B-mercaptoethanol, 1 mm EDTA, 50% ethanol) and stored at - 20°C. For use in nick translation, dCTP and dTTP were diluted 1:50 in 50% ethanol and dGTP was diluted 1:10. Two microlitres of each of the diluted unlabelled nucleotides was added to an eppendorf tube and dried in a dessicator for 15 - 20 mins (until completely dry). The following solutions were added in the order described to the dried nucleotides: 15 ul of pure DNA; distilled water to maintain volume at 20 ul; 2 ul '10x' buffer (50 ug BSA, 50 ul NT buffer (1 M Tris, pH 7.8; 0.1 M $MgCl_2$), 0.7 ul B-mercaptoethanol); and 2 ul of DNase stock solution (1 mg/ml) diluted 10^{-7} in distilled water. Three microlitres of ^{32}P dATP was added and the mixture incubated at 15°C for 20 - 30 minutes, depending on probe size. Two microlitres of a 1:10 dilution of DNA polymerase I stock solution (20 u/ul) (BRL) was added and the reaction mixture reincubated for 90 minutes at 15°C. The reaction was then terminated by adding 8 ul of 0.25 M EDTA. Unincorporated nucleotides were removed from the probe by passing the DNA mixture through a 20 cm sephadex G-75 chromatography column (Biorad, California) (Figure 8.1). Excess elution buffer (1mM Tris, 0.25 M EDTA pH 8.0) was removed from the top of the column to just above the resin bed and the tubing clamped. The nick translated mixture was loaded on to the column with a p-200 pippeteman and the column allowed to run until the sample entered the top of the resin. After adding a small amount of buffer and allowing this to run into the resin bed, the column was refilled with buffer and the cap replaced.

FIGURE 8.1: APPARATUS FOR THE REMOVAL OF UNINCORPORATED NUCLEOTIDES FROM THE RADIOACTIVE PROBE



Eight 600 ul fractions were collected in eppendorf tubes and the radioactivity per labelled tube measured on a scintillation counter. Fractions corresponding to the first peak were stored at - 70 c until ready for use. (The column was allowed to wash for at least 30 minutes before reuse).

COLONY HYBRIDISATION

Bacterial strains were grown directly on 0.45 um nitrocellulose filters (Schleicher and Schuell) placed on Luria broth agar plates, and colonies lysed in situ by placing the filter, colony side up , on a sheet of 3 MM Whatman paper soaked in 10% filtered SDS in a pyrex petri dish. After 3 minutes the filter was transferred to a second sheet of 3 MM paper saturated with denaturing solution (0.5 m NaOH, 1.5 M Na Cl), and left for 5 minutes. The filter was then transferred to 3 MM paper soaked in neutralising solution (2 M NaCl, 1 M Tris-Cl pH 8.0) for a further 5 minutes before placing on a final piece of 3 MM paper that had been soaked in 2 x SSPE (0.36 M NaCl, 20 mm NaH_2PO_4, 2 mM EDTA pH 7.4). After 5 minutes the nitrocellulose was removed to a sheet of dry 3 MM paper (colony side up) and allowed to dry at room temperature for 30 to 60 minutes. It was then baked at 65°C overnight.

Filters were hybridised to P^{32} labelled probes in a manner similar to that described by Maniatis et al (1975). Dried blots were placed in heat sealable bags (Phillips sealobags) to which was added 25 ml of a prewash solution (for 100 mls: 5 mls 1 M Tris; 20 mls 5 M NaCl, 0.4 ml 0.25 M EDTA; 1 ml 10% SDS, 73.6 ml sterile distilled water). After

sealing, the bags were incubated at 42°C, with gentle shaking, for 1 hour. The prewash solution was poured off and 5 mls of preincubation solution (for 10 ml: 5 ml formamide, 2.5 ml 20 x SSC (3M NaCl; 0.3M Na citrate), 0.5 ml 2% SDS, 40 ul 0.25 M EDTA, 1 ml 10 x PM (0.2% Ficoll, 0.2% BSA, 0.2% polyvinyl pyrrolidone in 10 x SSC, 960 ul distilled water) was added. Any air bubbles were removed by squeezing, before resealing the bags and incubating, with gentle shaking, at 42°C for a further 3 hours. After this, the probe was prepared as follows; 250 ul of sonicated herring sperm DNA (2.5 mg/ml stock in sterile distilled water) was added to 2.5 ml of preincubation mixture in a glass scintillation vile. The ^{32}P labelled DNA probe was added to this and the solution boiled for 10 minutes on a hot plate in a boiling water bath. A corner of each bag was cut off and the preincubation mixture removed, before adding the probe solution. Care was taken to remove all air bubbles before resealing and incubating overnight at 42°C, with gentle shaking. The following day the bags were opened and the ^{32}P liquid waste removed. Blots were removed and submerged in a prewarmed wash buffer of 5 x SSC, 0.1% SDS, 1 mM EDTA. Incubation proceeded for 1 hour at 65°C, after which time the blots were quickly washed twice in 2 x SSC, with gentle agitation . The blots were air dryed on paper towels ready for autoradiography.

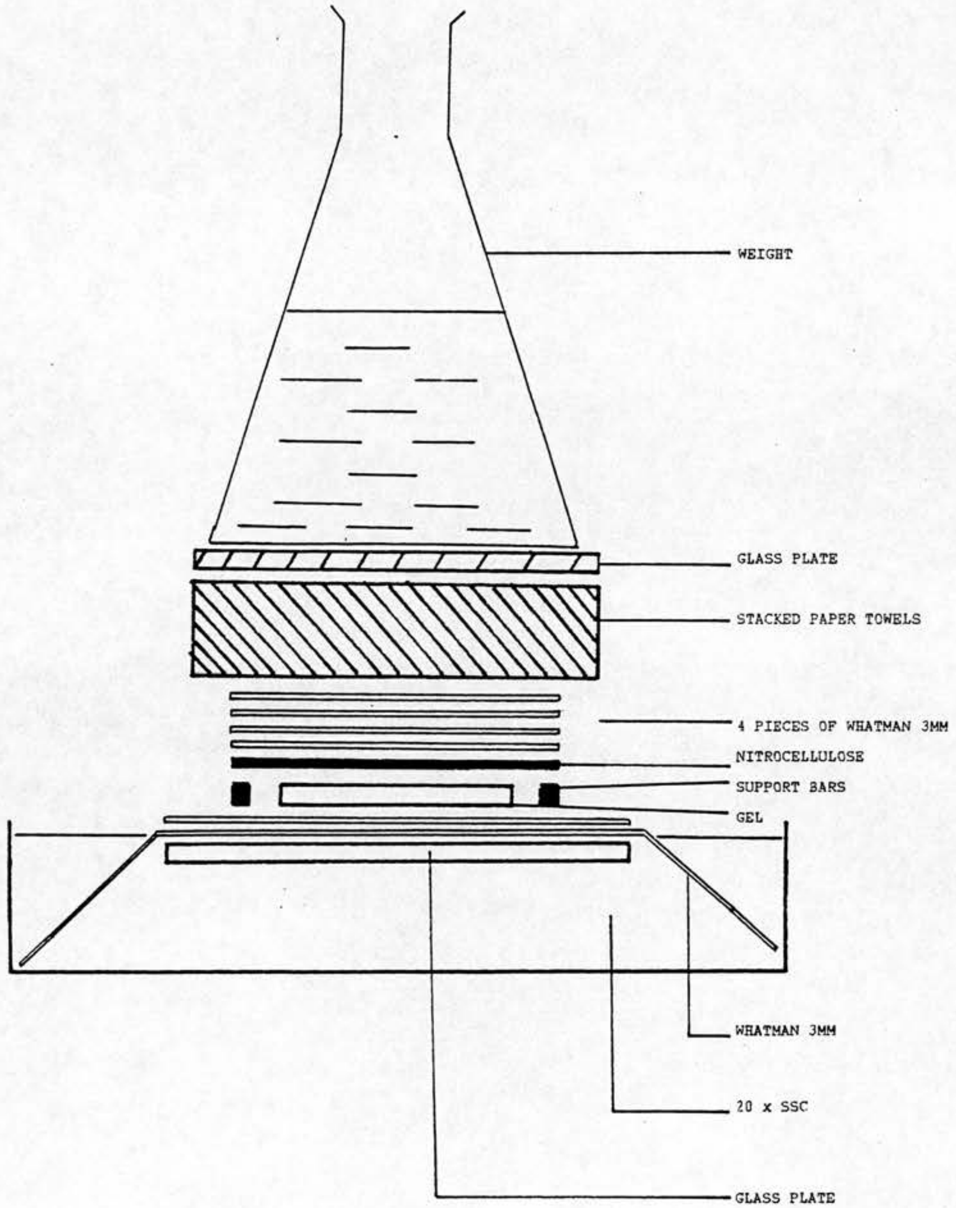
SOUTHERN HYBRIDISATION

Electrophoresed DNA was blotted from agarose gels to nitrocellulose by the method of Southern (1975). Ethidium bromide stained gels were photographed and excess DNA cut away with a razor blade. The remaining gel was immersed in denaturing solution (0.5 M NaOH, 1.5 M Na Cl) and

incubated at room temperature, with gentle shaking. After 45 minutes the solution was poured off and the gel neutralised by soaking in 1 M Tris, 2 M NaCl, adjusted to pH 5.5 with NaOH. The gel was left gently shaking in this solution for 45 minutes. Nitrocellulose (BA85 Schleicher and Schuell), cut 0.25 inches larger than the gel on all sides, was prepared by layering onto the surface of distilled water. Once wetted the nitrocellulose was transferred to the surface of 20 x SSC and immmersed for up to 20 minutes. The transfer apparatus (Figure 8.2) was set up as follows: a large glass tray was filled with 20 x SSC, on top of which was placed a glass plate the width of the tray, but shorter than the tray. A piece of Whatman 3MM filter paper was placed across the glass plate so that its ends extended down into the 20 x SSC. A second piece of filter paper the size of the glass plate was placed on top of this, and the whole unit was kept covered with saran (plastic) wrap until ready to use.

When ready to blot 4 pieces of Whatman 3MM paper were cut the size of the nitrocellulose. The gel was then placed on top of the apparatus and surrounded with plastic support bars. The nirocellulose was place on top of the gel and one of the pieces of filter paper, soaked in 20 x SSC, placed on top of this. The remaining pieces of filter paper were placed, dry, on top and covered with 2-3 inches of stacked paper towels. A glass plate was placed on top of the whole system with a weight on top and left overnight. The following day the paper towels and filter paper were removed and the nitrocellulose, with gel attached, inverted on to a paper towel. Well positions, date and samples, were marked on the nitrocellulose, before removing and discarding the gel. The nitrocellulose was washed in 2 x SSC for 5 - 10 minutes, with gentle shaking, before leaving to dry at room temperature, on top of paper

FIGURE 8.2: SOUTHERN BLOT APPARATUS



towels. The nitrocellulose was baked overnight at 65 ^{o}C and was hybridised to ^{32}p labelled probes in the same manner as for the colony blots, except that the prewash step was omitted.

AUTORADIOGRAPHY

Air dryed blots were exposed to X-ray film (Kodak X - AR - OMAT) at -70°C for between 2 hours and 2 days depending on the activity of the probe.

RESULTS

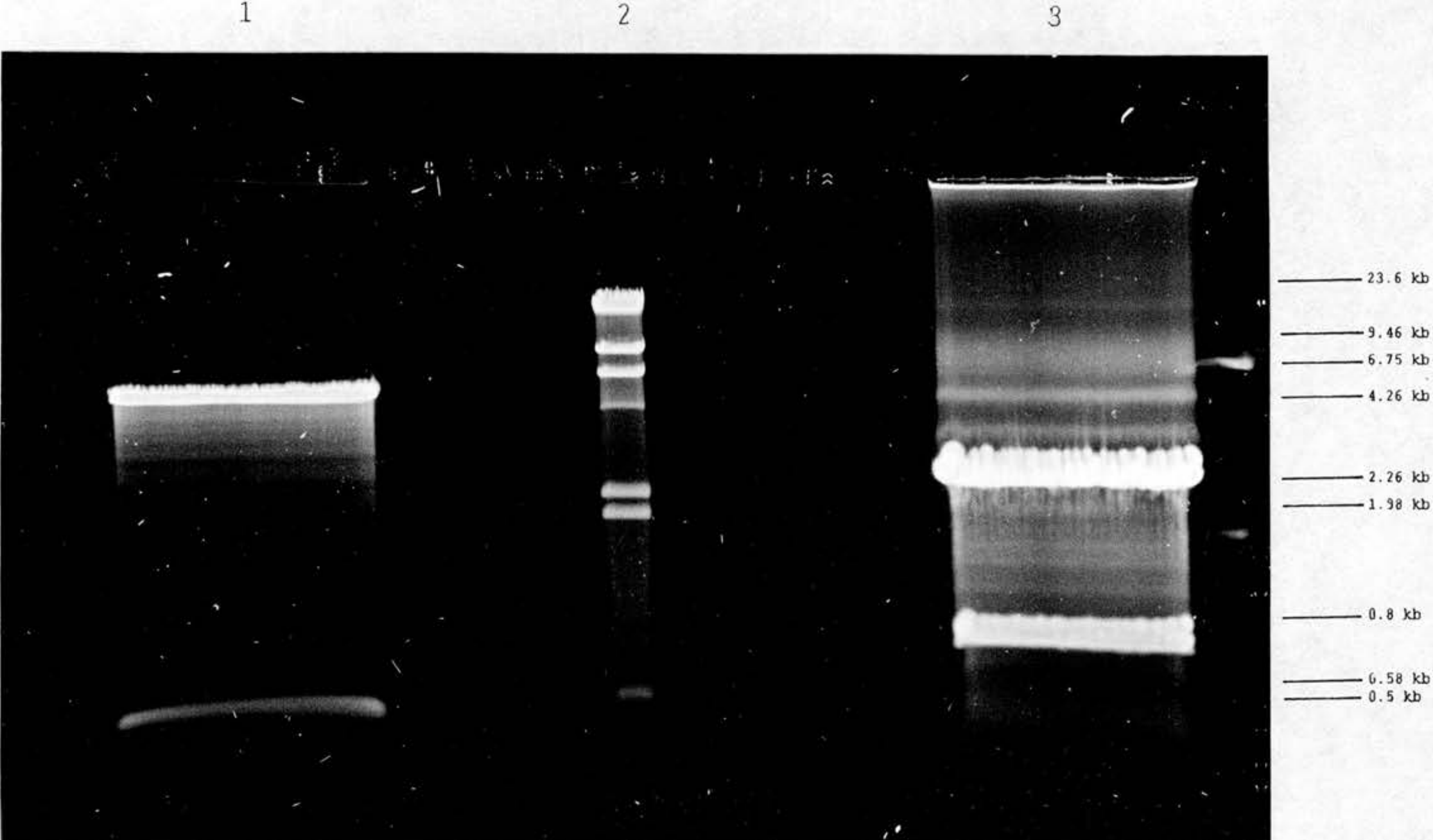
PREPARATION OF PROBES

Gene probes were prepared from large scale preparations (Chapter 3) of RP4, Sa-1, p872 and p700. RP4 and Sa-1 DNA was nick translated directly to a specific activity of 3.3×10^4 cpm/ul DNA solution. Plasmid p872 (100 ul) was digested overnight with 10 u of Hpa I in a total volume of 130 ul, and plasmid p700 (100 ul) was similarly digested with 10 u of EcoR1 in a total volume of 120 ul. Reaction mixtures were electrophoresed in the dark in a 0.7% agarose gel in borate buffer at 80 v for 2 hours (Figure 8.3). The 500 bp p872 fragment, containing the type I DHFR structural gene, and the 800 bp p700 fragment, were electroeluted from the gel (as described in chapter 3) and the fragments nick translated to a specific activity of 3×10^3 cpm/ul DNA solution according to the modified Maniatis method (1975), except unlabelled nucleotides were dried down onto the plasmid DNA to increase the efficiency of labelling.

DETERMINATION OF THE ORIGIN OF (Sa-1::Tn4135)[a] DNA BY COLONY HYBRIDISATION

In order to determine whether aberant excision of Tn4135 from RP4, resulting in the cotransfer of some of the RP4 genome to Sa-1, was responsible for the large size of the Sa-1::Tn4135[a] species (Chapters 5 and 6), this plasmid and suitable controls were probed with radiolabelled RP4 and Sa-1 DNA. Individual E coli colonies, harbouring the following plasmids, were grown directly on nitrocellulose filters: Sa-1, Sa-2, RP4,

FIGURE 8.3: AGAROSE GEL ELECTROPHORESIS OF RESTRICTED PROBE DNA



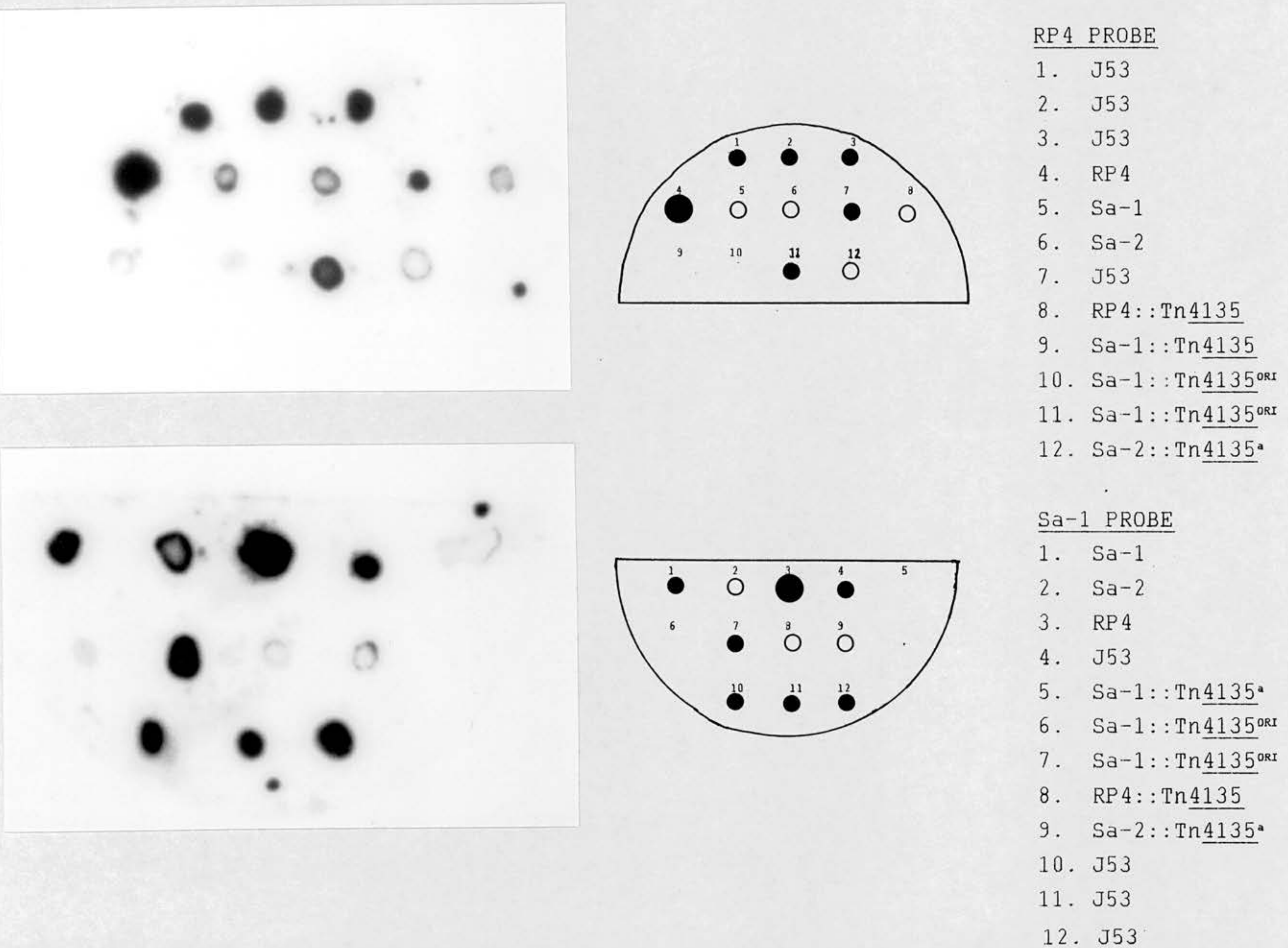
TRACK 1. Hpa I digest of p872, the type I probe
2. Hind III restricted lambda DNA
3. EcoRI digest of p700, the type II probe

RP4::Tn4135, Sa-1::Tn4135^{a}, Sa-1::Tn4135^{ORI}, and Sa-2::Tn4135^{a}, and E coli strain J53 was used as a control. Autoradiograms (Figure 8.4) were examined after 2 hours and indicated that both RP4 and Sa-1 probes unexpectedly hybridised to E coli J53 control DNA. Therefore no firm conclusions could be drawn from the other results. There would also appear to be a certain amount of cross reactivity between the RP4 and Sa-1 probes ie. the Sa-1 probe hybridised to RP4 yet did not hybridise to Sa-2. In order to determine the cross reactivity potential of the RP4 and Sa-1 probes, different E coli species were colony hybridised to the above probes. However, a lack of probe intensity and problems with X-ray film development, hampered the analysis of results and no conclusions could be drawn. (Colony hybridization lacks the specificity of Southern hybridization due to the larger amounts of 'unpurified' DNA present).

DETERMINATION OF THE ORIGIN OF (Sa-1::Tn4135)^{a} DNA BY SOUTHERN HYBRIDISATION

Due to the nonspecificity of the colony hybridisation results, the same plasmid species used in the previous section were restricted, and the DNA fragments probed with the RP4 and Sa-1 probes. DNA was prepared from the test strains by the large scale method and purified by a CsCl density gradient (Chapter 3). Plasmid DNA from Sa-1 containing strains was restricted with 9 u of Bgl II and 25 u of Pst I was utilised to digest DNA from RP4 containing cells. DNA samples (30 ul), including Hind III restricted lambda DNA, were subjected to agarose gel electrophoresis at 40v overnight (Figure 8.5: A(i) and B(i)) and the DNA fragments transferred

FIGURE 8.4: COLONY HYBRIDIZATION UTILISING RADIOLABELLED RP4 AND Sa-1 DNA



RP4 PROBE

1. J53
2. J53
3. J53
4. RP4
5. Sa-1
6. Sa-2
7. J53
8. RP4::Tn4135
9. Sa-1::Tn4135
10. Sa-1::Tn4135^{ORI}
11. Sa-1::Tn4135^{ORI}
12. Sa-2::Tn4135^{a}

Sa-1 PROBE

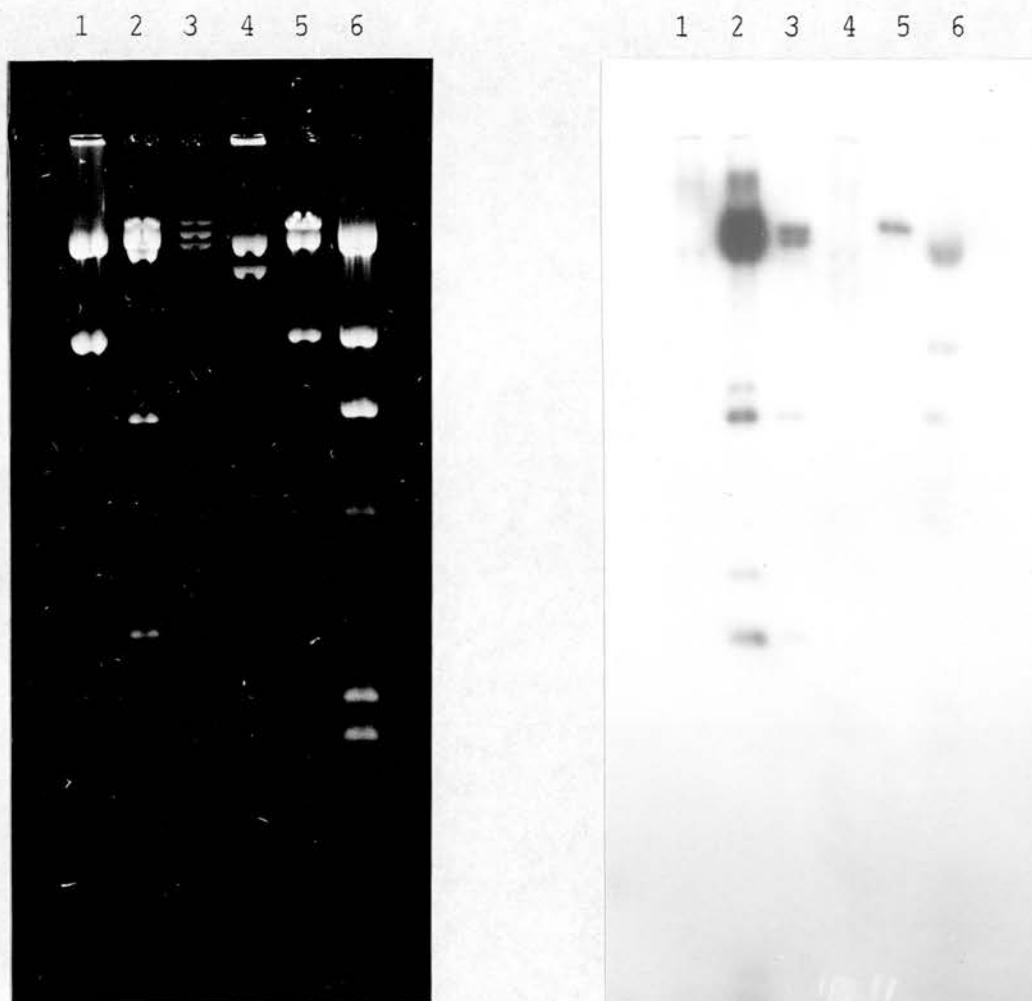
1. Sa-1
2. Sa-2
3. RP4
4. J53
5. Sa-1::Tn4135^{a}
6. Sa-1::Tn4135^{ORI}
7. Sa-1::Tn4135^{ORI}
8. RP4::Tn4135
9. Sa-2::Tn4135^{a}
10. J53
11. J53
12. J53

to nitrocellulose by the method of Southern (1975). Blots were hybridised with the RP4 and Sa-1 probes utilised in the colony hybridisation experiment, and exposed to X-ray film overnight (Figure 8.5: A(ii) and B(ii)). A comparison of the restriction and hybridisation patterns indicates that, although the probes show some cross hybridisation with lambda DNA, there is no cross hybridisation between RP4 and Sa-1. The RP4 probe, whilst not hybridising to Sa-1 or Sa-1::Tn4135^a containing samples, did hybridise to Sa-1::Tn4135^{ORI}. This result suggests that Tn4135 did not cotransfer some of the RP4 genome with it on transposition to Sa-1 but, instead, indicates that Sa-1::Tn4135^{ORI} posseses a region of DNA homologous to part of the RP4 genome. This finding, although contrasting with the expected results in which RP4 would only hybridize to Sa-1::Tn4135, supports the view that the transposons harboured by E coli J62 (Sa-1::Tn4135)^a and E coli J62(Sa-1::Tn4135)^{ORI} genuinely are different. Hybridisation with the Sa-1 probe (Figure 8.5: B(i) and (ii)) suggests that E coli J62(Sa-1::Tn4135)^a does not harbour the Sa-1 plasmid either.

CLASSIFICATION OF THE DHFR GENE OF Tn4135 BY SOUTHERN HYBRIDISATION

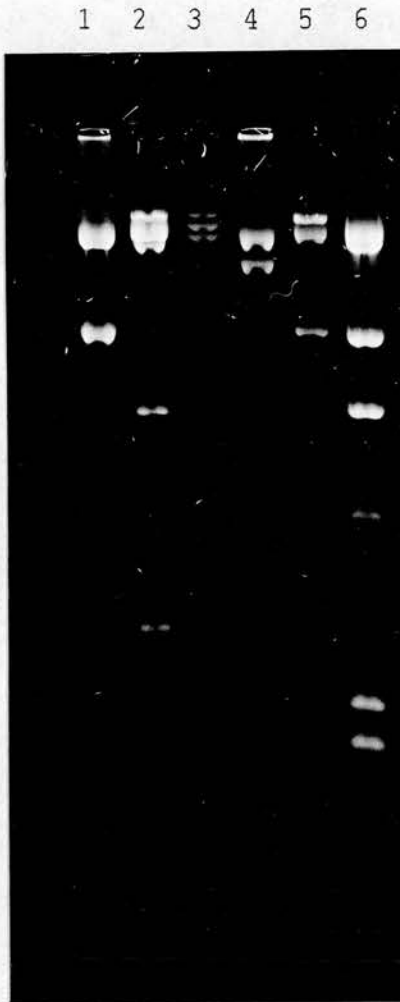
In order to determine the relatedness of the Tn7 and Tn4135 encoded DHFR genes, and to determine any differences between the origin of the E coli J62(Sa-1::Tn4135)^a and E coli J62(Sa-1::Tn4135)^{ORI} encoded genes, hybridisation was carried out utilising DHFR type I and type II gene probes. Plasmid DNA from E coli strains harbouring R751, R388, R483, RP4::Tn4135, Sa-2::Tn4135^a, Sa-1::Tn4135^a, Sa-1::Tn4135^{ORI}, RP4 and

FIGURE 8.5: PROBING OF Tn4135 CONTAINING STRAINS WITH LABELLED RP4 (A) AND Sa-1 (B) DNA



A (i) RESTRICTION PATTERN A (ii) HYBRIDIZATION PATTERN

TRACK 1. Bgl II digest of Sa-1
2. Pst I digest of RP4
3. Pst I digest of RP4::Tn4135
4. Bgl II digest of Sa-1::Tn4135[a]
5. Bgl II digest of Sa-1::Tn4135[ORI]
6. Hind III digest of lambda DNA



B (i) RESTRICTION PATTERN

B (ii) HYBRIDIZATION PATTERN

Sa-1 was prepared by the method of Takahashi and Nagano (1984), and blotted from 0.7% gels (Figures 8.6 and 8.8), run in borate buffer at 100v for 1.25 hours, onto nitrocellulose (Figures 8.7 and 8.9). A comparison of Figures 8.6 and 8.7 indicates that the type I probe is specific; hybridising to R483 but not R388. However, there is some unexpected cross reactivity with RP4. Homology was observed between this probe and RP4::Tn4135, indicating that this latter plasmid encodes a type I-like DHFR gene and is therefore related to Tn7. The (Sa-1::Tn4135)^{a} and (Sa-2::Tn4135)^{a} plasmids also encode a type I DHFR but there was no hybridisation of this probe to strains harbouring (Sa-1::Tn4135)^{ORI}. These observations again indicate that the Tp resistance of the *E coli* J62(Sa-1::Tn4135)^{a} and *E coli* J62(Sa-1::Tn4135)^{ORI} strains is mediated by different enzymes, and that the original pig isolate must therefore harbour two different DHFR genes. The results of hybridisation with the type II probe (Figures 8.8 and 8.9) are not conclusive, owing to the non specificity of this probe, so it was not possible to positively determine whether (Sa-1::Tn4135)^{ORI} harboured a type II enzyme. However, the lack of strong positive hybridisation would suggest that there is little homology between the R67 probe and the Sa-1::Tn4135 ^{ORI} encoded enzyme.

ANALYSIS OF THE TRIMETHOPRIM RESISTANCE MEDIATED BY THE ORIGINAL PIG ISOLATE

The previous results suggest that the original pig isolate harboured two different DHFR genes (possibly residing on different plasmids), and in view of the fact that the genes mediating Tp resistance in *E coli* J62(Sa-1::Tn4135)^{a} and *E coli* J62(Sa-1::Tn4135)^{ORI} appeared

FIGURE 8.6: AGAROSE GEL ELECTROPHORESIS OF Tn4135 CONTAINING STRAINS

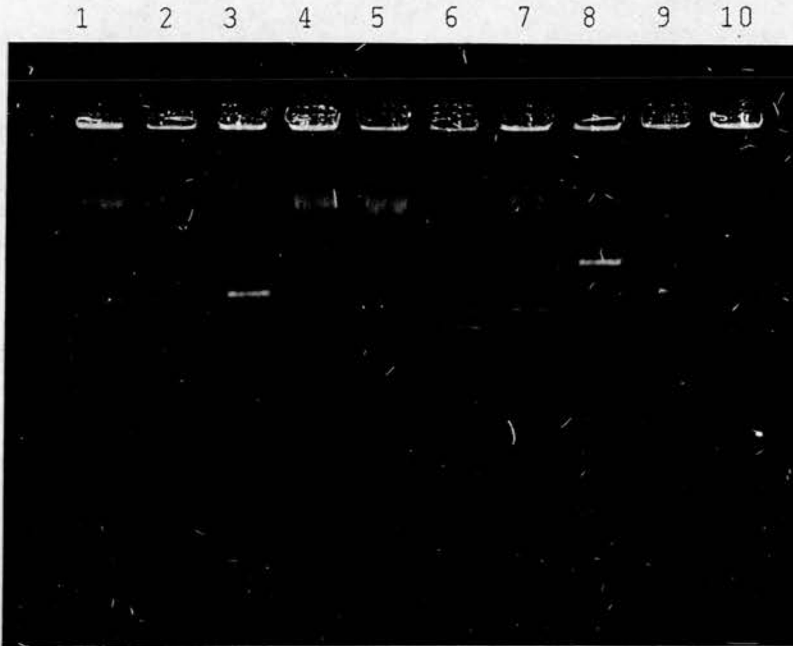


FIGURE 8.7: PROBING Tn4135 CONTAINING STRAINS FOR THE TYPE I DHFR

TRACK 1. RP4
2. Sa-1
3. Sa-1::Tn4135[a]
4. Sa-1::Tn4135^{ORI}
5. Sa-1::Tn4135^{ORI}
6. Sa-2::Tn4135[a]
7. RP4::Tn4135
8. R483::Tn7
9. R388
10. R751

FIGURE 8.8: AGAROSE GEL ELECTROPHORESIS OF Tn4135 CONTAINING STRAINS

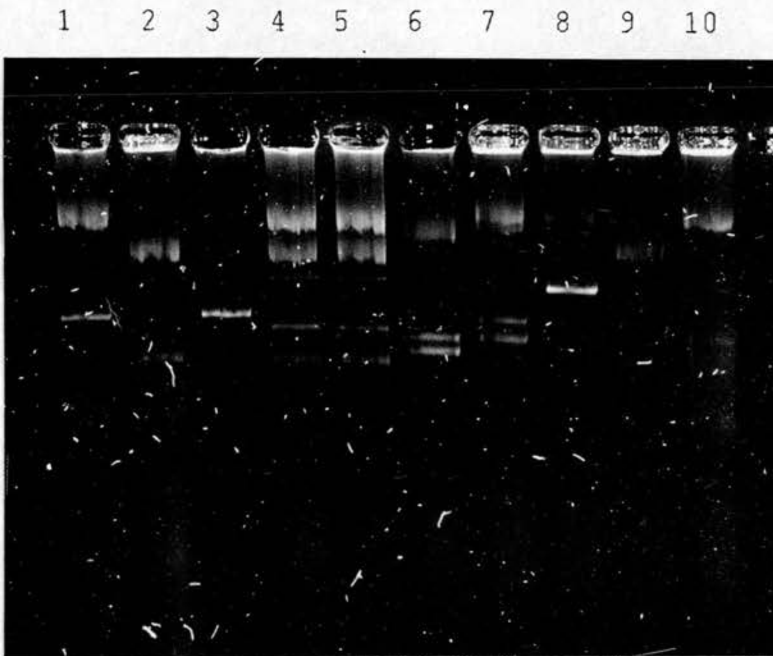
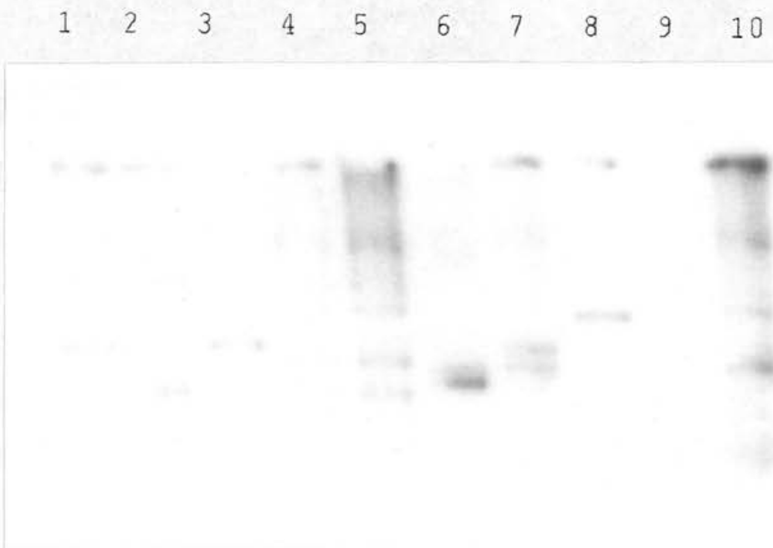


FIGURE 8.9: PROBING Tn4135 CONTAINING STRAINS FOR THE TYPE II DHFR



TRACK			
1.	RP4	6.	Sa-2::Tn<u>4135</u>[a]
2.	Sa-1	7.	RP4::Tn<u>4135</u>
3.	Sa-1::Tn<u>4135</u>[a]	8.	R483::Tn<u>7</u>
4.	Sa-1::Tn<u>4135</u>[ORI]	9.	R388
5.	Sa-1::Tn<u>4135</u>[ORI]	10.	R751

different, DNA from the original pig isolate, P-20, was probed with the type I and type II gene probes. Plasmid DNA was prepared from P-20 cells by the method of Takahashi and Nagano (Chapter 1), and transferred from agarose gels (Figure 8.10), run at 100 v for 1.25 hours in borate buffer, to nitrocellulose.

Hybridisation with the type I probe (Figure 8.11) indicated that the smaller plasmid bands encode a type I DHFR, whilst no homology was observed between this probe and the larger plasmid band. Observations after type II probing, although not conclusive, would suggest that there is no homology between this probe and any of the P-20 plasmids. Thus the E coli J62(Sa-1::Tn4135)^{ORI} encoded gene would appear to have an evolutionary origin distinct from the type I and type II DHFR genes.

FIGURE 8.10: AGAROSE GEL ELECTROPHORESIS OF P-20 STRAINS

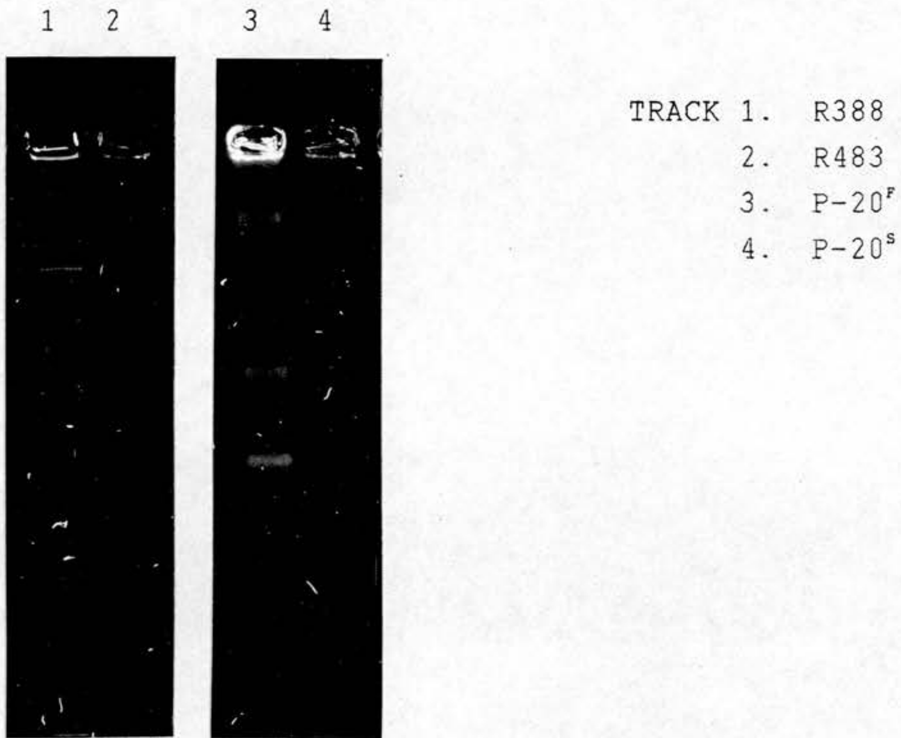
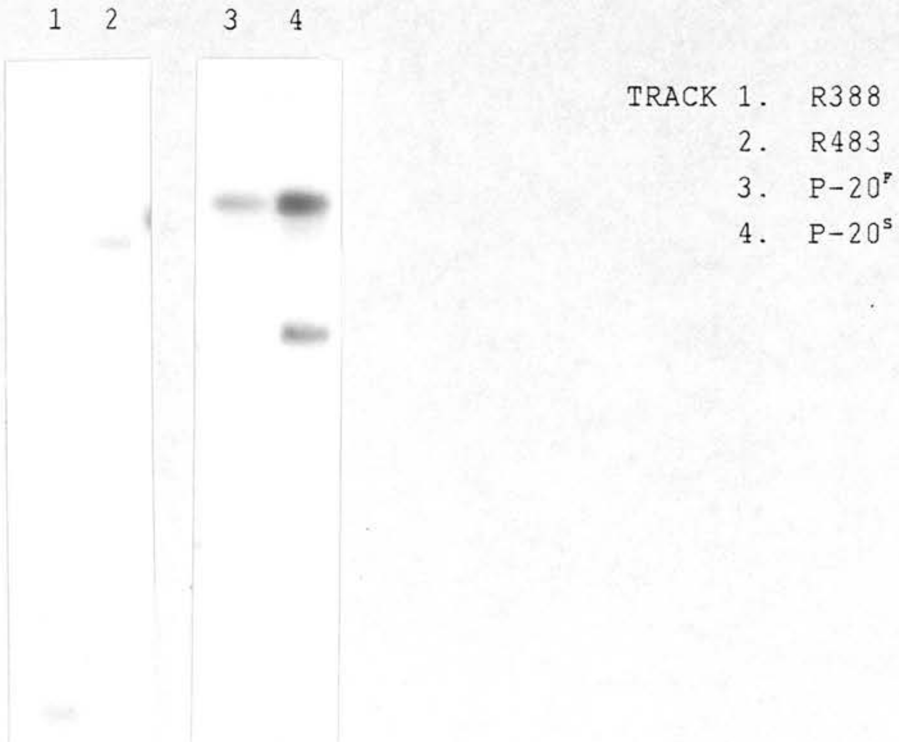


FIGURE 8.11: PROBING P-20 STRAINS FOR THE TYPE I DHFR



DISCUSSION

Nucleic acid hybridization has a number of applications in microbial diagnostics eg in detection of enterotoxigenic _E coli_ (Moseley _et al_, 1982), and viruses (Brandsma and Millar, 1980; Chou and Merigan, 1983) and in taxonomical studies of bacterial plasmids (Roussel and Chabbett, 1978). Although some references to the development of this technique for detecting _Tn7_ in bacterial strains have been made (Elwell _et al_, 1980; Burchall _et al_, 1982; Fling _et al_, 1982), it is only recently that epidemiological studies on the spread of _Tn7_ have made use of DNA hybridization.

The probes used to monitor this spread of Tp resistance have varied: Datta _et al_ (1981) utilised the whole of CoEl::_Tn7_ and Fling _et al_ (1982) used the plasmid pFE506 as a type I probe. Both these probes contain the type I DHFR gene, whereas Pulkkinen _et al_ (1984) develop ed a type I probe, containing the _Bam_ HI fragment of _Tn7_, which lacked the type I structural gene but which was still specific and able to detect the type I DHFR. In this study of _Tn4135_ , probes contained the type I structural gene of _Tn7_, from a pBR322 derivative, and an 800 bp ECoRl fragment of R67 capable of detecting the type II DHFR (Elwell, personal communication), but their specificity was not absolute. Although differentiating between the type I (R483) and the type II (R388) enzymes, the type I probe appeared to cross hybridise with RP4. Although this type I probe is known to cross hybridize with the _E coli_ chromosome (Fling and Richards, 1983; Simonsen _et al_, 1983), this lack of specificity is more likely to be due to contaminating pBR322 DNA in the probe, as suggested by Pulkinnen _et al_

(1984), or possibly as a result of non-optimal hybridization conditions. The specificity of the hybridization reaction on filters can be affected by two types of artefacts: non specific binding of the probe DNA to the filter and non specific hybridization of probe DNA to the DNA sequences bound to the filter (Caro _et al_, 1984). Single stranded DNA binds efficiently to filters and if this happens during the hybridization reaction it will cause a 'background' that can reach high levels. A number of methods have been devised to reduce this ' background' (Denhardt, 1966), including carrying out the reaction in the presence of a denaturing agents, such as formamide (McConaughty _et al_, 1969). The second type of background, hybridizing of probe DNA to heterologous sequences of the DNA bound to the filter, results from the accidental presence of short homologous sequences on both DNA's for any one of a number of causes (insertion sequences, evolutionary relationship etc.). The conditions of the hybridization reaction will determine how extensive such a spurious homology has to be before it contributes significantly to the background. Therefore, in choosing hybridization conditions, a balance must be found between maximum specificity and maximum efficiency (McConaughy _et al_, 1969). Probe concentration and sequence complexity , temperature, solvent and salt concentration will all effect the rate and precision of probe hybridization. This latter explanation, with RP4 and the type I probe containing an identical sequence - possibly an IS element - is the more likely, since probing of RP4::Tn4135 DNA revealed a similar hybridization pattern, all be it more intense. Problems with 'background' hybridization also arose with the type II probe, as suggested by Elwell (personal communication). However, despite the non-specificity of the probes, this technique was able to confirm that the Tn_4135_ transposon residing in RP4, Sa-1::Tn_4135_[a] and Sa-2::Tn_4135_[a] encoded a Tn_7_-like

type I DHFR, and that this enzyme differred from that encoded by Sa-1::Tn4135[ORI]. The lack of strong positive hybridization with the type II probe, although not conclusive, would suggest that the latter DHFR has an origin distinct from the type I or type II enzymes. This view was confirmed by probing of the P-20 isolate with both probes; only the type I probe hybridized.

Colony hybridization was utilised to detect the presence of RP4 sequences in the Sa-1::Tn4135[a] plasmid, but problems arose with the specificity of this method: the RP4 and Sa-1 probes appeared to be non-specifically hybridizing to plasmid-free E coli J53 cells. Whilst suggesting that the initial colony hybridisation technique described by Grunsten and Hogness (1975), and subsequently modified by Gergen et al (1979), was not sufficiently sensitive to detect small genes in large naturally occurring plasmids of low copy number (such as RP4 and Sa-1), Maas (1983) indicated that nonspecific hybridization could be decreased by reducing the quantity of probe used. Although producing a weaker signal this would presumably dilute out contaminant DNA. However, because of the relatively large size of the Sa-1 and RP4 probes it is conceivable that the E coli chromosome might contain sequences, such as IS elements, that are also present in RP4 and Sa-1. Therefore a more likely solution to the problem would be to remove the chromosomal DNA and restrict the sample plasmid DNA, to increase the specificity of the hybridization.

Despite the cross reactivity with lambda DNA, Southern hybridization did improve the specificity of the experiments, and confirmed previous antibiotic sensitivity results that E coli J62(Sa-1::Tn4135)[a] did not contain any sequences specific to RP4 or Sa-1. This data suggests that the molecular species of E coli J62(Sa-1::Tn4135)[a] contains no plasmid DNA and may, therefore, be an autonomously replicating R-determinant

(Clowes, 1972; Rownd et al, 1978; Wiedemann ,1981) consisting of spontaneously amplified copies of the Tp resistance gene. This would contradict reports by Clewell et al (1974) who were unable to detect poly r-dets in the amplification of Pam I, although Perlman and Rownd (1975), using improved techniques, demonstrated both monomers and polymers of r-dets in cultures of transitioned cells. In contrast, the hybridization of probes to E coli J62(Sa-1::Tn4135)^{ORI} DNA, and, in particular, the RP4 probe, would appear to indicate that this transposon contains a sequence specific to the RP4 plasmid. When (Sa-1::Tn4135)^{ORI} DNA was probed with RP4 DNA only the larger of the two bands hybridized, as compared with both bands after Sa-1 probing. Non specific hybridization can be ruled out due to the fact that RP4 does not hybridize to Sa-1 alone.

The results mentioned support the concept that E coli J62(Sa-1::Tn4135)^{a} and E coli J62(Sa-1::Tn4135)^{ORI} contain different DHFR genes and thus the original pig isolate, P-20, contains two Tp resistance genes of distinct evolutionary origins. One of these genes is related to Tn7 but the other, encoded by Sa-1::Tn4135^{ORI}, showed no sequence homology with either the type I or type II DHFR 's and therefore awaits further characterisation.

CHAPTER 9

BIOCHEMICAL AND GENETIC ANALYSIS OF Tn402

INTRODUCTION

R751 is a member of the incompatibility group P-1 plasmids (Jobanputra and Datta, 1974); other members include RK2, RP1 and RP4 - which are probably identical to one another (Burkhardt et al, 1979). This group of plasmids was first identified in Pseudomonas aeruginosa as a result of an investigation into the agents responsible for carbenicillin resistance in infections in a hospital burns unit (Lowbury et al, 1969), although R751, itself, was found in a strain of Klebsiella aerogenes (Jobanputra and Datta, 1974). A wide range of Gram negative bacteria have now been found to harbour plasmids of this incompatibility group and it is this broad host range or promiscuous property that has excited an interest for study (Datta and Hedges, 1972; Olsen and Shipley, 1975; Beringer, 1974; Cho et al, 1975). There is speculation that in the past this group of plasmids could have been responsible for genetic exchange between otherwise unrelated bacterial species. Inc P group plasmids are invaluable in initiating the genetic analysis and manipulation of potentially important bacteria and have been used, along with their derivatives, as broad host range cloning vehicles (Jacob and Grinter, 1975; Meyer et al, 1975; Hedges et al, 1976). The plasmid R751 has been used primarily as an Inc P-1 group plasmid for incompatability testing (Datta, 1974; 1977).

The most extensively studied of the Inc P group plasmids are RK2, RP4 and RP1 (Barth and Grinter, 1977; Meyer et al, 1977b; Figurski and Helsinki, 1979; Thomas et al, 1980), which all carry resistance genes for Ka, Ap and Tc (Ingram et al, 1973). The plasmid R751, although

approximately the same molecular size (51.4 kb compared with 56.4 kb - Meyer et al, 1977a; Burkhardt et al, 1978), does not encode the same drug resistances (Jobanputra and Datta, 1974). It carries a gene for trimethoprim resistance only, which was found to reside on a transposon, Tn402 (Shapiro and Sporn, 1977).

The genetic organization of R751 has been studied by Meyer and Shapiro (1980) and Ward and Grinsted (1982). The restriction map, although varying slightly between the two latter papers, was found to bear no resemblance to that of RK2 (RP1, RP4) (Meyer et al, 1977c; Grinsted et al, 1977, 1978; Barth and Grinter, 1977; Depicker et al, 1977) but, like RK2, the genes for replication and self transfer are located at positions which are physically separated from one another. These positions are relatively free from restriction sites (Meyer and Shapiro, 1980), compared with regions encoding the antibiotic resistance genes (Meyer et al, 1977c, Thomas et al, 1980). The lack of restriction sites is consistent with the idea that the restriction map reflects the evolution of the broad host range plasmids (Ward and Grinsted, 1982). Recognition sites for restriction enzymes might be expected to be lost through evolution of these plasmids, if it is assumed that the class II enzymes are important in degrading foreign DNA. Regions lacking large numbers of restriction sites would have been conserved in the evolutionary process and, as a result, contain genes coding for essential functions. On the other hand, regions containing many such sites, eg. DNA encoding antibiotic resitance, would have been acquired more recently, possibly by reciprocal recombinant events or by transposition eg. Tn402.

The origins of the trimethoprim resistance gene of R751, and whether in fact it does lie on a transposon, are still disputed. Despite the fact that Shapiro and Sporn (1977) have shown that Tn402 can transpose to

phage lambda, there have been no reports of its transferability to other replicons (Amyes, 1979; Goldstein et al, 1986). The lack of resistance to streptomycin of strains harbouring R751 would indicate a dissimilarity from the more common trimethoprim resistance encoding transposon, Tn7 (Barth et al, 1976). Tn402 is also smaller than Tn7: 7.5 kb (Shapiro and Sporn, 1977) as compared with 14 kb for Tn7 (Barth et al, 1976), and there is no genetic evidence that the transposons are related. Tennhammer-Ekman and Skold (1979) also indicated that the DHFR genes carried by Tn7 and Tn402 were distinct and therefore possessed different origins. Attempts to establish the origin of R751, and thus Tn402, have included hybridization studies with DNA from other Inc P plasmids (Ward and Grinsted, 1982) and examination of the DHFR produced by Tn402, in comparison with DHFR's from R388, R483::Tn7 and R67bis. The Studies of the DHFR have included biochemical analysis (Pattishall et al, 1977; Amyes and Smith, 1978; Tennhammer-Ekman and Skold, 1979), hybridization of the gene with Type I and II gene probes (Fling et al, 1982), use of reacting sera to DHFR (Fling and Elwell, 1980) and isoelectric focusing of the DHFR (Broad and Smith, 1982). As mentioned in chapter 7, the exact classification of DHFR to type is still controversial in some cases, although the features of the type I and type II enzymes are distinct. Whilst R388 has been classified as a variant of type II (it possesses the resistance properties of a type I enzyme (Amyes and Smith, 1976) but is synthesised in amounts typical of the type II), R751 has been classified both as a type I (Amyes and Smith, 1978) and a type II (Fling and Elwell, 1980). Tennhammer-Ekman and Skold (1979) indicated from their results that R751 did not satisfactorily fit into either classification, therefore suggesting a putative type III classification for this enzyme. Although not classifying to enzyme type, these authors showed that R751, like R388,

had properties similar to R483, the only difference being the amount of enzyme produced. However, Amyes (1986) is in agreement with Broad and Smith (1982) that R751's properties are sufficiently similar to the type II enzyme that it can be classified as a variant type II enzyme (like R388). The type II class would thus consist of Type IIa (R67bis) and Type IIb (R751) (Amyes, 1986).

Much of the confusion in the classification of R751, and the apparent variations in the properties of this plasmid may have resulted from the extremely low production of this enzyme (Amyes and Smith, 1978). However, major advances have now been made in the ability to clone genes into multicopy plasmid vectors with a concomittant increase in the gene product. Therefore, in order to re-evaluate the properties of the DHFR encoded by R751, the trimethoprim resistance gene was cloned into the multicopy plasmid pBR322.

MATERIALS AND METHODS

The experimental procedures for resistance testing and plasmid transfer are as indicated in Chapter 3, and transposon transfer was affected as described in Chapter 4. The techniques used for phage lysis are as in Chapter 5 and for the biochemical analysis of the Tn402 DHFR, as in Chapter 7.

PLASMID DNA EXTRACTION AND GEL ELECTROPHORESIS

Plasmid DNA was prepared from overnight broth cultures by the method of Takahashi and Nagano (1984) (see Chapter 1). Restriction digestion was performed with Pst 1 (NBL Enzymes Ltd) for 3 hours by method 11 (chapter 1). Samples were electophoresed in Buffer A (chapter 1) either on 0.7 % horizontal agarose gels (chapter 1) or on 1 % agarose minigels (Uniscience Ltd), for the times and voltages listed.

DNA CLONING - (Hatfield Polytechnic, 1985)

Aliquots (40 ul) of restricted cloning vector and sample were placed in eppendorf tubes and the restriction enzyme inactivated by incubating at 70°C in a water bath for 15 minutes. The contents of one tube were transferred into the other with a micro-pipette and mixed well. Forty microlitres were transferred back into the first tube and 5 ul of ligase additive (NBL Enzymes Ltd) added to each tube. Five microlitres of T4 DNA

ligase (NBL Enzymes Ltd) was added to one tube (ligase +ve) and 5 ul of distilled water added to the other (ligase -ve). The tubes were maintained at 4°C overnight to allow ligation to proceed. Approximately 5 ul was removed from each tube for analysis by agarose gel electrophoresis.

BACTERIAL TRANSFORMATION

Bacterial transformation was carried out as indicated in Chapter 3, utilising 50 ul of unrestricted cloning vector, the remainder of the ligase -ve mixture and the remainder of the ligase +ve mixture. A control, containing no added DNA, was also set up.

RESULTS

TRANSPOSITION OF Tn402

a. Transfer of Tn402 from R751 to Sa (Figure 9.1)

The plasmid Sa-1 was introduced into E. coli J62(R751::Tn402) in a 5 hour standard mating, selecting for trimethoprim and kanamycin resistances. (The transfer frequency is shown in Table 9.1, mating 1). After promoting transposon transfer, by subculture, a single transconjugant colony was used as a recipient in a mating with E. coli J53(RP4). Selection was made on DM plates containing the supplements for strain J62 and the three antibacterial drugs ampicillin, trimethoprim and kanamycin (Table 9.1, mating 2). Transconjugants were subcultured twice in nutrient broth and restreaked on the same DM selection plates as before. Colonies were tested for their resistance to unselected markers and a colony displaying the characteristics of a J62 strain harbouring RP4 Sa and Tn402 (ie. carrying the resistance determinants to Ap, Ka, Tc, Sm/Sp) was identified. (The presence of RP4 in the strain should have eliminated R751, a plasmid of the same incompatibility group. This strain was used as a donor in a standard mating with E coli J53. Selection was carried out on DM plates containing the supplements for strain J53 plus either trimethoprim alone (selection for the transposon), streptomycin alone (selection for plasmid Sa) and the two drugs together (Table 9.1; mating 3). Resistances of transconjugants were again checked after subculturing and a colony was identified and purified which conferred

FIGURE 9.1: FLOW DIAGRAM FOR THE TRANSFER OF Tn502 FROM R751 TO Sa-1

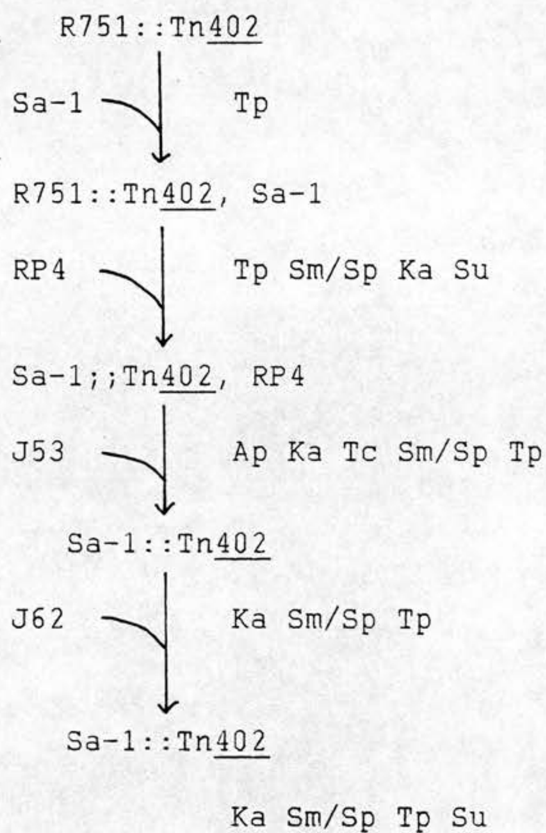


TABLE 9.1: TRANSFER FREQUENCIES FOR THE TRANSFER OF Tn402 FROM R751 TO Sa

EXPERIMENT	MATING	SELECTION MEDIA	TRANSFER FREQUENCY
1	Introduction of Sa	Ka Tp	5.78×10^{-3}
2	Introduction of RP4	Ka Tp Ap	3.02×10^{-3}
3	Transfer to <u>E coli</u> J53	Tp	4.03×10^{-3}
		Sm	7.65
		Tp Sm	4.03
4	Transfer to <u>E coli</u> J62-2	Sm Rif	1.26
		Tp Rif	0.84
		Tp Sm Rif	2.53

resistance to Ka, Sm/Sp, Tp, the markers for Sa and Tn402. This strain was used as a donor to transfer Sa::Tn402 back into E coli J62-2. Selection was performed on DM plates with the J62 supplements to which had been added rifampicin and either trimethoprim, streptomycin or trimethoprim and streptomycin (Table 9.1; mating 4). The transconjugants were purified and found to confer resistance to Ka, Sm/Sp, Su and Tp. This inferred the presence of plasmid Sa-1 into which had been inserted Tn402.

b. Analysis of E. coli J6-2(Sa-1::Tn402) for the Loss of R751 by Phage Lysis

1. Lysis with Stock PRR1 and PR4 Phage

In order to establish if the original R751 plasmid had been eliminated E coli J62(R751), E coli J53(RP4), E coli J53(Sa-1), E coli J62(R7K) and E coli J62(Sa-1::Tn402) were grown up overnight in Nutrient broth (4.5 ml) and diluted as appropriate. The phages PRR1 (neat and 10^{-2} dilutions) and PR4 (10^{-6} dilution) were utilized in phage overlays of the above cultures and the plaque counts per ml are shown in Table 9.2. Unexpectedly, PRR1 lysed Inc W plasmid-carrying strains, as well as Inc P, which raised doubts as to the specificity of this phage.

TABLE 9.2: PLAQUE COUNTS FOR PRR1 AND PR4 LYSIS

STRAIN	PRR1 pfu/ml	PR4 pfu/ml
E coli J62(R751)	8.16×10^5	1.13×10^9
E coli J53(RP4)	Confluent	Confluent
E coli J53(Sa-1)	XS	3.00×10^7
E coli J62(R7K)	XS	$< 10^7$
E coli J62(Sa-1::Tn402)	2.56×10^5	1.60×10^9

11. Lysis with Purified PRR1

As a result of the PRR1 retitering experiment (Chapter 6), which indicated that PRR1 genuinely lysed cells containing the Inc W plasmid, albeit at an eop which was 800 times lower than its ability on Inc P plasmid containing strains, the above lysis experiment was repeated with a newly purified phage PRR1 preparation (Chapter 6); looking for differences in eop (Table 9.3). Despite the lack of specificity of the phage, PRRR1 lysed Sa-1 containing strains although the plaque counts were 100 fold lower than for strains harbouring RP4. E coli J62(Sa-1::Tn402) counts compare more favourably with RP4 and R751 than with Sa-1, suggesting that an Inc P plasmid may still be present within this cell.

c. Analysis of E.coli J6-2(Sa-1::Tn402) for the Loss of R751 by Transfer to Pseudomonas

Due to the lack of phage PRR1 specificity an alternative approach to determining the presence/absence of an Inc P plasmid in E coli J62(Sa-1::Tn402) was adopted: transfer to P aeruginosa. Inc P plasmids are easily transferred to this strain and stably maintained, whilst Inc W plasmids are either not transfered at all, or not as readily. A comparison of the transfer frequencies of Sa-1, R751, RP4 and Sa-1::Tn402 to P aeruginosa, should thus establish the origin of the DNA of the latter plasmid species.

However, as indicated in Chapter 6, an inability to find suitable selection markers for the incomming plasmids and P aeruginosa, whilst selecting against the E coli donor cells, prevented any conclusive results being obtained. Selection with Ka, naladixic acid and sulphonamide all

TABLE 9.3: PLAQUE COUNTS FOR PRR1 AND PR4 LYSIS USING PURIFIED PHAGE

STRAIN	PRR1	PR4
E coli J62(R751)	2.4×10^8	8.00×10^7
E coli J53(RP4)	9.9×10^9	9.00×10^8
E coli J53(Sa-1)	1.0×10^7	4.00×10^7
E coli J62(Sa-1::Tn402)	8.2×10^9	2.38×10^9

proved unsuitable due to the high intrinsic resistance of P aeruginosa to Ka, the unexpected resistance of E coli to Nalidixic acid at 10 ug/ml and the poor selectibility potential of Su.

d. Minimum Inhibitory Concentration(MIC) for Trimethoprim of E. coli J62(Sa-1::Tn402)

In order to check that the Tp resistance of E coli J62(Sa-1::Tn402) was due to a plasmid/transposon mediated gene, and not a chromosomal mutation, the trimethoprim MIC of this strain along with E coli J53(Sa-1) and E coli J62(R751) were determined (Table 9.4).

E coli J62(Sa-1::Tn402) has a Tp MIC of >1000 ug/ml indicative of a plasmid/transposon mediated resistance gene, but from the phage lysis and Pseudomonas transfer experiments it is not possible to determine whether R751 is still present, or whether Tn402 has transposed to Sa-1.

THE CLONING OF Tn402 INTO pBR322

Due to difficulties in obtaining enough DHFR from R751, to determine accurately enzyme properties and therefore assign enzyme type, Tn402 DNA was cloned into PBR322 following the protocol of Hatfield Polytechnic (1985). pBR322 and R751 DNA were restricted with Pst I, utilising various restriction mixture compositions (Table 9.5), ligated, and E coli C600

TABLE 9.4: TRIMETHOPRIN MINIMUM INHIBITORY CONCENTRATIONS

STRAIN	Tp MIC (ug/ml)
E coli J53(Sa-1)	< 2.5
E coli J62(R751)	> 1280
E coli J62(Sa-1::Tn*402*)	> 1280

TABLE 9.5: VARIATIONS IN THE COMPOSITION OF THE RESTRICTION MIXTURE

	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3
VOLUME OF INITIAL pBR322 CULTURE	4.5 ml	4.5 ml	4.5 ml
VOLUME OF INITIAL R751 CULTURE	4.5 ml	4.5 ml	9.0 ml
VOLUME OF DW IN WHICH NEWLY ISOLATED pBR322 DNA RESUSPENDED IN	60.0 ul	150.0 ul	150.0 ul
VOLUME OF DW IN WHICH NEWLY ISOLATED R751 DNA RESUSPENDED IN	60.0 ul	34.0 ul	34.0 ul
VOLUME OF pBR322 DNA RESTRICTED	15.0 ul	15.0 ul	15.0 ul
VOLUME OF R751 DNA RESTRICTED	30.0 ul	30.0 ul	30.0 ul
VOLUME OF RESTRICTION BUFFER	6.0 ul	6.0 ul	6.0 ul
VOLUME OF _Pst_ I ENZYME	1.0 ul	2.0 ul	2.0 ul
NUMBER OF _Pst_ I UNITS	6.0 UNITS	40.0 UNITS	40.0 UNITS
TOTAL VOLUME	60.0 ul	50.0 ul	50.0 ul

cells transformed with the resulting DNA. Transformants were examined for the presence of Tn402 DNA inserted into pBR322.

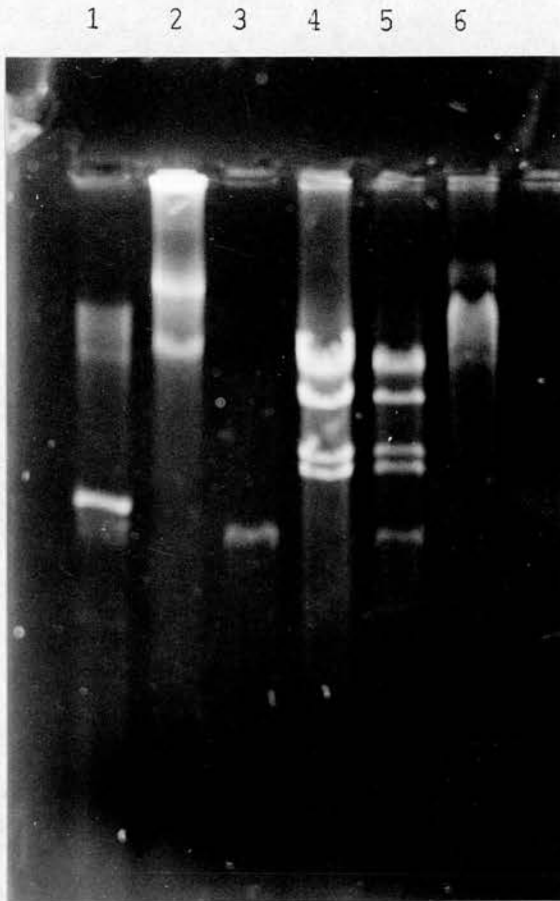
a. Examination of Ligation Mixtures

To check that R751 and pBR322 restricted DNA had ligated, before carrying out any transformation, cut and uncut DNA (5 ul) from PBR322 and R751 were examined, along with an aliquot (4 ul) from the ligase +ve and ligase -ve DNA mixture, by agarose gel electrophoresis on a submerged minigel system. One percent gels were run for 1.5 hours at 100 v. Ligase +ve, ligase -ve and restricted pBR322 DNA samples produced by experiment 1 (Table 9.5), failed to produce any plasmid bands. However, plasmid DNA was present in all samples in experiment 2 (Table 9.5), but the bands were very faint. An increase in the concentration of R751 DNA in the restriction mixture (Table 9.5; Experiment 3) improved the sharpness of bands (Figure 9.2) and indicated that R751 and PBR322 DNA had been restricted by Pst 1 and that the DNA ligase was functioning properly (Tracks 5 and 6).

b. Examination of Ligation Products by Bacterial Transformation

Ligase + DNA from each of the experiments 1 - 3 was used to transform E coli C600 cells, selecting for transformants on LB plates containing Ap,Tp, and Tc incividually, and Ap Tp together and Tc Tp together. (Transformations utilising ligase -ve DNA, uncut pBR322 DNA and no DNA were also set up to act as controls). pBR322 DNA transformed in experiment 1, despite the lack of DNA visible on the gel, as did DNA from the ligase -ve and +ve samples, but the DNA fragment of R751 carrying

FIGURE 9.2: AGAROSE GEL OF THE DIFFERENT STAGES IN THE LIGATION OF R751 AND Tn402 DNA FROM EXPERIMENT 3



TRACK 1. Uncut PBR322

2. Uncut R751

3. Pst I restricted PBR322

4. Pst I restricted R751

5. Ligase -ve sample

6. Ligase +ve sample

Tp resistance had not been picked up, as there was no growth on the Tp plates. The conditions of experiment 2 greatly increased the chances of PBR322 picking up the Tp containing fragment, and the number of transformants per ml of undiluted sample are shown in Table 9.6. However, Ap resistant transformants unexpectedly appeared to be as equally prevalent as Tc resistant transformants. The resistances of transformants from the Tp Tc and the Tp only plates were therefore checked by restreaking on plates containing appropriate antibacterial drugs. All transformants from the Tp Tc plates were found to be Ap resistant, as well as Tp and Tc resistant (Table 9.7). Table 9.8 indicates the number of transformants obtained per ml on each selective medium under the conditions of experiment 3. The number of ligase +ve transformants growing on the Ap plates is again comparable to those growing on Tc, but some of the transformants from the ligase +ve plates, when restreaked out to check their resistances markers (Table 9.9), were found to be Ap sensitive, as expected. Those transformants resistant to Tp and Tc but sensitive to Ap were indicative of pBR322 with Tn402 inserted into the Ap resistance gene.

c. Examination of DNA from a Number of Transformed Colonies

In order to check transformants for the insertion of Tn402 into pBR322, DNA from possible Tn402 containing transformants plus suitable controls were examined by agarose gel electrophoresis. The 0.7 % gels were run for 16 hours at 50 v. Preparations from experiment 2 failed to show any evidence of Tn402 cloning. Transformants 3 and 4 (Table 9.7) contained R751 DNA only. However, a DNA preparation of transformant C6

TABLE 9.6: NUMBER OF C600 TRANSFORMANTS PER ml FOR EXPERIMENT 2

NATURE OF TRANSFORMING DNA	AVERAGE NUMBER OF TRANSFORMANTS PER ml FOR EACH SELECTIVE MEDIUM				
	Tp	Tc	Ap	Tp Ap	Tp Tc
NO DNA - CONTROL	-	-	-	-	-
50 ul UNCUT pBR322 DNA	-	6.48×10^3	8.1×10^2	-	-
LIGASE -VE	1.2×10^2	5.0×10^1	-	-	3.0×10^1
LIGASE +VE	1.3×10^2	5.9×10^2	5.0×10^2	2.0×10^1	2.3×10^1

TABLE 9.7: RESISTANCE MARKERS OF TRANSFORMANTS FROM EXPERIMENT 2

TRANSFORMANT	SELECTION PLATE	Ap	Tc	Tp
T-1	Tp Tc	+	+	+
T-2	Tp Tc	+	+	+
T-3	Tp Tc	+	+	+
T-4	Tp	-	-	+
T-5	Tp	+	-	+
T-6	Tp Tc	+	+	+

TABLE 9.8: NUMBER OF C600 TRANSFORMANTS PER ml FOR EXPERIMENT 3

NATURE OF TRANSFORMING DNA	AVERAGE NUMBER OF TRANSFORMANTS PER ml FOR EACH SELECTIVE MEDIUM			
	Tp	Ap	Tc	Tp Tc
NO DNA - CONTROL	-	-	-	-
50 ul UNCUT pBR322 DNA	-	3.20×10^3	3.13×10^3	-
LIGASE -VE	-	1.04×10^3	5.88×10^2	2.00×10^1
LIGASE +VE	1.88×10^2	2.31×10^2	1.85×10^3	1.50×10^2

TABLE 9.9: RESISTANCE MARKERS OF TRANSFORMANTS FROM EXPERIMENT 3

STRAIN	Tp	Tc	Ap	Tp Tc
E coli J53	-	-	-	-
E coli J62(R751)	+	-	-	-
E coli C600(pBR322)	-	+	+	-
E coli J53(RP4)	-	+	+	-
CLONE 1	+	+	+	+
2	+	+	-	+
3	-	-	-	-
4	+	-	+	-
5	+	-	-	-
6	+	+	+	+
7	-	-	-	-

from experiment 3 (Figure 9.3; Track 8) indicated that DNA had been successfully cloned into pBR322. Repeated DNA preparations from clones 2 and 6 (Figure 9.4) indicated that clone 2 (Track 3) also contained pBR322 with an insert. The minimum inhibitory concentration of Tp for clones 2 and 6 was $>$ 1000 ug/ml compared with $<$ 2.5 ug/ml for pBR322 without the insert.

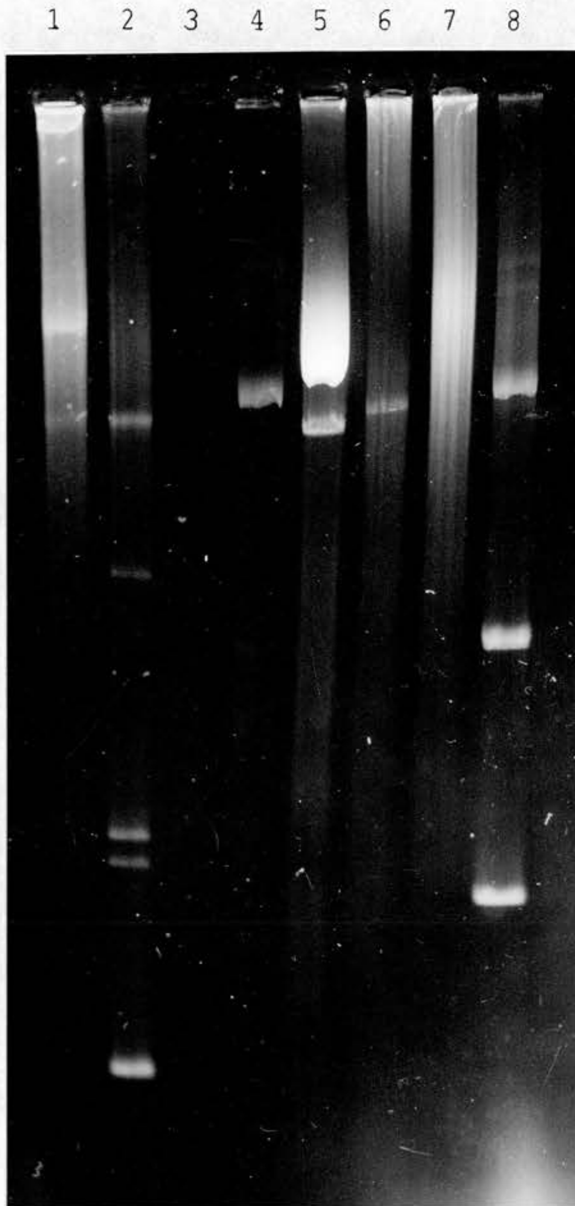
BIOCHEMICAL ANALYSIS OF THE DIHYDROFOLATE REDUCTASES OF R751, Sa-1::Tn402 AND pBR322 CLONES.

The DHFRs of R751, Sa-1::*Tn402* and the pBR322 clones (including clone-H - Young unpublished results) were isolated, purified and their properties examined, to establish enzyme type.

a. Separation of Chromosomal and Plasmid Enzymes

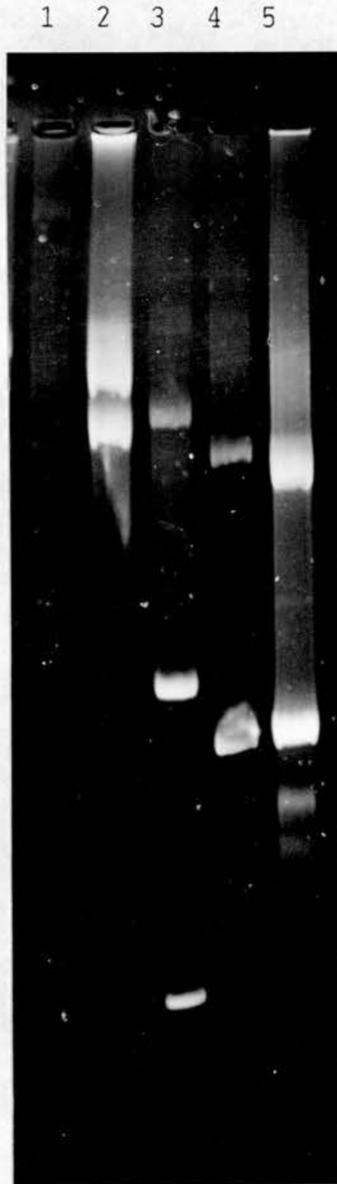
E. coli J62(R751) and *E. coli* J62(Sa-1::Tn402) were cultured in 5 litres of nutrient broth, whilst the clones were cultured in both nutrient broth, and Isosensitest broth containing trimethoprim at 10 ug/ml. DHFR was prepared from each culture and purified by gel filtration on Sephadex G-75 (For purification tables see Appendix 9.1). Complete separation of the trimethoprim sensitive and trimethoprim resistant DHFR's was achieved for each culture (Fig 9.5) and the elution patterns for each bacterial strain were in most respects identical.

FIGURE 9.3: EXAMINATION OF DNA FROM TRANSFORMANTS FROM EXPERIMENT 3



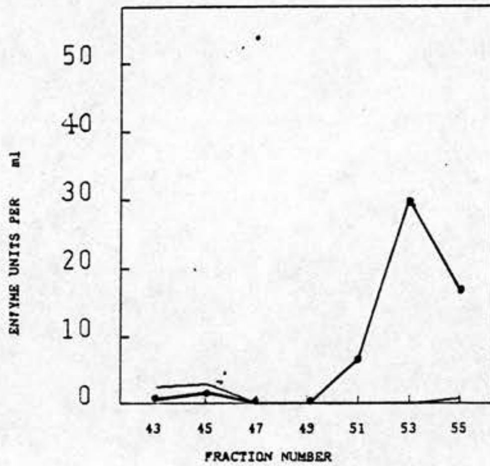
TRACK
1. R751
2. PBR322
3. -
4. Clone 1
5. Clone 2
6. Clone 4
7. Clone 5
8. Clone 6

FIGURE 9.4: AGAROSE GEL ELECTROPHORESIS OF DNA FROM CLONES 2 AND 6 (EXPERIMENT 3)



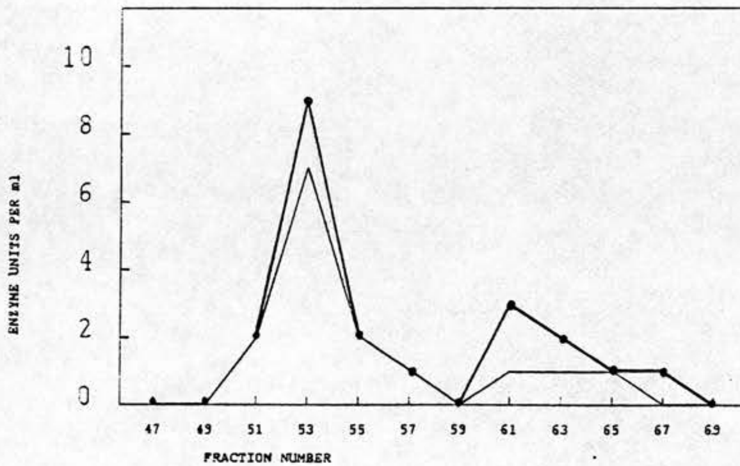
TRACK 1. PBR322
2. R751
3. Clone 2
4. Clone 6
5. Clone H-C

FIGURE 9.5 DIHYDROFOLATE REDUCTASE ACTIVITIES (EXPRESSED AS ENZYME UNITS PER ML) IN FRACTIONS OBTAINED AFTER GEL FILTRATION FOR THE Tn402 ENZYME IN DIFFERENT STRAINS

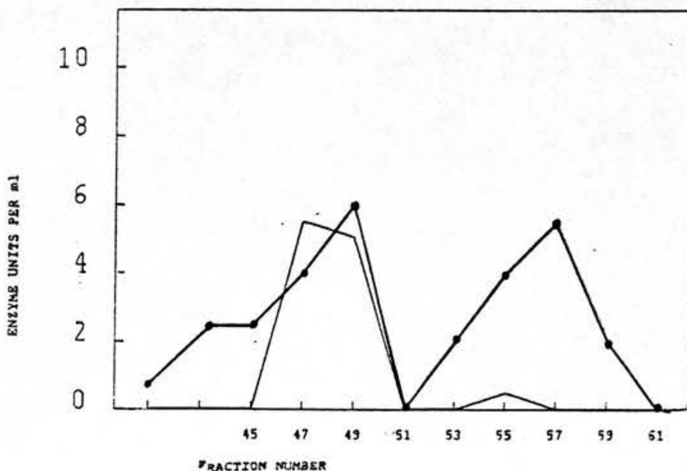


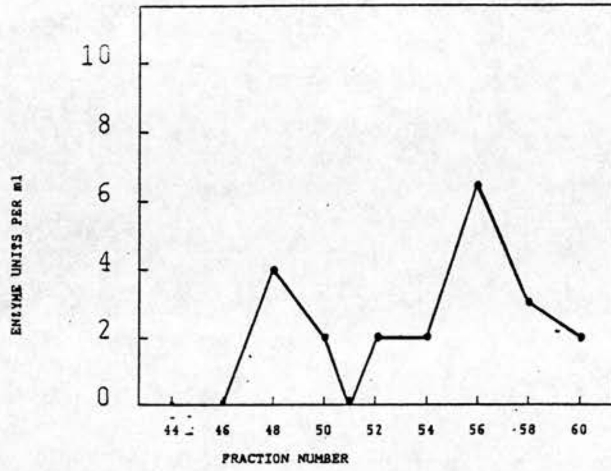
E. coli J62(R751::Tn402)

E. coli J62(Sa-1::Tn402)

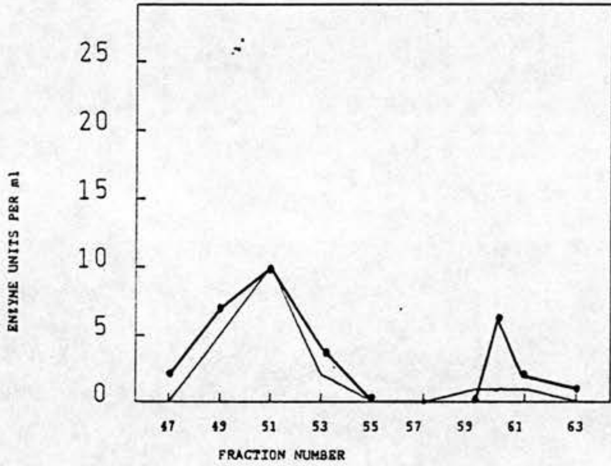


E. coli C600(PBR322::Tn402)
CLONE H-C



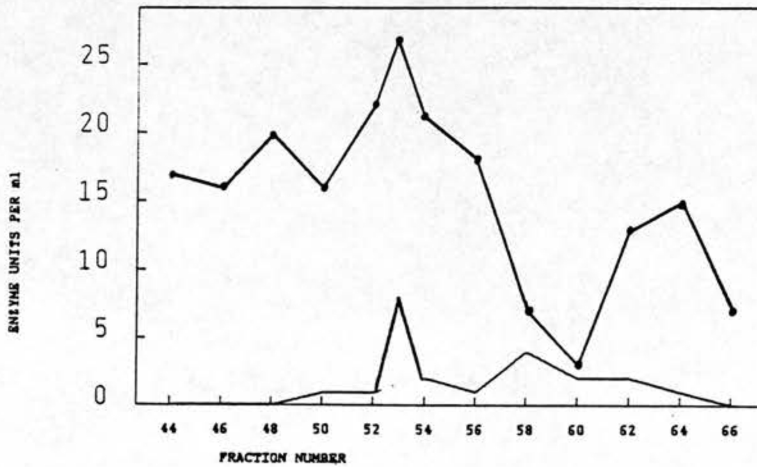


E. coli C600(PBR322::Tn402)
CLONE T-3



E. coli C600(PBR322::Tn402)
CLONE C-3

E. coli C600(PBR322::Tn402)
CLONE C-6



Fractions were assayed in the presence (—) and absence (•—•) of 4×10^{-6} M Tp

b. Molecular Weights

Molecular weight estimations from the gel filtration elution volumes indicated the expected molecular weight of approximately 21,000 for the chromosomal enzyme (Amyes and Smith, 1974). The trimethoprim resistant DHFR's (Table 9.10) varied from 32,000 to 37,000.

c. Specific activity

The specific DHFR activities of the Tn402 enzyme in each strain were measured at pH 6.0 in phosphate buffer A. Table 9.10 indicates the specific activities of the crude samples (before gel filtration purification). Cloning into PBR322 increases the specific activity of the Tn402 enzyme between 10 and 20 fold.

d. Inhibition of the Tn402 Enzyme by Anti-folate Compounds

Each DHFR was assayed in 40 mM sodium phosphate buffer, pH6.0 in the absence and presence of increasing concentrations of trimethoprim and methotrexate. The concentration required to give 50 % inhibition (ID_{50}) were determined for each strain (Table 9.10).

TABLE 9.10: PROPERTIES OF THE Tn402 DIHYDROFOLATE REDUCTASES ISOLATED FROM DIFFERENT STRAINS UNDER VARYING CULTURE CONDITIONS

STRAIN	CLONE	CULTURE CONDITIONS	MOLECULAR WEIGHT	SPECIFIC ACTIVITY	TRIMETHOPRIM ID_{50}	METHOTREXATE ID_{50}	TIME (S)	SOURCE
E coli J62(R751::Tn402)		51 NB	36307	0.468	1.30×10^{-4}	1.047×10^{-3}		
E coli J62(Sa-1::Tn402)		51 NB	35000	2.700	1.05×10^{-5}	3.470×10^{-6}	55	This Work
E coli C600(pBR322::Tn402)	H-C	51 NB	35000	3.720	8.90×10^{-6}			Young - unpublished results
E coli C600(pBR322::Tn402)	H-C	51 ISO + Tp		4.650				
E coli C600(pBR322::Tn402)	T-3	51 NB	32360	4.650				This Work
E coli C600(pBR322::Tn402)	C-2	11 ISO + Tp	34276	10.850	2.30×10^{-5}		>240	This Work
E coli C600(pBR322::Tn402)	C-6	400 ml ISO + Tp	37583	8.600	1.25×10^{-7}	8.700×10^{-7}	164	This Work

e. Heat Sensitivity

Where sufficient enzyme was purified, heat sensitivities were determined (Table 9.10). The Tn402 clone enzymes were more stable at 45°C than the DHFR produced by Sa-1::Tn402. Insufficient enzyme was available to test the enzymes of the other strains.

DISCUSSION

In 1977 Shapiro and Sporn identified a new transposable element determining trimethoprim resistance, Tn402, that was able to insert into the bacteriophage λ . This transposon, originating in R751 (Jobanputra and Datta, 1974), differred from Tn7 (Barth et al, 1976) in that it was smaller and lacked the streptomycin resistance determinant. Unlike Tn7, which transposes readily between replicons (Barth et al, 1978; Hassan and Brevet, 1983; Taylor, 1983; Ouartsi et al, 1985), Tn402 has not been transposed from other plasmid sources except R751 (Goldstein et al, 1986) and has not been shown to integrate into the bacterial chromosome (Amyes, 1979).

Because of the need to compare the small trimethoprim transposon, Tn4135 (chapter 4), with Tn7 and Tn402, to try and determine relatedness, all transposons were required in a common background. The Inc W plasmid Sa (Watanabe et al, 1968; Ward and Grinsted, 1982) was chosen and the results would appear to indicate that Tn402 is transferable to this plasmid, contrary to previously published reports. Trimethoprim and streptomycin resistance markers were transferred at the same frequency indicating their coexistence on the same piece of DNA. However, because of the uncertainty surrounding the ability of Tn402 to transfer, and because of the possibility that R751 had not been eliminated from the cell, transconjugants were carefully checked for resistance markers. However, since R751 carries no resistance markers of its own, other than trimethoprim, its loss from a bacterial cell can not easily be checked by antibiotic resistance testing, therefore, phage lysis and transfer into P aeruginosa were employed to distinguish between cells harbouring R751 and

those that did not. However, as indicated in chapter 6, the lack of specificity of phage PRR1 and the inability to provide suitable selection markers for the transfer of plasmids into P aeruginosa, (Su is not a good selection marker as background growth interferes with results), prevented any confirmation of the transfer potential of Tn402 to Sa-1. (The phage experiments served only to confirm the observation that PRR1 is capable of lysing Sa-1 containing cells, contrary to published reports, but at a 100 fold lower eop, than lysis of RP4 containing cells.) Although the principle of the Pseudomonas transfer experiment appeared to be sound, since RP4 was successfully transferred to P aeruginosa and Sa-1 did not transfer, the reason for the failure of R751 to transfer to P aeruginosa is unclear.

In addition to the uncertainties surrounding the transferability of Tn402, there is still confusion as to the nature of the DHFR encoded by this transposon. Previous studies have shown that the mechanism of plasmid associated trimethoprim resistance results from the synthesis of novel DHFRs which are highly resistant to trimethoprim (Amyes and Smith, 1974). These enzymes appear to be quite distinct from the chromosomal specified enzymes, on the basis of molecular weight and enzyme characteristics (Amyes and Smith, 1976), but their origins and evolutionary relationships with one another are unclear. Of the DHFRs characterised, probably the greatest controversy surrounds that of R751. Amyes and Smith (1978) postulated that the remarkable resemblence they found between the physical properties of R-factors (including R483::Tn7 and R751::Tn402) was due to one gene spreading through the bacterial population. In contrast to these findings, Tennhammer-Ekman and Skold (1979) reported that the DHFR genes mediated by Tn7 and Tn402 were distinct from each other, on the basis of inhibition data, pH profiles and heat lability curves, and therefore have different origins.

Examination of the DHFR mediated by R751::Tn402 in this study revealed similarities with previously published results (Amyes and Smith, 1978). The Tp ID_{50} of 1.3 x 10^{-4} M for this strain was in agreement with that found by Amyes and Smith (1978) (Table 9.11); a value indicative of a type I enzyme (Table 9.12). However this result is in sharp contrast to the findings of Tennhammer-Ekman and Skold (1979) and Broad and Smith (1982) who found Tp ID_{50}s to be 100 fold higher (2 x 10^{-2} M) and thus similar to the type II. In the present study, the inhibitor profile for methotrexate (ID_{50} 1 x 10^{-3} M) compares favourably with more recent views (Amyes, 1986) and suggests that R751 mediates a type II enzyme. As seen from Table 9.11 this contradicts the initial findings of Amyes and Smith (1978). The reason for this variation in enzymic properties could lie in the relative amounts of enzyme produced. The classical type I DHFR mediated by R483::Tn7 is produced in quantities several fold higher than the chromosomal enzyme (Pattishall et al, 1977), making the examination of it's properties relatively easy. The type II (prototype R67bis) however is synthesised in about the same amount, or less, as the chromosomal enzyme. It is this low yield, coupled with losses during purification, that hamper the accurate determination of properties. The low level of the R751::Tn402 mediated DHFR produced in this study (Specific activity 0.46 - Table 9.10) is typical of a type II enzyme and is in agreement with all other published results (Amyes and Smith, 1978; Tennhammer-Ekman and Skold, 1979; Broad and Smith, 1982). It was because of this problem in obtaining enough activity to work with, and because of the difficulties in directly comparing results from different workers, that a method for increasing the production of the DHFR of R751::Tn402 sort. [Different criteria, as well as different purification and assay conditions, have been used to classify the enzyme type of the R751::Tn402 mediated DHFR:

TABLE 9.11: PROPERTIES OF THE Tn402 DIHYDROFOLATE REDUCTASE DETERMINED BY DIFFERENT RESEARCH WORKERS

RESEARCH TEAM	SPECIFIC ACTIVITY	Tp ID_{50} (M)	Mtx ID_{50} (M)	TEMPERATURE SENSITIVITY	PI	SUBUNIT STRUCTURE	ENZYME CLASSIFICATION
AMYES AND SMITH (1978)	1.34	1.2×10^{-4}	5×10^{-6}	2.1			Type I
TENNHAMMER-EKMAN AND SKOLD (1979)	0.90	$10^{-1}/10^{-2}$	1×10^{-3}	15.0			Type III?
FLING AND ELWELL (1980)						9000 d	Type II
BROAD AND SMITH (1982)		2×10^{-2}		15.0	7.2		Different from Type I and II
AMYES (1986)		2×10^{-2}	1×10^{-3}	12.0	7.2		Type IIb
THIS STUDY R751	0.40	1.3×10^{-4}	1.1×10^{-3}				
THIS STUDY CLONES	3.70-109	$10^{-5}/10^{-6}$	8.7×10^{-7}	4.0			

TABLE 9.12: PROPERTIES OF THE TYPE I AND TYPE II DHFRs

PROPERTY	TYPE I R483::Tn7	TYPE II R67bis
SPECIFIC ACTIVITY	13.6	0.85
Tp ID_{50}	5.7×10^{-5} M	7.0×10^{-2} M
Mtx ID_{50}	5.6×10^{-6} M	1.1×10^{-3} M
TEMPERATURE SENSITIVITY	0.5 minutes	12 minutes
PI	6.4	5.5
SUBUNIT STRUCTURE	18000 d	9000 d

REFERENCES: Pattishall et al, 1977; Amyes and Smith, 1978; Tennhammer-Ekman and Skold, 1979; Broad and Smith, 1982.

early investigations were based purely on sensitivity of enzymes to inhibitors, temperature sensitivity and specific activity measurements, whilst more recently subunit structure and serology (Fling and Elwell, 1980) as well as iso-electric focusing (Broad and Smith, 1982) have been used. The former two techniques have classified the R751::Tn402 enzyme to type II: it has a similar subunit structure (four identical 9000 d units) to other type II DHFRs and also shows immunological cross reaction with serum raised against Type II DHFRs. In contrast, iso-electric focusing results (Broad and Smith, 1982) concluded that the R751::Tn402 enzyme was different from both the type I and the type II enzymes: it had a pI value of 7.2 compared with PI's of 6.4 and 5.5 for the type I and type II respectively (Table 9.11).].

DHFR production can be increased by cloning of the relevant gene into a multicopy plasmid such as pBR322 (Bolivar and Bachman, 1979) or, if the gene resides on a transposon, by transposing it to pBR322, and using transformation to facilitate the movement of the plasmid in and out of the bacterial cell. Required clones could then be identified by antibiotic selection or phage lysis. However, because of the reported inability of Tn402 to transpose to other plasmids (Amyes, 1979) and because of the problems encountered with the specificity of phage lysis, Tn402 was 'cloned' into PBR322. Cloning initially depends on the ability to restrict both the vector and the insert DNA such that the ends of both can be ligated together. Recircularisation of linear DNA improves uptake by bacterial cells as demonstrated by transforming E coli C600 with ligated (ligase +) and unligated (ligase -) DNA (Table 9.8). There is, in general, a 10 fold increase in the number of transformants obtained with ligated DNA as compared with unligated DNA, although this varied slightly with experiment and therefore conditions. (The restriction conditions and

the relative proportions and absolute concentrations of plasmid vector and insert fragment are important if effective cloning is to be maximised; as demonstrated by comparing the results of experiments 1 - 3.) For insertion of DNA the plasmid vector must be opened up, by a single cut, with an enzyme that also restricts the DNA for cloning. Tn402 is conveniently flanked by Pst I sites (Figure 9.6) and this same enzyme restricts pBR322 once, within the Ap resistance gene (Figure 9.7). The presence of suitable resistance markers on the vector DNA eg Ap and Tc on pBR322 (Figure 9.6) aids easy completion of the cloning procedure ie the transformation of cells with the ligated DNA and the selection of desired clones. (Insertion of DNA into a resistance site inactivates the resistance gene such that transformed clones are no longer resistant to the particular antibiotic. On the basis of this insertional inactivation, pBR322 clones that have picked up the Pst I fragment, encoding Tp resistance (ie.Tn402), would be expected to be Tp and Tc resistant but sensitive to Ap. E coli C600 cells that had picked up recircularised pBR322 DNA without an insert would appear Tc resistant, Ap resistant and Tp sensitive and C600 cells transformed by pBR322 DNA with a Pst I fragment insert lacking the Tp resistance gene, would appear Tc resistant and Ap and Tp sensitive. Examination of the transformants from experiment 2 and 3, however, revealed transformants that were Tp and Ap resistant (Tables 9.5 and 9.8). It would appear therefore, that either the Tn402 fragment has been taken up into the cell and inserted into another region of pBR322, thus leaving the Ap gene intact, (unlikely as pBR322 only has one Pst I site), or R751 and pBR322 have recircularised and both plasmids have been taken up intact into E coli C600. Examination of the DNA from a number of transformants, however, did not indicate that R751 and pBR322 were present together in the same cell. Either, R751 was present alone

FIGURE 9.6: RESTRICTION MAP OF R751 (WARD AND GRINSTED, 1982)

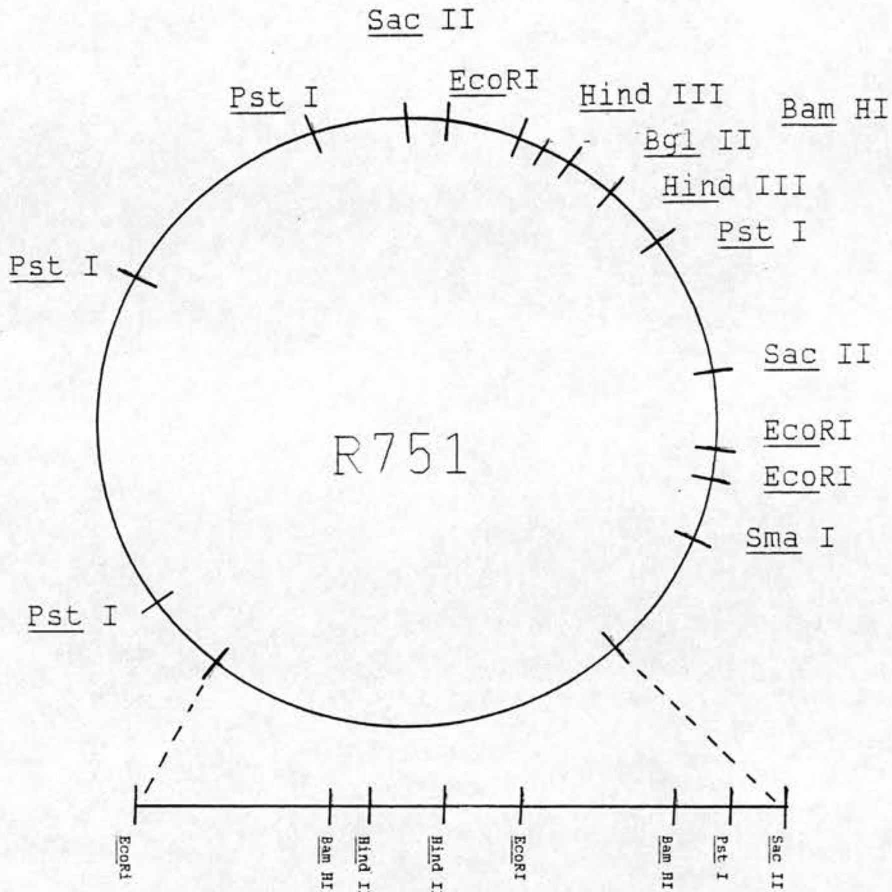
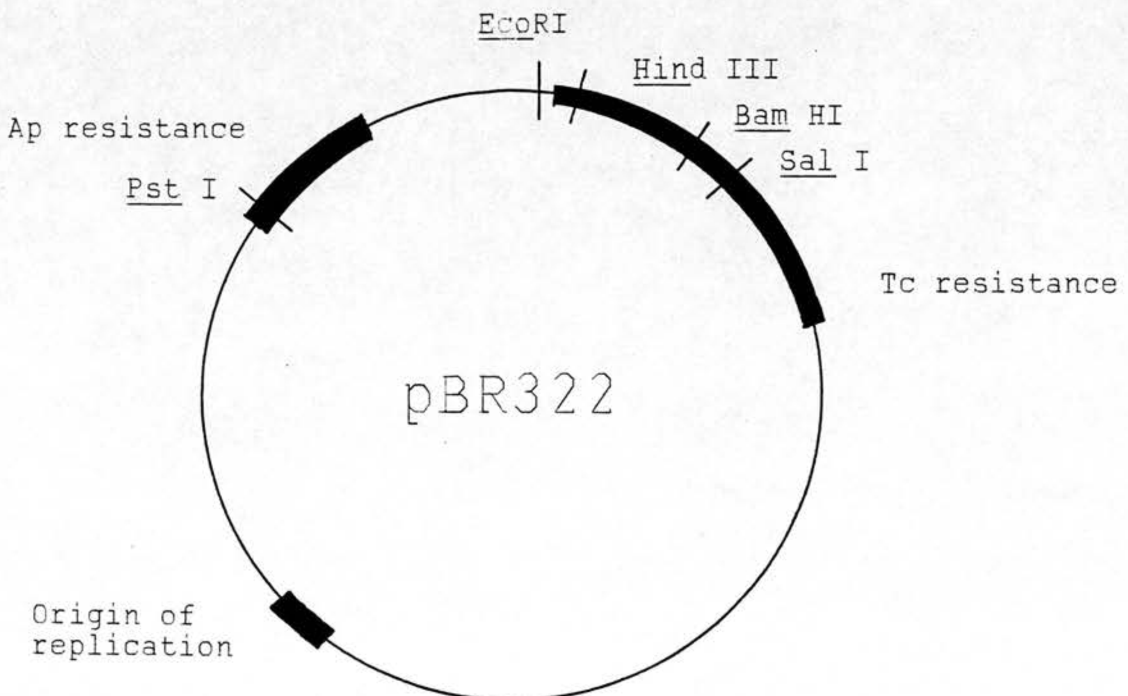


FIGURE 9.7: RESTRICTION MAP OF PBR322



(clones T-3 and T-4: Experiment 2) or clones contained pBR322 with an insert (clones C-2 and C-6: Experiment 3). An alternative hypothesis to explain the prevalence of Ap resistance amongst transformants is that R751::Tn402 encodes an Ap resistance gene. The majority of Inc P-1 plasmids are resistant to Ap (Ingram et al, 1973) but R751 is reported to be resistant to Tp only (Shapiro and Sporn, 1977). However, Reid (1986) reported that R751 conferred a significant degree of Cb and Ap resistance to E coli J62-2 and J62-1 during routine sensitivity testing. It was concluded that the plasmid R751 promoted a mutation, probably in the J62 chromosome, which allowed the expression of an SHV-1 B-lactamase (Reid, Simpson, Harper and Amyes - unpublished results) that was otherwise silent. The mechanism by which the R751 plasmid caused this mutation was unclear but its presence was necessary to maintain the mutation. The clones, however, only possess a small portion of R751 DNA, that of the Tn402 region, suggesting that if this mutation is responsible for the appearance of Ap resistant clones, then the genes causing it lie on the Tn402 DNA region. Since the ampicillin resistance of PBR322 is encoded by the TEM-1 β-lactamase, the origin of the unexpected ampicillin resistance of transformants could be traced. Analytical iso-electric focusing would distiguish between clones harbouring the TEM-1 and SHV-1 β-lactamases.

Biochemical analysis of the DHFR from a number of the clones confirmed the initial findings of the examination of R751::Tn402. The enzyme yields, although not as high as expected, were increased from between 10 and 25 fold (Table 9.10) enabling a more accurate determination of properties. Trimethoprim ID_{50}s were consistent with those of Amyes and Smith (1978) and R751::Tn402 from this study, suggesting the enzyme belongs to the type I class. The Mtx ID_{50}s, although differing

considerably from those of R751::Tn402 (this study), were also in agreement with Amyes and Smith (1978). The high sensitivity of clone 6 to methotrexate and trimethoprim (Table 9.10) suggests that a chromosomal mutation may be involved. The E coli chromosomal DHFR has a $TpID_{50}$ of 1.1×10^{-8} and a Mtx ID_{50} of 1.4×10^{-9} (Amyes and Smith, 1978). However, the molecular weight of this species (Table 9.10) is consistent with a plasmid-encoded enzyme (35000 d) and the DNA profiles indicated the presence of pBR322 plus an insert (Figure 9.5). The elution profile of this clone vairies slightly from the other clones in that a secondary peak is visible around fraction 48, corresponding to a molecular size of 48000. This could be part of a broad peak or may correspond to a type IV enzyme (Young and Amyes, 1985), indicating the presence of two DHFR genes in R751. The presence of two genes could explain the variation in properties observed for the plasmid. However, the likelihood of a 7.5 kb piece of DNA encoding two trimethoprim resistance genes is small and this phenomenon is only observed in clone 6. The temperature sensitivities of the Tn402 mediated DHFR do not vary significantly between authors and are consistent with a type II enzyme.

It would thus appear, contrary to the evidence of all other reports on R751 (Review Amyes, 1986), except for Amyes and Smith (1978), that the DHFR of R751 possesses mainly type I properties but is synthesised in amounts characteristic of a type II. It's temperature sensitivity is indicative of the type II class also. As Tennhammer-Ekman and Skold (1979) suggest, R751 may therefore belong to a class of it's own. The PI values (Broad and Smith, 1982) certainly substantiate this view. The fact that R751 possesses properties of both the type I and the type II classes, may indicate an evolutionary origin intermediary between the class I and the class II.

DISCUSSION

DISCUSSION

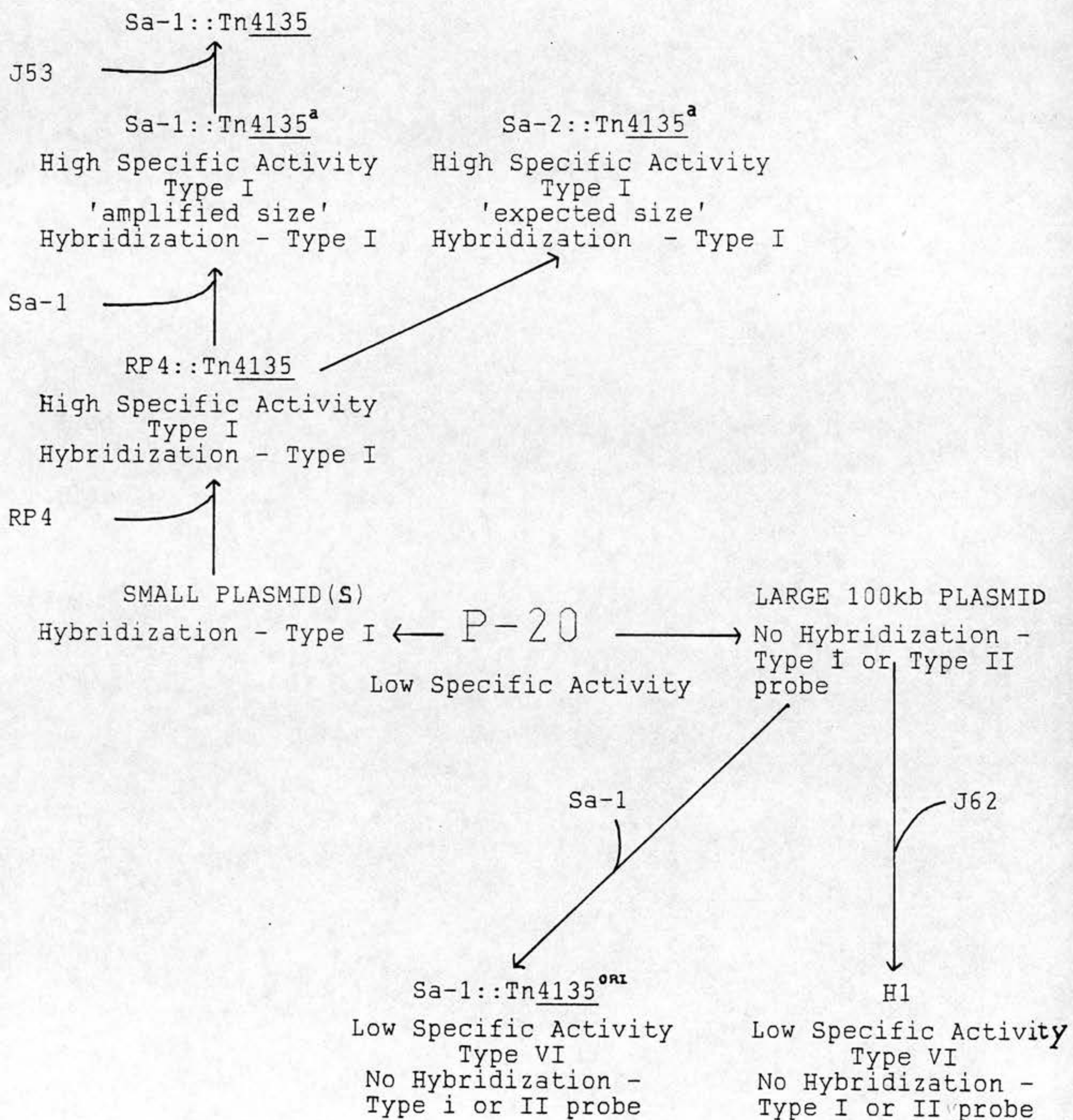
Since the first report of the 14 kb transposon, Tn7, encoding Tp and Sm/Sp resistances (Barth et al, 1976), the incidence of Tp resistance, especially high level resistance, within the enterobacteriaceae has risen markedly (Amyes et al, 1978; Datta et al, 1981; Amyes et al, 1981; Fling et al, 1982; Towner et al, 1982). Examination of many of these isolates has revealed the presence of Tn7 within both the bacterial chromosome and R-plasmids of many different incompatibility groups. However, more recent surveys have indicated that other transposons and Tp resistance genes may also be contributing to the epidemiology of Tp resistance (Amyes, 1986; Goldstein et al, 1986; Papadopoulou et al, 1986; Sundstrom et al, 1987; Young et al, 1987). Kraft et al (1984) suggested that the reduction in the proportion of plasmids carrying linked TpSp resistance (presumptive evidence of Tn7) and the appearance of plasmids carrying Tp, TpTc and Tp Km, was further evidence of the spread of other Tp transposons in the plasmid population. Whilst DHFR's were originally thought to belong to two classes; the type I and the type II (Pattishall et al, 1977), it has now become clear that this enzyme displays a greater evolutionary diversity than at first envisaged (Table iii). Type III and IV enzymes have now been isolated and characterised (Fling et al, 1982; Young and Amyes 1986a), and recent work by Sundstrom et al (1987) has suggested the evolution of a type V enzyme. This continuing evolution of Tp resistance genes is reinforced by the discovery of a possible type VI enzyme in this thesis.

TABLE iii: THE DIVERSITY OF THE DHFR ENZYME

DHFR	PLASMID	TRANSPOSON	SPECIFIC ACTIVITY	Tp ID_{50} uM	TD 50 uM	MOLECULAR WEIGHT	REFERENCE
Ia	R483	Tn7	13.6	57	0.5	35000	Pattishall et al, 1977
		Tn4132	4.5	32	1.2	24500	Young and Amyes, 1985a/b
IIa	R67bis		0.85	70000	>12.0	35000	Pattishall et al, 1977
IIb	R751	Tn402	0.07	20000	>12.0	34000	Amyes, 1986
III	pAZI		2.0	2.1		16900	Fling et al, 1982
IV	pUK1123		600.0	0.2	>12.0	46700	Young and Amyes, 1986a
V	pLMO44		121.0	10-100	>5.0		Sundstrom et al, 1987
VI	Sa-1	Tn4135ORI	<1.0	0.02	>10.0	34673	This Thesis
SI	pSKI		129.6	50	>12.0	19700	Young et al, 1987
CHROMOSOME				0.02	>12.0	21000	Amyes and Smith, 1976

Whilst examination of an enterobacterial strain, P-20, from a porcine faecal isolate revealed an array of plasmids, the presence of two distinct Tp resistance genes was also indicated (Figure ii); one mobilsised by RP4 (RP4::Tn4135 - unpublished results) and the other mobilised directly by Sa-1 (Sa-1::Tn4135)^{ORI} - this thesis). The presence of two different R-genes within the same plasmid has been indicated previously, although it is likely in this case, that the genes in question lie on different plasmids of the P-20 strain: the 100 kb plasmid and one of the smaller plasmids. The fact that P-20 possesses multiple plasmids may be indicative of the transfer potential of this strain. Kraft et al (1983) indicated that isolates that transfered resistance, tended to be those that carried multiple bands, possibly because an increase in the number of plasmids concomitantly increases the probability that one would be conjugative. Possession of a conjugative plasmid would also make more likely the mobilisation of non-conjugative plasmids and this might explain why many transconjugants acquired several plasmids. The presence of both large and small (probably non transmissible) plasmids in the same strain is therefore suggestive of there being two methods of transfer for the Tp resistance genes: transposition (possibly from the large plasmid) and mobilisation of the small non-transferable plasmids. This availability of more than one system for transfer has been suggested by Towner et al (1982) and Papadopoulou et al (1986), and may account for the rapid dissemination of resistance to Tp in bacteria isolated from human and veterinary specimens. The results suggest that the small plasmid bands are not only mobilizable, but readily integrate and excise themselves from both plasmid and chromosome in an aberrant fashion, resulting in considerable variability in the size of plasmid bands and indicating a certain amount of instability. This instability may be a necessary prerequisite for the adaptation to an ever changing environment. The

FIGURE ii: THE TRIMETHOPRIM RESISTANCE GENES OF THE ORIGINAL PIG ISOLATE, P-20



integration and excision could not be confirmed, however, due to the inability to transfer any of the original pig plasmids to a Rec A strain, but if these mechanisms are in existance, they are probably mediated by Tn4135 ifself or by IS sequences (Kleckner, 1981). Alternatively Tn4135 or IS elements could mediate the oligimerisation of some of the smaller bands, which would help to explain the observed variations in plasmid size.

Genetic and biochemical studies revealed that RP4::Tn4135 was very similar to RP4::Tn7, despite differences in transposon size (3 -6 kb as compared with 14 kb for Tn7 - Barth et al, 1976) and apparent intermittant expression of Sm/Sp resistance. This strain was found to encode a type I DHFR, with properties indistinguishable from Tn7, and was therefore thought to have evolved from Tn7 by deletion in a similar, but not identical, manner to the small Tp transposon Tn4132 (Young, 1984). Hybridisation confirmed the presence of a type I enzyme and located the gene to one of the small plasmid bands of the original P-20 strain. The appearance of Tp resistance genes on small, presumably nontransferable plasmids, was suggested by Towner (1981) and Towner et al (1982) to be one reason for the decrease in the proportion of isolates that transferred Tp resistance. The variable expression of Sm/Sp resistance can be explained by deletion(s) in the promoter region and subsequent integration of the transposon adjacent to a plasmid or chromosomal promoter, or an IS sequence that could 'switch on' expression. The ability of IS sequences to act as novel 'switches' has been documented by Glansdorff et al (1980), and it is therefore possible that insertion of an IS element directly into a transposon, such as Tn4135, could result in the switching off of Sm/Sp expression also. Loss of such an IS element on transfer between replicons, or as a result of other cellular changes, would result in the re-expression of the resistance genes.

Characterisation of *E coli* J62(Sa-1::Tn4135)^{ORI}, revealed distinct differences between the Tp resistance element of this strain and that of *E coli* J62(RP4::Tn4135). Restriction patterns were different, with only RP4::Tn4135 possessing the characteristic two internal *Hind* III fragments of Tn7 (Datta *et al*, 1979). Sa-1::Tn4135^{ORI} DNA more closely resembled that of the HI transconjugant, which contains the approximately 100 kb large plasmid of the P-20 strain. Enzymic analysis of the DHFR encoded by Sa-1::Tn4135^{ORI}, although hampered by the inability to produce large quantities of active enzyme, again implicated a different origin for this enzyme compared with that of RP4::Tn4135. (It may be necessary to clone the DHFR gene of Sa-1::Tn4135^{ORI} into a multicopy plasmid , such as ColEl, to obtain larger quantities of this enzyme for confirmation of properties.). The specific activity ($<$1 unit) and temperature sensitivity (TD_{50} $>$ 10 minutes) results more closely resemble that of a type II enzyme (Pattishall *et al*, 1977). However, despite a molecular size representative of a plasmid encoded enzyme, the sensitivity of this enzyme to Tp was 100 fold higher than that of a type I enzyme and 10^6 fold greater than that typical of a type II (Tennhammer Ekman and Skold, 1979), and in this respect the enzyme more closely resembles the chromosomal DHFR (Amyes and Smith, 1976). This finding, coupled with the failure of both type I and type II probes to hybridise with Sa-1::Tn4135^{ORI} DNA suggests that this enzyme is distinct from all other characterised DHFR's. The results mentioned support the concept that Sa-1::Tn4135^{ORI} (and thus P-20 - probably the large 100 kb plasmid) encodes a type VI enzyme, which may have an evolutionary origin intermediary between the chromosomal and the type II DHFR. Young (1984) has suggested that the 8 kb p699 non-self transmissible plasmid, isolated by Fling *et al* (1982) may also be an evolutionary intermediate, formed by

mobilisation of part of the bacterial chromosome carrying a spontaneously derived mutant DHFR gene, encoding an enzyme similar to that isolated from J53Tp1K^R. DNA sequence analysis or hybridisation studies between the chromosomal and Sa-1::Tn4135^{ORI} encoded enzymes would be required to confirm this hypothesis and determine the degree of relatedness. Preliminary hybridization studies of Sa-1::Tn4135^{ORI} did however, reveal that the transposon possesses sequences homologous to those of the Inc P plasmid, RP4. This would help to explain the inability to transfer this transposon from Sa-1 to RP4, by introduction of the Inc P plasmid into E coli J62(Sa-1::Tn4135)^{ORI}, and might also explain why introduction of RP4 into P-20 only picked up the type I enzyme.

Regarding the fact that the transfer of Tn4135 from RP4 to Sa-1 (Sa-1::Tn4135^a) resulted in the formation of an unexpectedly large species (70 kb as opposed to 35 kb), it may be concluded that more than one mechanism may be involved in the formation of this species. Transfer studies, coupled with resistance testing and molecular size analysis suggested that such a large species could have been generated as a result of:-

i. multiple transposition leading to insertional inactivation of Su and Ka resistance,

ii. a natural amplification,

iii. transposon mediated transfer of some or part of the RP4 genome across to Sa-1, resulting in the formation of a composite transposon

iv. fusion of the RP4 and Sa-1 DNA, followed by deletion of non-essential DNA (such as resistance markers).

Multiple insertion is doubtful due to the large number of insertions required to increase the size to 70 kb and because such an event would result in a drastic increase in the number of restriction fragments, as

observed for Tn4132 (Young, 1984), and this does not appear to have occurred. Preliminary biochemical studies of the Sa-1::Tn4135[a] encoded DHFR suggested that amplification was the cause of the large species and high specific activity: slight variations in molecular size and specific activity could be acounted for by variations in the degree of amplification. The fact that restriction fragments of Sa-1::Tn4135[a] DNA did not sum to 70 kb either, suggestd that some fragments may have been amplified. However, the stability of this species on transfer and in drug free medium would tend to go against this hypothesis. In addition, the finding that the DHFR of Sa-1::Tn4135[ORI] differed from Sa-1::Tn4135[a], not only in specific activity, but also in $TpID_{50}$ and temperature sensitivity and was therefore a different enzyme, ruled out the possibility that one DHFR was an amplified form of the other. Although incompatibility studies would tend to favour hypothesis iii, or at least the presence of an Inc P function in Sa-1::Tn4135[a], hybridisation with whole RP4 DNA probes, indicated no homology. Whilst suggesting that RP4 DNA was not present, this does not rule out the posibility that the transposon itself encodes an Inc P function, not directly homologous to that of RP4, which would prevent the cohabitation of RP4 and Sa-1::Tn4135[a] in the same cell, as observed. The incompatibility results also indicate that Sa-1 is not present either, as suggested by resistance testing, and this is confirmed by the lack of hybridisation of an Sa-1 probe to E coli J62(Sa-1::Tn4135)[a]. Although replicon fusion, mediated by Tn4135 and followed by deletion of the resistance gene regions of RP4 and Sa-1, would account for the lack of Su and Ka resistance and the unusual size, it would not explain the lack of RP4 and Sa-1 probe hybridisation. The formation of an autonomously replicating r-determinant, as described by Chandler et al (1982) and Clerget et al

(1982) in the absence of plasmid DNA, could explain the above phenomena, although why this should happen in the absence of selection pressure is unclear. Alternatively Sa-1::Tn4135[a] may have fused with the E coli chromosome, a not uncommon phenomonon (Bennet et al, 1986)), resulting in the generation of homologous sites such that re-excision of Tn4135 would bring about co-excision of some of the E coli chromosome to generate a species of 70 kb. In favour of this hypothesis are reports of certain R-plasmids in E coli (Nugent, 1981) and Haemophilus (Stuy, 1980) which prefer to be integrated in the chromosome. However, although Watson and Scaife, 1980) have shown that insertion of RP4 derivatives containing the att region of lambda, into the bacterial chromosome of E coli, results in loss of expression of their incompatibility function, it is generally believed that integration of R plasmids into the chromosome of E coli is not usually accompanied by the complete loss of expression of plasmid function, as would appear to be the case here (Nugent, 1981). Ie. although incompatability testing indicates Sa-1 is absent, the integration of Sa-1::Tn4135[a] into the chromosome would not be expected to result in loss of resistance markers as well. Never-the-less, several authors have indicated that the formation of R' plasmids, by recombination of Inc P plasmids (notably R68.45) with the bacterial chromosome, can result in structural instability of the R-plasmid while the chromsosomal markers remain stable (Haas and Holloway, 1976,1978). In this instance the whole plasmid is not lost as a unit, but plasmid markers are lost progressively during bacterial replication, until either the remnant stabilises as a more or less permanent function of the original plasmid, or the whole plasmid is lost (Godfrey et al, 1980). Such deletions are thought to occur by intramolecular recombination between regions of homology. It is conceivable, therefore , that a similar situation may have occurred here

with Sa-1, resulting in the eventual loss of the whole of this latter plasmid and the subsequent aberrant excision of Tn4145 from the chromosome. This could give rise to a 70 kb species lacking RP4 and Sa-1 DNA, but containing chromosomal and transposon DNA. Replicon fusion might also help to explain the initial inability to detect transferable Tp resistance in P-20, (hence the need to mobilise the resistance genes with RP4), whilst subsequent studies were able to show transfer between RP4 and Sa-1. If it is assumed that the type I DHFR gene of RP4::Tn4135 originated from the small 3-6 kb plasmid of P-20, as suggested by hybridisation, and that this small plasmid is unable to transfer on its own, its mobilisation by RP4 and subsequent fusion with this plasmid and/or Sa-1 would lead to a wider host range and thus transfer potential. In addition, if Tn4135 has evolved from Tn7 by deletion, its presence on a non transmissable plasmid may be a further evolutionary step, resulting in the availability of two mechanisms for the transfer of one gene.

In conclusion, the transposons of bacteria are diverse according to criteria such as DNA sequence and structure of transposition products, and have probably evolved along a number of separate pathways in many different species. The evolution of Tp transposons is no exception to this, and the isolation and characterisation of two distinct Tp resistance transposons and their gene products from the enterobacterial P-20 isolate, only serves to illustrate this continuing diversity.

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APPENDIX

APPENDIX 1.1 THE ELECTROPHORETIC MOBILITIES AND MOLECULAR WEIGHTS OF R1, R6K, RP4 AND Sa PLASMID DNA PREPARED BY A NUMBER OF DIFFERENT METHODS

Birnboim and Doly

PLASMID	MOLECULAR SIZE (Kb)	LOG. MOLECULAR SIZE	DISTANCE TRAVELLED
R1	90	1.954	0.70
RP4	52	1.716	0.45
R6K	38	1.580	1.15
Sa	33	1.519	1.1

Kado and Liu

PLASMID	MOLECULAR SIZE (Kb)	LOG. MOLECULAR SIZE	DISTANCE TRAVELLED
R1	90	1.954	
RP4	52	1.716	0.40
R6K	38	1.580	2.60
Sa	33	1.519	

Takahashi and Nagano

PLASMID	MOLECULAR SIZE (Kb)	LOG. MOLECULAR SIZE	DISTANCE TRAVELLED
R1	90	1.954	2.40
RP4	52	1.716	2.70
R6K	38	1.580	3.40
Sa	33	1.519	3.53

Ish-Horowitz and Burke

PLASMID	MOLECULAR SIZE (Kb)	LOG. MOLECULAR SIZE	DISTANCE TRAVELLED
R1	90	1.954	3.00
RP4	52	1.716	3.20
R6K	38	1.580	3.69
Sa	33	1.519	

APPENDIX 1.2 THE ELECTROPHORETIC MOBILITIES AND MOLECULAR WEIGHTS OF R1, RP4, R6K AND Sa PLASMID DNA PREPARED BY THE METHODS OF TAKAHASHI AND NAGANO, AND ISH-HOROWITZ AND BURKE, ON A NUMBER OF SEPARATE OCCASIONS

Takahashi and Nagano

PLASMID	R1	RP4	R6K	Sa
MOL SIZE	90	52	35	33
LOG. MOL SIZE	1.954	1.716	1.580	1.519
EXP 1	2.4	2.7	3.4	3.53
EXP 2	2.0	2.2	2.4	2.3
EXP 3	1.5	1.8	2.6	2.6
EXP 4	2.4	2.7	3.4	3.53
EXP 5	2.8	3.2	3.8	4.2
EXP 6	2.6	2.8	3.7	4.1
EXP 7	2.6	3.1	3.8	4.2
EXP 8	2.1	3.0	3.6	4.0
EXP 9	3.5	4.2	4.7	5.3

Ish-Horowitz and Burke

PLASMID	R1	RP4	R6K	Sa
MOL SIZE	90	52	35	33
LOG. MOL SIZE	1.954	1.716	1.580	1.519
EXP 1	3.0	3.2	3.8	
EXP 2	1.6	1.8	1.8	
EXP 3	2.8	3.5	3.35	3.7
EXP 4	3.2	3.4	3.7	

APPENDIX 7.1: PURIFICATION TABLES

R483::Tn7

SAMPLE	VOLUME	PROTEIN CONCENTRATION	DHFR ACTIVITY PER ml	SPECIFIC ACTIVITY	TOTAL DHFR	% RECOVERY	PURIFICATION
BULK	11.5	55.255	1510	27.33	17365	100	1
STREPTOMYCIN SULPHATE	11.5	45.580	1670	36.64	19205	111	1.34
0-50% $(NH_4)_2SO_4$	5.5	31.605	1100	34.80	6050	34.8	1.27
PELLET	1.9	46.225	1440	31.15	2736	15.8	1.14
SEPHADEX FRACTION	1.9	4.300	143	33.26	271.7	1.6	1.22

RP4::Tn7

SAMPLE	VOLUME	PROTEIN CONCENTRATION	DHFR ACTIVITY PER ml	SPECIFIC ACTIVITY	TOTAL DHFR	% RECOVERY	PURIFICATION
BULK	15.5	20.425	340	16.65	5270	100	1
STREPTOMYCIN SULPHATE	15.5	22.790	400	17.55	6200	118	1.05
0-50% $(NH_4)_2SO_4$	7.5	19.135	420	21.95	3150	60	1.32
PELLET	1.0	56.975	1820	31.94	1820	35	1.92
SEPHADEX FRACTION	2.3	8.6	30	3.49	69	1	0.21

RP4::Tn4135

SAMPLE	VOLUME	PROTEIN CONCENTRATION	DHFR ACTIVITY PER ml	SPECIFIC ACTIVITY	TOTAL DHRR	% RECOVERY	PURIFICATION
BULK	25.0	13.33	290	21.75	7250	100	1
STREPTOMYCIN SULPHATE	25.0	20.21	430	21.28	10750	148	0.97
0-50% $(NH_4)_2SO_4$	12.0	10.75	350	32.56	4200	57.9	1.50
PELLET	2.0	38.70	2200	56.85	4400	60.7	2.61
SEPHADEX FRACTION	1.5	14.19	155	10.92	232.5	3.2	0.50

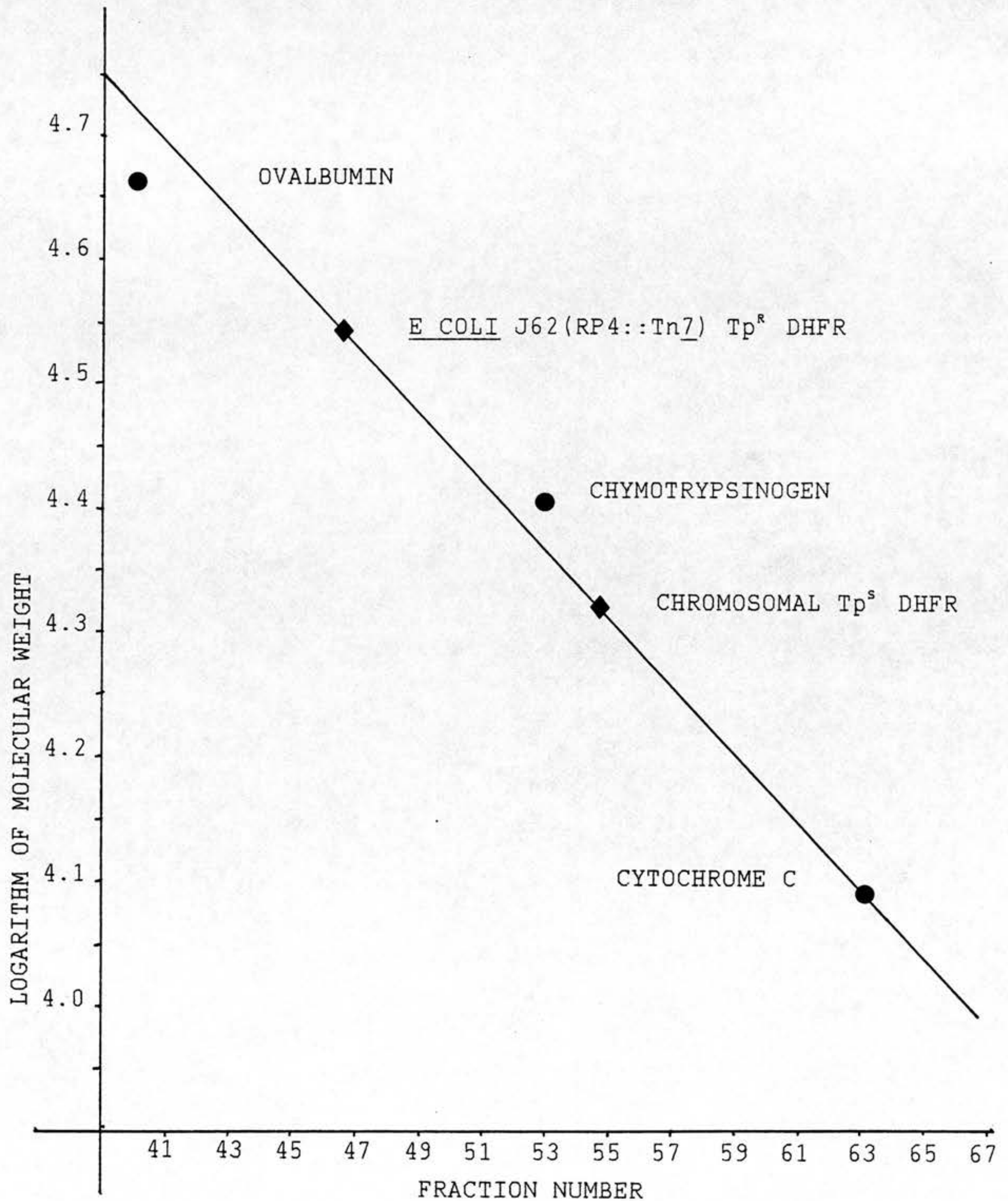
(Sa-1::Tn<u>4135</u>)[a]

SAMPLE	VOLUME	PROTEIN CONCENTRATION	DHFR ACTIVITY PER ml	SPECIFIC ACTIVITY	TOTAL DHFR	% RECOVERY	PURIFICATION
BULK	11.5	32.465	300	9.24	3450	100	1
STREPTOMYCIN SULPHATE	11.0	23.865	220	9.22	2420	70	0.99
0-50% $(NH_4)_2SO_4$	5.0	16.125	300	18.60	1500	43	2.01
PELLET	1.6	34.400	780	22.67	1248	36	2.45
SEPHADEX FRACTION	1.9	0.559	34	60.82	64.6	2	6.58

(Sa-1::Tn<u>4135</u>)[ORI]

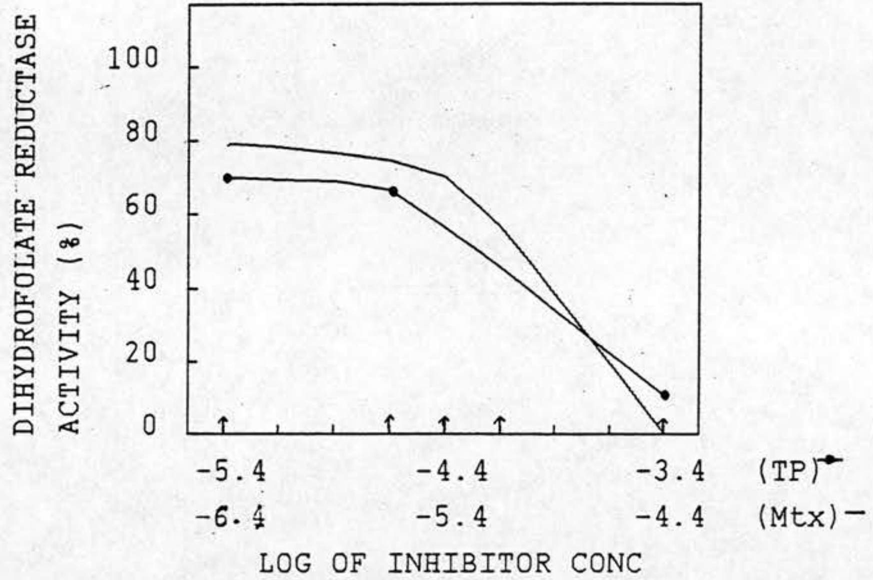
SAMPLE	VOLUME	PROTEIN CONCENTRATION	DHFR ACTIVITY PER ml	SPECIFIC ACTIVITY	TOTAL DHFR	% RECOVERY	PURIFICATION
BULK	63.0	90.3	60	0.66	3780	100	1
STREPTOMYCIN SULPHATE	67.0	68.8	60	0.872	4020	106	1.32
0-50% $(NH_4)_2SO_4$	30.0	60.2	120	1.993	3600	95.2	3.02
PELLET	5.0	81.7	120	1.469	600	15.8	2.23
SEPHADEX FRACTION	1.9	12.04	49	4.070	93.1	2.4	6.17

APPENDIX 7.2: EXAMPLE MOLECULAR WEIGHT ESTIMATION OF DHFR AS MEASURED BY GEL FILTRATION ON SEPHADEX G-75

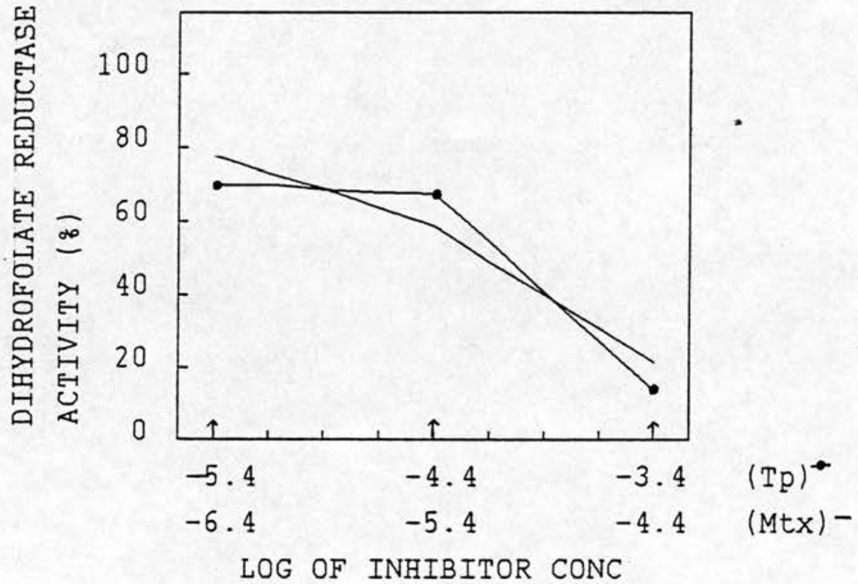


APPENDIX 7.3: INHIBITOR PROFILES FOR TRIMETHOPRIM AND METHOTREXATE

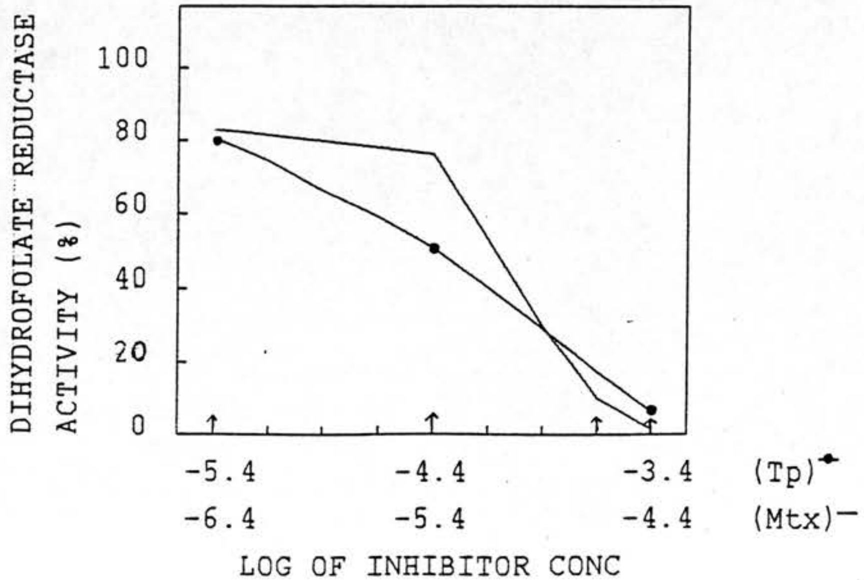
R483::Tn7



RP4 .Tn7



RP4::Tn4135



APPENDIX 7.4 DETERMINATION OF $TpID_{50}$'s AND $MtxID_{50}$'s FOR THE DHFR's OF R483::Tn7, RP4::Tn7 AND RP4::Tn4135

a. R483::Tn7

Tp CONC(M)	LOG Tp CONC	ENZ u/ml	% ACTIVITY
0	-	93	100
4×10^{-6}	-5.4	65	69.89
4×10^{-5}	-4.4	52	55.91
4×10^{-4}	-3.4	10	10.75

Mtx CONC(M)	LOG Mtx CONC	ENZ u/ml	% ACTIVITY
0	-	93	100
4×10^{-7}	-6.4	74	29.57
4×10^{-6}	-5.4	65	69.89
4×10^{-5}	-4.4	0	0

b. RP4::Tn7

Tp CONC(M)	LOG Tp CONC	ENZ u/ml	% ACTIVITY
0	-	67	100
4×10^{-6}	-5.4	47	70.15
4×10^{-5}	-4.4	45	67.16
4×10^{-4}	-3.4	9	13.43

Mtx CONC(M)	LOG Mtx CONC	ENZ u/ml	% ACTIVITY
0	-	67	100
4×10^{-7}	-6.4	52	77.61
4×10^{-6}	-5.4	39	58.21
4×10^{-5}	-4.4	14	20.90

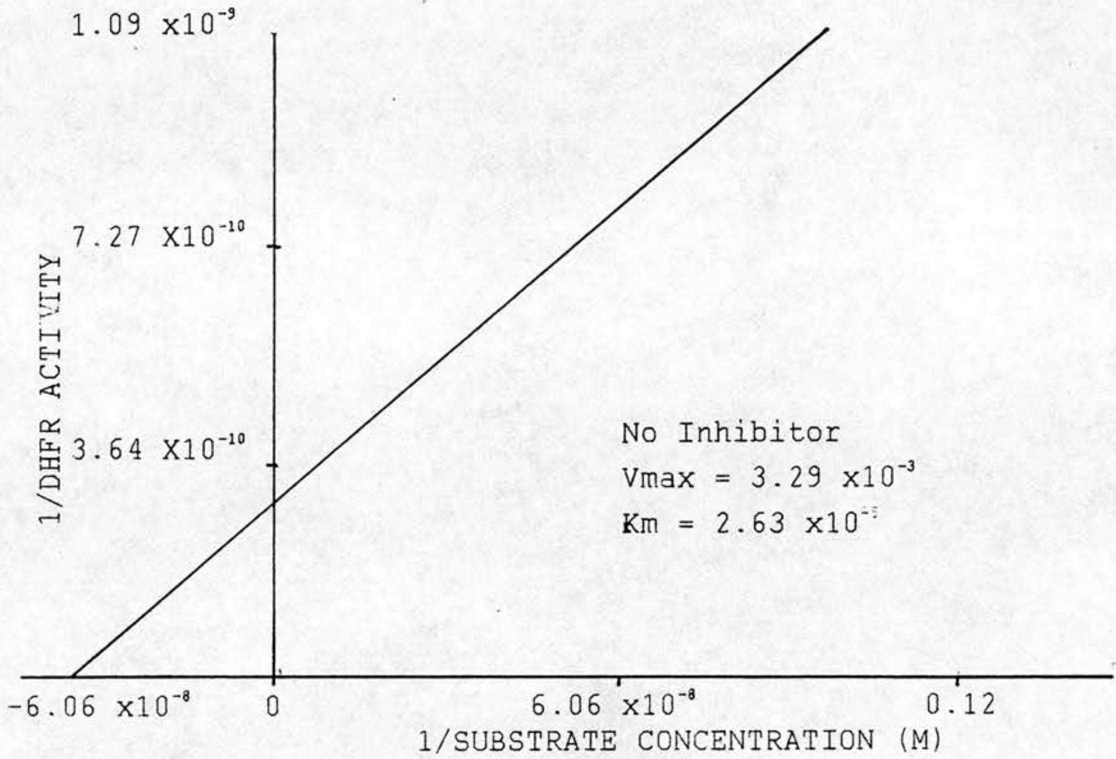
c. RP4::Tn4135

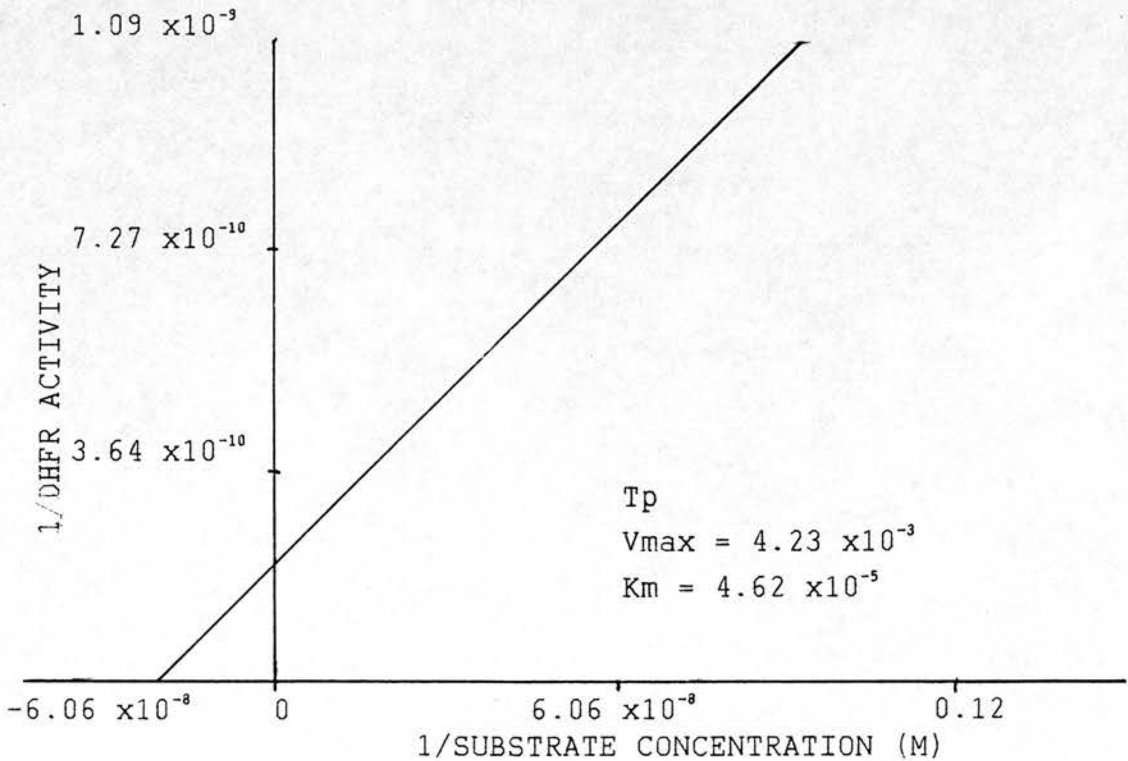
Tp CONC(M)	LOG Tp CONC	ENZ u/ml	% ACTIVITY
0	-	144	100
4×10^{-6}	-5.4	116	80.56
4×10^{-5}	-4.4	73	50.69
4×10^{-4}	-3.4	9	6.25

Mtx CONC(M)	LOG Mtx CONC	ENZ u/ml	% ACTIVITY
0	-	144	100
4×10^{-7}	-6.4	120	83.33
4×10^{-6}	-5.4	110	76.39
4×10^{-5}	-4.4	2	1.39

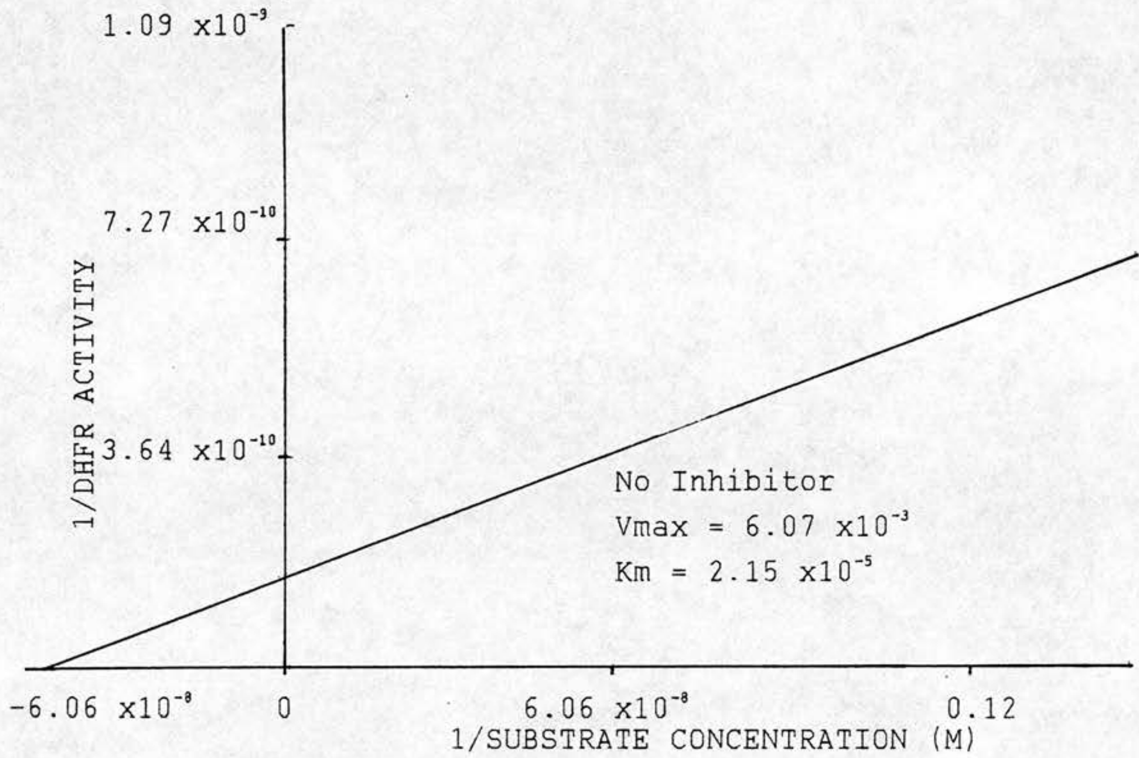
APPENDIX 7.5: DETERMINATION OF MICHAELIS MENTEN KINETICS

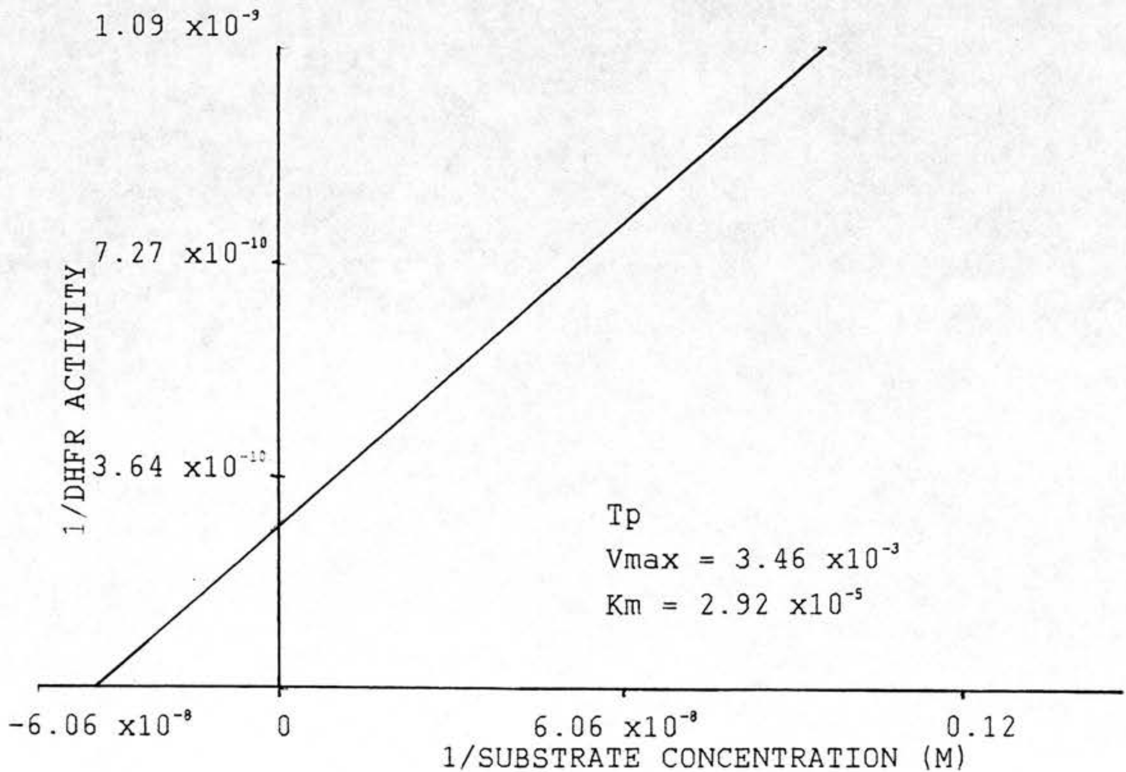
R483::Tn7



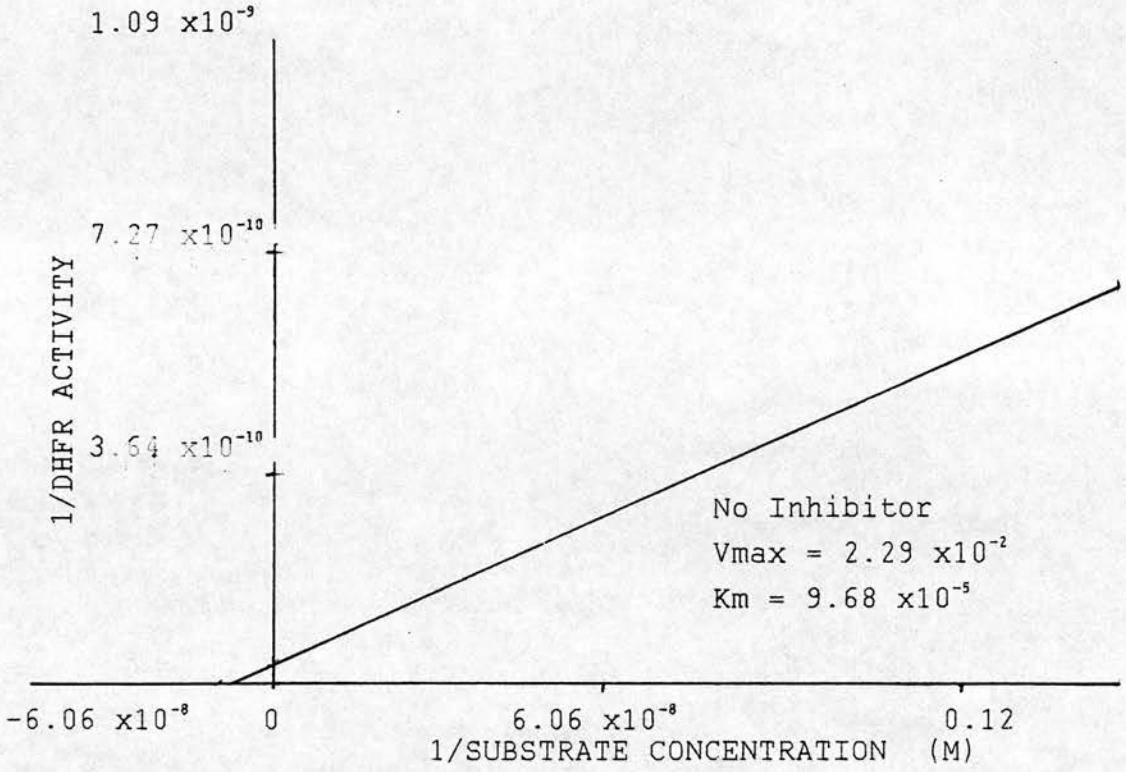


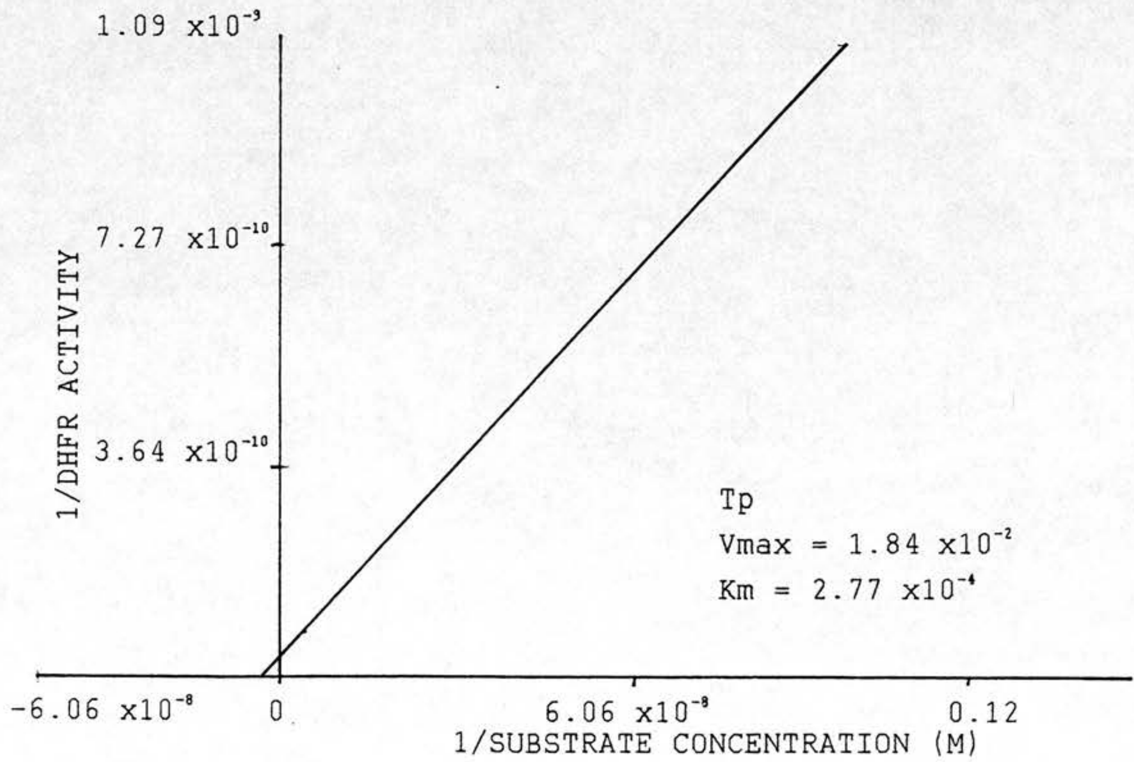
RP4::Tn7





RP4::Tn4135





APPENDIX 9.1: PURIFICATION TABLES FOR THE DHFRs OF Tn402 HARBOURING STRAINS

R751::Tn402

SAMPLE	VOLUME	PROTEIN CONCENTRATION	DHFR ACTIVITY PER ml	SPECIFIC ACTIVITY	TOTAL DHFR	% RECOVERY	PURIFICATION
BULK	11.0	42.780	20	0.468	220	100	1
STREPTOMYCIN SULPHATE	11.0	46.870	60	1.280	660	300	2.74
0-50% $(NH_4)_2SO_4$	5.5	27.950	100	3.579	550	250	7.65
PELLET	2.4	43.000	200	4.651	480	218	9.94
SEPHADEX FRACTION	2.2	2.150	3	1.390	6.6	3	2.97

Sa-1::Tn402

SAMPLE	VOLUME	PROTEIN CONCENTRATION	DHFR ACTIVITY PER ml	SPECIFIC ACTIVITY	TOTAL DHFR	% RECOVERY	PURIFICATION
BULK	11.0	29.67	80	2.70	880	100	1
STREPTOMYCIN SULPHATE	11.0	24.51	120	4.90	1320	150	1.815
0-50% $(NH_4)_2SO_4$	6.0	12.04	110	9.14	660	75	3.385
PELLET	0.9	16.56	60	3.62	54	6.14	1.341
SEPHADEX FRACTION	2.0	32.25	9	0.279	18	2.70	0.103

CLONE-H (NB)

SAMPLE	VOLUME	PROTEIN CONCENTRATION	DHFR ACTIVITY PER ml	SPECIFIC ACTIVITY	TOTAL DHFR	% RECOVERY	PURIFICATION
BULK	5.0	64.5	240	3.72	1200	100	1
STREPTOMYCIN SULPHATE	4.5	37.84	120	3.17	540	45	0.85
0-50% $(NH_4)_2SO_4$	2.0	23.65	90	3.81	180	15	1.02
PELLET	1.5	8.60	100	11.62	150	12.5	3.124
SEPHADEX FRACTION	2.0	3.23	7.5	2.33	15	1.25	0.626

CLONE-H (ISO + Tp)

SAMPLE	VOLUME	PROTEIN CONCENTRATION	DHFR ACTIVITY PER ml	SPECIFIC ACTIVITY	TOTAL DHFR	% RECOVERY	PURIFICATION
BULK	20.0	45.15	210	4.65	4200	100	1
STREPTOMYCIN SULPHATE	20.0	51.17	220	4.30	4400	104.8	0.92
0-50% $(NH_4)_2SO_4$	10.0	32.465	100	3.08	1000	23.81	0.66
PELLET	2.2	80.62	110	1.86	242	5.76	0.40
SEPHADEX FRACTION							

CLONE 3

SAMPLE	VOLUME	PROTEIN CONCENTRATION	DHFR ACTIVITY PER ml	SPECIFIC ACTIVITY	TOTAL DHFR	% RECOVERY	PURIFICATION
BULK	5.0	34.40	160	4.65	800	100	1
STREPTOMYCIN SULPHATE	4.2	23.65	340	14.38	1428	178.5	3.09
0-50% $(NH_4)_2SO_4$	1.5	16.77	400	23.85	600	75	5.13
PELLET	2.0	10.75	280	26.05	560	70	5.60
SEPHADEX FRACTION	2.1	2.60	5.5	2.115	11,55	1.44	0.45

CLONE 2

SAMPLE	VOLUME	PROTEIN CONCENTRATION	DHFR ACTIVITY PER ml	SPECIFIC ACTIVITY	TOTAL DHFR	% RECOVERY	PURIFICATION
BULK	24.0	25.80	280	10.85	6720	100	1
STREPTOMYCIN SULPHATE	24.0	25.37	190	7.49	4560	67.85	0.69
0-50% $(NH_4)_2SO_4$	13.0	18.28	380	20.79	4940	73.51	1.916
PELLET	2.0	25.37	110	4.336	220	3.27	0.400
SEPHADEX FRACTION	1.9	2.15	10	4.65	19	0.20	0.429

CLONE 6

SAMPLE	VOLUME	PROTEIN CONCENTRATION	DHFR ACTIVITY PER ml	SPECIFIC ACTIVITY	TOTAL DHFR	% RECOVERY	PURIFICATION
BULK	16.0	43.0	370	8.6	5920	100	1
STREPTOMYCIN SULPHATE	10.0	6.02	420	69.77	4200	71	8.11
0-50% $(NH_4)_2SO_4$	3.5	2.15	390	181.4	1365	23	21.09
PELLET	2.0	3.23	440	136.4	880	14.8	15.86
SEPHADEX FRACTION	1.9	2.80	27	9.66	51.3	0.8	1.12