GENETIC ASPECTS OF ANTIBIOTIC RESISTANCE, HAEMOLYSIN AND BACTERIOCIN PRODUCTION IN ENTEROCOCCI

Unkles, Shiela E.

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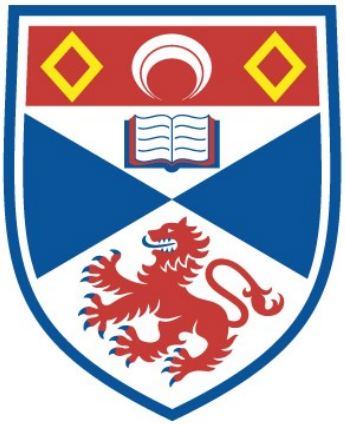

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GENETIC ASPECTS OF ANTIBIOTIC RESISTANCE, HAEMOLYSIN AND BACTERIOCIN PRODUCTION IN ENTEROCOCCI.

SHIELA E. UNKLES

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SUMMARY

A previous survey of enterococci had identified five strains of Streptococcus faecalis (K55 and SB94) - two subspecies liquefaciens (K60 and K88) and one zymogenes (K87) - and two S. faecium strains (K46 and SB69) which were resistant to tetracycline and streptomycin but susceptible to gentamicin. All the S. faecalis strains and K46 were in addition resistant to erythromycin but only the S. faecium strains were penicillin and ampicillin resistant.

The minimal inhibitory concentrations of a further six antibiotics were determined. These values confirmed that in S. faecalis strains, erythromycin resistance was accompanied by resistance to lincomycin and pristinamycin IA, a phenotype typical of macrolide - lincosamide - streptogramin B - type (MLS) antibiotics resistance. The erythromycin resistant K46 however, although resistant to lincomycin, was pristinamycin susceptible and so the basis of resistance is unknown. S. faecalis K60, K87 and SB94 were resistant to kanamycin and neomycin as was S. faecium K46 but all strains were susceptible to spectinomycin. The phenotypes were consistent with resistance mediated by enzymic modification of streptomycin with adenyltransferase (6) and of kanamycin and neomycin with phosphotransferase (3') (5")-III.

Erythromycin and tetracycline resistances were expressed constitutively in all strains. Only one S. faecalis (K88) was found to be chloramphenicol resistant and as is typical of Gram-positive bacteria, resistance was inducible.

The ability to produce bacteriocin was restricted to β-haemolytic strain K87 and to strain SB94. Subsequent results indicated that strain K87 probably produced more than one bacteriocin, the activity of which was repressed in the parental strain but which, in derivatives, could be

enhanced by the presence of streptomycin.

Evidence for the location of resistance, haemolysin and bacteriocin genes was sought from study of the transfer characteristics and stability of markers and from examination of the plasmid content of parental strains and their derivatives. The well characterised S. faecalis subspecies zymogenes strain DS5 (Clewell et al., 1982b) was included for comparison in transfer and curing experiments.

All the S. faecalis strains aggregated in response to a cell free filtrate of a plasmid free recipient strain JH2-1, indicating the presence of at least one conjugative plasmid although the low transfer frequencies of most resistance genes in broth matings suggested that response was not necessarily encoded by antibiotic resistance plasmids. Transfer of β-haemolytic activity and all resistance markers was observed after broth matings but the range of transfer frequencies between strains was wide. Furthermore, the incidence of transfer could be variable particularly in the transfer of DS5 erythromycin resistance and all K87 antibiotic resistances which seemed to be dependent on the production of active donor bacteriocin. Matings of S. faecalis strains carried out on membrane filters were only marginally more efficient in terms of transfer frequencies but were superior with regard to reproducibility of transfer. No antibiotic resistance transfer from S. faecium donors was observed after broth matings and only SB69 tetracycline resistance transferred after filter mating at very low frequency. Several resistance determinants and those encoding β-haemolysin were found to be capable of retransfer indicative of association with genes specifying conjugative ability.

Analysis of transconjugant phenotypes revealed that the tetracycline resistance gene of K55, the streptomycin resistance gene of K88 and β-

haemolytic activities were always transferred alone but some resistance markers were usually co-transferred with other donor markers. Conversely, transconjugants of K60, K87 and SB94 matings selected on any aminoglycoside were always co-resistant to the other aminoglycosides and to erythromycin while transfer of K88 chloramphenicol and, probably, erythromycin resistances required simultaneous transfer of tetracycline resistance.

The stability of antibiotic resistance and β-haemolytic activity was tested after storage for 10 to 12 months or treatment with novobiocin. Unlike the situation after resistance transfer, although aminoglycoside resistances of K60, K87 and SB94 were lost simultaneously, concomitant susceptibility to erythromycin was not necessarily observed. Extremely high frequency loss of haemolytic activity, as high as 96% in DS5, was obtained but tetracycline resistance was eliminated from strain SB94 only. Penicillin and ampicillin were never eliminated from S. faecium strains.

Several methods of plasmid isolation were carried out and the most effective for the strains in this study, was found to be that of Dunny et al. (1981b). Nevertheless, plasmid DNA was always extremely difficult to isolate from strains K60 and K46 and was never observed in samples from strain SB69. It was assumed that in the latter strain, resistance was determined by the chromosome, tetracycline being transferred as a conjugative transposon. Including possible linear chromosomal DNA, the remaining strains contained between 4 and 6 bands in agarose gels ranging in size from 5 to 60kb. However, attempts to correlate the bands observed in transconjugants and cured derivatives with the strain phenotypes were unsuccessful as often there appeared to be no difference in the plasmid

content of strains with different phenotypes.

To aid interpretation, DNA samples were digested with restriction endonucleases EcoRl and Pstl and several plasmid species were identified. Strain K55 contains a conjugative plasmid pSK552 of approximately 24kb encoding MLS resistance and a large conjugative plasmid pSK551 which could be responsible for the pheromone response of K55 and, although probably cryptic, may specify tetracycline resistance. In transconjugants and cured derivatives of K60, the MLS determinant is most likely located on a conjugative 68kb plasmid, pSK601, which specifies pheromone response, and a tetracycline resistance gene on a 47kb plasmid, pSK602, which is non-conjugative but which can be efficiently mobilised. Strain K87 appears to harbour two conjugative plasmids encoding haemolysin-bacteriocin and possibly another specifying a different bacteriocin. In addition, two small, cryptic, non-conjugative plasmids, pSK871 and pSK872 were identified as were plasmids with similar characteristics to pSK871 in strain K88 (pSK881) and SB94 (pSK941). In strain K88 a plasmid of 23.5kb, pSK882, probably specifies tetracycline resistance and is capable of transfer but only on membrane filters.

Although transfer and curing data may have implied plasmid encoded resistance in many cases, with the above exceptions, the locations of the remaining determinants are speculative.

DECLARATION

I hereby declare that the following thesis is based on work carried out by myself, that the thesis is of my own composition and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry and Microbiology, University of St. Andrews, under the direction of Drs. D. Thirkell and J.R. Kinghorn.

STATEMENTS

I hereby declare that Shiela E. Unkles has spent nine terms in research work under my direction, that she has fulfilled the conditions of Ordinance General No. 16 and that she is qualified to submit this thesis for the degree of Doctor of Philosophy.

The research was undertaken from the date of my admission as a Research Student in October, 1981 under Ordinance General No. 16.

Access to the thesis in the University Library shall be governed by any regulations approved by the Library Committee.

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LIST OF ABBREVIATIONS

Bcn	=	bacteriocin production
BHI	=	brain heart infusion
BYGT	=	BHI-yeast extract-glucose-Tris broth
ccc	=	covalently closed circular
cfu	=	colony forming units
CIA	=	clump inducing agent
DST	=	diagnostic sensitivity test
EDTA	=	ethylenediaminetetraacetic acid, disodium salt
αHly	=	α-haemolysin production
Hly	=	β-haemolysin production
kb	=	kilobase pairs
MIC	=	minimal inhibitory concentration
MLS	=	macrolide, lincosamide, streptogramin B-type
N2GT	=	Oxoid nutrient broth No.2-glucose-Tris broth
PBP	=	penicillin binding protein
SDS	=	sodium dodecyl sulphate
TBE	=	Tris-borate-EDTA buffer
TE	=	Tris-EDTA buffer
Tn	=	transposon
Tris	=	tris(hydroxymethyl) aminomethane
v/v	=	volume for volume
w/v	=	weight for volume

Antibiotics

Ap	=	ampicillin
Cm	=	chloramphenicol
Em	=	erythromycin
Km	=	kanamycin
Lm	=	lincomycin

Nm = neomycin

Pn = benzyl penicillin

Pr = pristinamycin IA

Sm = streptomycin

Tc = tetracycline

INTRODUCTION

THE GENUS STREPTOCOCCUS

Streptococci are Gram-positive spherical or ovoid cells which form pairs or chains in broth culture. They are non-motile, facultative anaerobic bacteria and, on exposure to air, grow readily at 37°C on complex media containing fermentable carbohydrate.

A general classification of the streptococci was provided by Sherman (1937), with minor modifications by Parker (1983). Members of the pyogenic group, which includes many human and animal pathogens, usually produce an extracellular enzyme which lyses red blood cells and consequently, colonies on blood agar medium are characteristically surrounded by a clear zone in which erythrocytes have been completely lysed due to β-haemolysis. Unlike the pyogenic group the enterococci, so named because they occur mainly in the intestinal tract of man and animals, can grow over a wide range of pH and temperature (10 to 45°C) and have the group D antigen. Lactic streptococci are characterised by their low temperature range - they grow well at 10°C but cannot grow at temperatures in excess of 40°C. The cells of pneumococci are typically paired and the species is subdivided into a large number of types based on the antigenic specificity of its capsular polysaccharides. On media containing blood, colonies are surrounded by a zone of "greening", termed α-haemolysis, caused by oxidation of haemoglobin to methaemoglobin. The category of 'other streptococci' includes many strains originally classified as 'viridans' streptococci by Sherman (1937) due to their α-haemolytic reaction on blood agar.

A second widely used classification of the genus is based on Lancefield group specific antigens known as C substances (Lancefield, 1933). These are acid extractable polysaccharides or teichoic acids, attached to the peptidoglycan of the cell wall, which form a specific precipitate with rabbit antiserum to killed whole bacterial cells. Generally, members of a group

are biochemically uniform or contain recognisable biotypes with a characteristic host range and ability to cause particular diseases. However, many strains do not possess a group antigen and the classification is further complicated by the fact that group antigens which characterise certain pyogenic bacteria are occasionally found in members of otherwise distinct species.

Additionally, many streptococci possess type specific antigenic proteins such as the M (recently cloned in E. coli; Burdett and Beachey, 1984), T and R antigens of Lancefield group A Streptococcus pyogenes. Multiple, distinct antigenic types of these proteins exist but only one of each type is present in an individual organism, allowing further differentiation of strains into a number of serotypes.

As a result of their activities in the production of foods and as agents of human and animal disease, the streptococci are a group of major economic importance. The Lancefield group N lactic streptococci are particularly important in the dairy industry where they are commonly used as starter cultures in the production of butter and cheese (Lawrence and Thomas, 1979). Lactic streptococci are among those bacteria which occur normally in milk and are responsible for souring due to the production of lactic acid which lowers the pH sufficiently to cause coagulation of milk proteins.

The most common causative agents of human streptococcal disease are the pyogenic streptococci of which group A S. pyogenes is most frequently involved (Cybulska and Jeljaszewicz, 1970; Miyamoto et al., 1978; Chattopadhyay et al., 1981). A normal inhabitant of the pharynx, skin and rectum, S. pyogenes can cause suppurative infections such as pharyngitis, scarlet fever and impetigo as well as related non-suppurative diseases such as acute glomerulonephritis and acute rheumatic fever. S. agalactiae (group B) are a common cause of serious infections, including meningitis in neonates and infants (Yow, 1974; McCracken, 1976) as well as serious puerperal infections and skin infections among diabetics (Anthony and Concepcion, 1975). Additionally, group B

organisms are an important cause of bovine mastitis (Berghash et al., 1983). Although less common pathogens, groups C and G streptococci can cause a variety of serious infections such as endocarditis and septic arthritis (Mohr et al., 1979; Cudney and Albers, 1982).

The drugs of choice in the therapy of pyogenic streptococcal infections are the penicillins although other antibiotics such as lincomycin are used in cases of penicillin allergy. Generally, susceptibility to the penicillins is uniform but occasionally strains are isolated showing drug tolerance which can adversely affect the response to therapy (Severin and Wiley, 1976; Rolston et al., 1982). The incidence of resistance to macrolides, lincosamides and chloramphenicol is low although some highly resistant strains have been isolated (Cybulska and Jeljaszewicz, 1970; Baker et al., 1976) and there does appear to be some relationship between the geographical distribution of resistant isolates and high useage of antibiotics (Dixon and Lipinski, 1982). Tetracyclines should never be used for treatment of streptococcal infections due to the high frequency of resistance - as high at 90% in some studies (Baker et al., 1976; Fallon, 1981).

Alpha-haemolytic streptococci such as S. mitior, S. sanguis and S. mutans which normally inhabit the oral cavity, are the most common cause of infective endocarditis (Garvey and Neu, 1978) while the most widespread bacterial infection of man, dental caries, is thought to involve principally S. mutans (Hamada and Slade, 1980). As with the pyogenic streptococci, the drug used in therapy of endocarditis is penicillin in combination with streptomycin to produce a more rapid killing although complications can arise in cases of penicillin tolerance and high level streptomycin resistance (Bourgault et al., 1979; Farber et al., 1983).

The role of S. pneumoniae as the etiological agent of pneumonia is well known. It was assumed that pneumococci were uniformly susceptible to

penicillins but there is increasing evidence of resistance to these and many other drugs in clinical isolates (Jacobs et al., 1978; Tarpay et al., 1982; Michel et al., 1983).

Enterococci are commonly involved in urinary tract infections and can cause endocarditis. They are also frequently isolated from dental root canal infections and some strains have been shown to be cariogenic in germfree rats (Gold et al., 1975). Unlike the other streptococcal groups, the enterococci are intrinsically resistant to low levels of many drugs in common use (Taola et al., 1969) and consequently, synergistic combinations of penicillins or cephalosporins with aminoglycosides are recommended for serious enterococcal infections such as endocarditis. Increasingly, clinical isolates have been found resistant to synergism due to high level resistance to aminoglycosides (Finland, 1979; Carlier and Courvalin, 1982; Murray et al., 1983) and resistance to penicillins and cephalosporins (Finland, 1979; Krogstad and Moellering, 1982; Elliot et al., 1983). High level resistance to many other drugs including tetracycline, erythromycin and chloramphenicol has also been shown to be increasingly common (van Embden et al., 1977; Finland, 1979; Romero et al., 1979).

MODE OF ACTION OF ANTIBIOTICS

Antibiotics have been divided into five general groups according to the biochemical site on which they have a primary effect (Gale, 1963) - those acting on 1. energy metabolism 2. the function of bacterial membranes 3. protein synthesis 4. nucleic acid metabolism 5. the synthesis of peptidoglycan. An understanding of the molecular basis of antibiotic action has depended on the level of understanding of the basic biochemical processes involved and at present the level of knowledge in many cases is insufficient to provide definitive statement on the different modes of antibiotic action.

Much of the work on the activity of antibiotics has been carried out using Gram-negative bacteria in particular Escherichia coli and hence, in extrapolating results to Gram-positive organisms, the fundamental differences in cell envelope compositions must be considered. There are several reviews available on the structures of Gram-negative and Gram-positive cell envelopes (Rogers et al., 1980; Salton, 1983). Briefly, Gram-negative bacteria possess an outer and an inner cytoplasmic membrane enclosing an aqueous region known as the periplasmic space. The outer membrane provides a major hydrophobic barrier to passive diffusion of many compounds although low molecular weight hydrophilic molecules can diffuse through transmembrane pores formed by proteins called porins (Nikaido and Nakae, 1979). In contrast, Gram-positive bacteria have no outer membrane and consequently are much more susceptible to certain hydrophobic antibiotics such as rifamycin. The inner membrane of Gram-negative bacteria broadly corresponds to the cytoplasmic membrane of Gram-positive species. Structural integrity of both Gram-negative and Gram-positive bacteria is maintained by peptidoglycan located in the periplasmic space of Gram-negative organisms and surrounding the cytoplasmic membrane of Gram-positive organisms. Peptidoglycans are polymers of N-acetylglucosamine and N-acetylmuramic acid and, attached to the carboxyl groups of N-acetylmuramyl residues are peptides, which are often

crosslinked via a peptide bridge in Gram-positive bacteria and which are specific to the bacterial species. In Gram-negative organisms there is generally only one layer of peptidoglycan while in Gram-positive species, the structure is multi-layered and has associated with it a number of other polymers such as teichoic or teichuronic acids. In contrast to the molecular seiving action of porins, it is unlikely that peptidoglycans form a barrier to molecules less than 100,000 daltons.

Of relevance to this introduction, is a presentation of the current state of knowledge into mechanisms of action of the antibiotics to which resistance has been encountered in this group of study organisms.

Beta-lactam antibiotics

This group of antibiotics includes the penicillins, cephalosporins, cephamycins and the newer β-lactams such as thienamycin and clavulanic acid. β-lactams are inhibitors of peptidoglycan synthesis and consequently are inactive against resting cells and bacteria which lack cell walls such as mycoplasmas. They appear to have similar but not identical activities and there can be a wide spectrum of responses to a particular antibiotic. For example, among the streptococci, S. pneumoniae is both killed and lysed by penicillin, S. pyogenes is killed but not lysed and S. sanguis is neither killed nor lysed (Horne and Tomasz, 1977).

In studies of Staphylococcus aureus, Tipper and Strominger (1965) showed that penicillin inhibited the action of transpeptidase, an enzyme which removes the terminal D-alanine residue of N-acetylmuramyl pentapeptide and links the remaining tetrapeptide to another part of the peptidoglycan structure i.e. the enzyme which catalyses the formation of cross-links. They suggested that penicillin might act as a structural analogue of the transpeptidase substrate acyl-D-alanyl-alanine resulting in competitive inhibition of the enzyme leading to the formation of a "weak" cell wall and eventually wall

rupture and cell lysis. However, the anomalies that some β-lactams such as mecillinam do not inhibit transpeptidation (Matsuhashi et al., 1974) and that visible morphological distortion is not necessarily related to the degree of cross-linking (Katz and Martin, 1970) suggested that the situation was more complex.

Interest in the bactericidal action of β-lactams is now centered on penicillin binding proteins (PBP). These proteins which can specifically bind β-lactams, are found in the cytoplasmic membranes of both Gram-negative and Gram-positive organisms and have been shown to represent multiple forms of the transpeptidases and carboxypeptidases involved in peptidoglycan synthesis (Spratt, 1983). The profiles of PBPs vary between species of bacteria with usually five or more PBPs present which have variable binding affinities for different β-lactams. Additionally, the pattern of binding to PBPs and hence the effect of β-lactams, has been found to be heavily dependent on growth conditions (Fontana et al., 1983). The functions and interaction of PBPs with β-lactams are reviewed by Gale et al. (1981) and Spratt (1983).

A simplified explanation for the killing of Gram-positive bacterial cells by β-lactams, given by Lancini and Parenti (1982) is that β-lactams inhibit the function of one or more enzymes (PBPs) involved in peptidoglycan synthesis resulting in growth inhibition. A signal of unknown biochemical nature causes the release of teichoic acids into the medium resulting in the activation of autolytic enzymes which hydrolyse covalent bonds of the cell wall. Loss of structural integrity is followed by eventual osmotic lysis.

Tetracyclines

The first tetracycline to be introduced, chlortetracycline, was isolated from Streptomyces aureofaciens and tetracycline itself was originally obtained by hydrogenation of chlortetracycline, but subsequently from cultures of several Streptomyces, while doxycycline and minocycline are semi-synthetic

derivatives. This family of antibiotics have a broad spectrum of activity and have been shown to block protein synthesis in bacterial and mammalian systems (Rendi and Ochoa, 1961; Franklin, 1963). They are bacteriostatic at low concentrations but at high concentrations can affect a number of cellular processes, such as membrane permeability and DNA synthesis, and have a bactericidal effect (Levy, 1981).

In E. coli, uptake of tetracyclines has been shown to occur in two stages - an energy-independent phase and an energy-dependent phase. The energy-independent phase reflects the passive diffusion of the antibiotics through the outer membrane either via porins as in the case of the hydrophilic molecules for example tetracycline or by direct diffusion in the case of more hydrophobic molecules such as minocycline (Chopra and Ball, 1982; McMurray et al., 1982). The energy-dependent accumulation of tetracyclines, which has been shown to occur in Gram-positive bacteria (Dockter et al., 1978) including S. faecalis (Lindley et al., 1984) as well as Gram-negative bacteria (Chopra and Howe, 1978), corresponds to the active, carrier mediated transport of the antibiotics through the cytoplasmic membrane. In E. coli transport is inhibited by energy poisons, uncouplers and depolarisers but not by inhibitors of membrane bound ATPase, which implies that proton motive force is the energy source for transport. Similar studies in S. faecalis have shown that tetracycline influx is dependent on the transmembrane pH gradient component of the proton motive force, the drug being transported as an anion protonated to neutrality and bound to a neutral carrier (Lindley et al., 1984; Munske et al., 1984). It is thought that the membrane carrier is probably a protein but the identity of the carrier is not known (Chopra and Ball,1982).

Tetracyclines chelate metal ions particularly divalent cations such as magnesium, although the relevance of such an activity to the inhibitory effects of the antibiotics was unclear (Tritton, 1977). On the basis of stoichiometry, Munske and coworkers (1984) have recently suggested that the magnesium

ion has a role in transport by binding to the apo-carrier molecule to produce a functional carrier. Tetracycline inhibits protein synthesis by binding to ribosomes. At high drug concentrations, up to 300 drug molecules may bind to one ribosome but it appears that only one binding site is important and that only one drug molecule is necessary for inhibition of a single ribosome in E. coli (Tritton, 1977). The most significant binding site in terms of inhibition of protein synthesis is probably on the 30S subunit of 70S ribosomes. It has been shown that tetracycline blocks the A site of ribosomes thus preventing the binding of aminoacyl - tRNA and subsequent translocation and peptide elongation (Gale et al., 1981). Tetracycline also inhibits the synthesis of guanosine tetraphosphates and pentaphosphates normally synthesised in response to the presence of uncharged tRNAs at the A site and responsible for the stringent control of rRNA, tRNA and ribosomal protein operons.

Aminoglycoside-aminocyclitol antibiotics

The aminoglycoside-aminocyclitols are a large group of structurally related substances produced naturally by members of the genera Streptomyces, Micromonospora and Bacillus or by semisynthetic processes. They can be divided into several major groups on the basis of structural similarity - the streptomycin group which includes dihydrostreptomycin and bluensomycin, the neomycin group, the kanamycin group which includes amikacin and tobramycin, the gentamicin group and the groups represented by hygromycin, apramycin, spectinomycin and kasugamycin (Price, 1981; Foster, 1983). All show a wide spectrum of antimicrobial activity but are inactive against strict anaerobic bacteria (Bryan and Kwan, 1981) and against some facultative anaerobes for example S. faecalis (Bryan and van den Elzen, 1977). Unlike other inhibitors of protein synthesis, most are bactericidal to susceptible strains although a few are bacteriostatic. The killing of susceptible bacterial cells is accompanied by a wide range of pleiotropic effects which have led to

considerable difficulties in defining the lethal event.

Aminoglycoside accumulation in susceptible cells has been shown to occur in three phases (Bryan and van den Elzen, 1977). The first is a rapid energy-independent (EIP) binding of the polycationic molecules of aminoglycoside to negative charges (lipopolysaccharides or lipoteichoic acids) on the cell surface followed by passive diffusion through porins in the outer membrane of Gram-negative species (Nakae and Nakae, 1982) or the interstices of the cell wall in Gram-positive bacteria (Bryan and Kwan, 1983). At least part of EIP reflects binding to the cytoplasmic membrane. The first energy-dependent phase (EDPI) corresponds to the slow active transport of the drug across the cytoplasmic membrane, a process requiring electron transport, and although the nature of the transporter species is not known, cytochrome aa_3 and/or quinones have been implicated (Bryan and Kwan, 1983; McEnroe and Taber, 1984). The energy required for uptake was shown to be provided by the transmembrane electrical potential component of the transmembrane electrochemical proton gradient (proton motive force) and the lethal effects of the drugs were found to be proportional to the magnitude of this component (Mates *et al.*, 1983; Eisenberg *et al.*, 1984). After transport across the cytoplasmic membrane, some antibiotic molecules bind to ribosomes which act as a "binding sink". This increasing binding causes cessation of protein synthesis and signals the acceleration of aminoglycoside energy-dependent accumulation (EDPII). Throughout EIP and EDPI, cells remain viable although the cytoplasmic membrane becomes leaky to potassium ions (Dubin *et al.*, 1963). The onset of EDPII coincides with the onset of lethality, i.e. the inability of cells to form colonies, and further cytoplasmic membrane damage resulting in loss of permeability control for larger molecules (Bryan and van den Elzen, 1975; 1976; Hancock, 1981b). That EDPII is probably related to lethality is evident from the observations that the bacteriostatic antibiotics, spectinomycin and kasugamycin do not induce EDPII (Holtje, 1978) and that addition of

chloramphenicol to cells antagonises streptomycin killing and prevents EDP11 presumably by influencing events required for EDP1 (Hancock, 1981a, b). However, this may not be so for gentamicin uptake by Staph. aureus in which EDP11 was not necessarily associated with the lethal effect (Mandel et al., 1984).

The 30S subunit of 70S ribosomes is the main target for binding of aminoglycosides. Streptomycin and dihydrostreptomycin bind to a single site while the neomycins, kanamycins and gentamicins interact with multiple sites. The binding, which is reversible, results in a number of effects one of which is the phenomenon of misreading i.e. incorporation of the wrong amino acid during translation (Gorini, 1974; Tai et al., 1978). Misreading caused by streptomycin in vitro has been shown to be non-random - only one base in a given mRNA codon is misread and that base, located at the 5'-terminal or internal position of the codon, is almost always a pyrimidine (Davies et al., 1966). The action of neomycin is more random involving multiple bases at any codon position. Misreading also occurs in vivo, albeit at a lower frequency (Hancock, 1981b), as demonstrated by the phenotypic suppression of nonsense and missense mutations in the presence of sub-lethal concentrations of streptomycin (Gorini and Kataja, 1965; Orias and Gartner, 1966; Whitfield et al., 1966). The aminocyclitol spectinomycin which is not bactericidal, does not cause misreading (Davies et al., 1965) but nevertheless it is unlikely that production of faulty proteins is directly responsible for the lethal effects of aminoglycosides (Hancock, 1981b).

Streptomycin can inhibit aspects of all phases of protein synthesis (reviewed by Gale et al., 1981) in addition to causing mistranslation of mRNA. In extracts of E. coli containing polysomes but no initiation factors, streptomycin caused a rapid decrease in the rate of protein synthesis reflecting an inhibitory effect on elongation (Wallace et al., 1973). Streptomycin also affects initiation by causing release of fmet-tRNA resulting in unstable

aberrant initiation complexes containing 70S ribosomes and mRNA (Wallace and Davis, 1973). In polypeptide chain termination assays, streptomycin inhibits release by preventing recognition of the release factors RF-1 and RF-2 (Scolnick et al., 1968). The effects of streptomycin on initiation, elongation and termination are probably pleiotropic events due to the same interaction of the antibiotic with the ribosome and may reflect dislocation of the ribosomal A and/or P sites (Gale et al., 1981).

As ribosome binding is reversible however, inhibition of protein synthesis per se is unlikely to account for the lethal effects of aminoglycosides (Hancock, 1981b). Instead, there is general agreement that lethality of aminoglycosides is related to membrane associated disruptions although the precise mechanism is still controversial. Hancock (1981b) has postulated that disruption of the DNA-membrane attachment site may be at least one of the lethal targets involved. Bryan and Kwan (1983) propose that lethality results directly from the loss of cytoplasmic membrane integrity and function due to the physical forces involved in the accumulation of the antibiotics.

Chloramphenicol

Chloramphenicol is a fermentation product of Streptomyces venezuelae and is one of the few commonly used antibiotics which can be produced synthetically. It is a bacteriostatic agent which inhibits protein synthesis in a wide range of Gram-positive and Gram-negative bacteria.

In contrast to the complex energy dependent transport systems of aminoglycosides and tetracyclines, there is little evidence in favour of active accumulation of chloramphenicol (Chopra and Ball, 1982) and instead, the antibiotic probably diffuses passively through porins of the outer membrane of Gram-negative organisms. Uptake of the drug through the cytoplasmic membrane is also likely to be due to passive diffusion with apparent intracellular concentration resulting from the binding of the drug to ribosomes

(Harvey and Koch, 1980).

At bacteriostatic concentrations, chloramphenicol has been shown to bind reversibly to one high affinity site on the 50S subunit of ribosomes and polysomes (Fernandez-Munoz et al., 1971; Pestka, 1974). At higher concentrations, the antibiotic also binds to a low affinity site on the 30S subunit (Lessard and Pestka, 1972) although this may not be significant in terms of bacteriostatic activity. Chloramphenicol inhibits the elongation step of protein translation and "freezes" polysomes preventing the release of peptidyl-tRNA. The drug has an increased affinity for ribosomes when the peptidyl-tRNA is located in the P site (Contreras and Vazquez, 1977) and it is thought that it inhibits the action of peptidyl transferase (located on the 50S ribosomal subunit) by preventing recognition of the enzyme acceptor substrate i.e. aminoacyl-tRNA (Gale et al., 1981).

The MLS group - macrolides, lincosamides and streptogramin B-type antibiotics

Although structurally unrelated these three classes of antibiotics will be discussed together in view of the similarities of antibacterial spectrum and mode of action. All are produced either naturally by members of the genus Streptomyces or by synthetic modification of the natural products. They are active mainly against Gram-positive organisms - most members of the Enterobacteriacae are intrinsically resistant to clinically achievable concentrations (Garrod et al., 1981) probably due to the impermeability of the outer membrane or ribosomal resistance (Costerton and Cheng, 1975; Tanaka and Weisblum, 1975).

The macrolide antibiotics contain a large lactone ring of between 12 and 16 atoms, to which various sugar residues are linked, and can be subdivided according to the sugar residues they contain - erythromycin, spiramycin, carbomycin, methymycin and lancamycin groups. Chemically, the lincosamides are completely different, being composed of a modified amino acid condensed with

a complex amino sugar. Lincomycin, a natural fermentation product of Streptomyces lincolnensis, and clindamycin, obtained by chemical modification of lincomycin, are the most commonly used antibiotics of this class (Vazquez, 1979). The streptogramins are, in most cases, produced as mixtures of two different components A and B. The A components are considered to be one molecular species consisting of a large polyunsaturated non-peptide ring and are known by a number of synonyms - mikamycin A, virginiamycin M_1, vernamycin A, ostreogrycin A, pristinamycin 11A and synergistin A. The B components are cyclic hexadepsipeptides containing uncommon amino acids (Vazquez, 1979; Gale et al., 1981). All the MLS antibiotics inhibit protein synthesis in susceptible bacteria and all are usually bacteriostatic although, in combination, the streptogramin components demonstrate marked synergism and can be bactericidal (Gale et al., 1981).

MLS antibiotics have been shown to bind reversibly to 50S ribosomal subunits and to ribosomes with 1:1 stoichiometry. Studies of reciprocal competition for the binding site have shown that the antibiotics are mutually exclusive reflecting common or overlapping sites on the ribosomes. Additionally, they prevent the binding of chloramphenicol to ribosomes (Fernandez-Munoz et al., 1971; Cocito and Di Giambattista, 1978). The antibiotics do not however prevent the binding of chloramphenicol to polysomes (Pestka, 1974) and indeed, cannot bind to polysomes when peptidyl-tRNA is present in either the A or P sites of the ribosomes (Contreras and Vazquez, 1977). The degradation of polysomes found on addition of many of these drugs has been interpreted as normal ribosome "run-off" (Cundliffe, 1969) followed by antibiotic blocking shortly after initiation of a new round of translation. The antibiotics have not been shown to cause inhibition of the initiation step itself, but, after the addition of a variable number of amino acid residues, prevent further elongation of the oligopeptide chain, possibly by steric hindrance (Vazquez, 1979; Gale et al., 1981). Recently, however,

Menninger and Otto (1982) have shown in E. coli that macrolides stimulate the release of peptidyl-tRNA from ribosomes, possibly during attempted translocation from the A to the P site, followed by ribosome dissociation from the message. The effect of this enhanced dissociation would be to reduce to a growth inhibitory level the probability of formation of functional proteins.

MECHANISMS OF BACTERIAL RESISTANCE TO ANTIBIOTICS

Clinically important bacterial resistance is most frequently determined by extrachromosomal DNA molecules (plasmids) or by transposons which are capable of integration into the bacterial chromosome or other DNA molecules (Foster, 1983). Many plasmids are capable of transfer from one host to another, a phenomenon which has undoubtedly contributed to the rapid spread of antibiotic resistance. Naturally occurring chromosomal mutations to resistance are encountered less frequently although there are some reports of these, for example, penicillin resistance of S. pneumoniae (Jacobs et al., 1978).

Resistance to antibiotics can theoretically arise from a number of different mechanism (Davies and Smith, 1978). These are 1. inactivation of the antibiotic, 2. decreased accumulation either by reduced permeability or active efflux of the antibiotic 3. alteration of the antibiotic target site, 4. provision of a by-pass for the particular inhibited metabolic step, 5. increased production of the structure or molecule inhibited by the antibiotic, 6. production of a drug antagonist, 7. utilisation of a completely different and alternative pathway which is not inhibited. The first four mechanisms are most commonly encountered while examples of the latter three mechanisms are either extremely rare or unknown. This introduction will be confined to a description of the clinically significant resistance mechanisms to the antibiotics of interest to this study.

Beta-lactam antibiotics

Resistance to β-lactams can occur by inactivation, increased impermeability and alteration of antibiotic target site or by combinations of these mechanisms. Most high level β-lactam resistance is determined by genes located on plasmids or transposons which encode β-lactamases causing inactivation of the antibiotics (Davies and Smith, 1978; Foster, 1983). The action

of β-lactamases on their substrates, penicillins and cephalosphorins, is kinetically complex but basically, it involves hydrolysis of the amide bond in the β-lactam ring via an acyl-enzyme intermediate, leading to the formation of inactive penicilloic acid, in the case of penicillin substrates, or various decomposition products, with cephalosporins (Sykes and Matthew, 1976; Fisher et al., 1980; Foster, 1983).

The enzymes are commonly produced by Gram-negative bacteria, particularly members of the Enterobacteriaceae and Pseudomonas, but are also important in resistance of the Gram-positive genera Staphylococcus, Clostridium and Bacillus. The need for the development of new penicillins and cephalosporins with reduced sensitivity to enzymatic hydrolysis has reflected the increasing dissemination of these resistance genes. In addition to plasmid and transposon encoded β-lactamases most, and probably all, Gram-negative bacteria produce chromosomally specified enzymes which contribute to intrinsic resistance (Richmond and Sykes, 1973; Sykes and Matthew, 1976) and which are specific for the bacterial species, correlating with taxonomic bacterial classification (Matthew and Harris, 1976). It has been suggested that β-lactamases are produced by all bacteria, their normal function being to hydrolyse a β-lactam structure present as a transitory cell wall intermediate (Sykes and Matthew, 1976). Furthermore, it has been shown that some low molecular weight PBPs have similar mechanisms of enzyme action to and regions of amino acid sequence homologous with certain classes of β-lactamases (Spratt, 1983).

A widely used general classification of Gram-negative β-lactamases is based on substrate specificity, inhibitor studies and physicochemical and immunological data (reviewed by Sykes and Matthew, 1976; Matthew et al., 1979; Matthew, 1979; Foster, 1983). Briefly, the majority of chromosomally encoded enzymes are cephalosporinases - they have low activity against penicillins - and some have been found to be inducible by β-lactam substrates. Plasmid

and transposon specified β-lactamases are predominantly penicillinases which are expressed constitutively and are usually produced at levels far in excess of those observed for chromosomally encoded enzymes. All β-lactamases of Gram-negative bacteria are located in the periplasmic space and expression of resistance therefore is dependent not only on β-lactamase production but also on outer membrane permeability and indeed, mutation of chromosomal genes encoding porins can result in increased resistance (Jaffe et al., 1982; Sawai et al., 1982). The synergistic interaction of enzyme production and outer membrane permeability is responsible for the phenomenon of crypticity of Gram-negative bacteria (Richmond and Curtiss, 1974).

The β-lactamases of Gram-positive bacteria, most of which are inducible by low substrate levels (Dyke, 1979; Magot, 1981; Foster,1983), can be chromosomally specified (Richmond and Sykes, 1973; Lampen et al., 1980) but are often encoded by plasmids in staphylococci (Richmond, 1965). They are predominantly penicillinases and all are secreted as extracellular enzymes (Sykes and Matthew, 1976; Foster, 1983). Studies on the penicillinases of bacilli have shown that they are synthesised as a precursor molecule with an N-terminal signal sequence necessary for transport through the cytoplasmic membrane and following proteolytic modification, soluble expenicillinase can be released (Simons et al., 1978; Cheng et al., 1982). Production of β-lactamases by Gram-positive organisms is a less widespread phenomenon than that of Gram-negatives and until recently was unknown in the genus Streptococcus. The only example thus far of a β-lactamase producing Streptococcus is a clinical isolate of S. faecalis which produced extracellular penicillinase and was capable of conjugal transfer of this ability to an S. faecalis recipient (Murray and Mederski-Samaroj, 1983). This may therefore represent the beginning of dissemination of β-lactamase genes in streptococci.

Increasingly common is the isolation of strains which do not owe their resistance to the production of β-lactamases. For example, the problem of

β-lactamase producing Staph. aureus was overcome by the use of methicillin which was resistant to hydrolysis by the plasmid specified β-lactamase but methicillin resistant strains, cross-resistant to other β-lactams, have since been isolated. It was proposed that this intrinsic resistance was due to mutations which caused either overproduction or decreased affinity of PBP 3, thought to be essential for viability in the presence of the drug (Brown and Reynolds, 1980; Hayes et al., 1981). However, subsequent evidence has shown that, unlike susceptible cells, methicillin resistant Staph. aureus produced a novel PBP 2a which required β-lactam concentrations in the region of the minimal inhibitory concentration to produce saturation while the other PBPs were saturated at much lower concentrations (Hartman and Tomasz, 1984). A similar alteration in PBP pattern has been described in S. pneumoniae (Tomasz et al., 1984), while alterations in the affinity of PBPs for β-lactam antibiotics have been shown to be important in the development of resistance in Neisseria gonorrhoeae (Dougherty et al., 1980), Pseudomonas aeruginosa (Godfrey et al., 1981), S. pneumoniae (Zighelboim and Tomasz, 1980; 1982) and Haemophilus influenzae (Parr and Bryan, 1984). In contrast to alterations in binding affinity, Eliopoulos and coworkers (1982) comparing the PBPs of naturally resistant and hypersusceptible mutants of S. faecium, found no difference in the PBP affinities. Further studies by Fontana et al. (1983b) showed that in fact, naturally insensitivity to penicillin in S. faecium was correlated with the overproduction of PBP 5 which reacted much more slowly than the other PBPs and which required 90% saturation with drug before cell growth was inhibited. Thus, it was postulated that as saturation required a period longer than that required for cell division and as PBP 5 was overproduced, cells could remain viable at high drug concentrations. Similar PBPs were found in β-lactam resistant S. faecalis, S. durans and S. lactis but not group A, B, C or G streptococci or S. pneumoniae. In the latter strains, resistance was probably due to decreased affinity of one or more PBP (Gutmann and Tomasz, 1982; Fontana et al., 1983b).

Tolerance of penicillins has clinical significance in the treatment of staphylococcal and streptococcal infections (Brennan and Durack, 1983; Hess et al., 1983). Unlike resistance in which the minimal inhibitory concentration of the drug is raised, tolerant organisms have virtually unchanged minimal inhibitory concentrations, but greatly elevated minimal bactericidal concentrations. In the streptococci, tolerance does not appear to be associated with change in the PBPs (Gutmann and Tomasz, 1982; Daneo-Moore and Pucci, 1982) but is instead probably dependent on autolytic enzyme function and regulation (Daneo-Moore and Pucci, 1982). In the future, with the increased use of β-lactamase stable antibiotics, tolerance of and resistance to β-lactams by alterations in PBPs may become as important clinically as resistance mediated by production of β-lactamases (Spratt, 1983).

Tetracycline

Tetracycline resistance is widely distributed among various Gram-negative and Gram-positive species. Among the Gram-negative bacteria, tetracycline resistance genes are usually located on plasmid molecules and resistance is inducible by low levels of the drug, although high level resistance in Proteus mirabilis and E. coli can be chromosomally encoded (Levy, 1981; George and Levy, 1983). On the basis of resistance phenotypes and DNA - DNA hybridisation studies, the tetracycline determinants have been divided into various classes designated A to E (Mendez et al., 1980; Levy, 1981) and recently, Marshall and coworkers (1983) have demonstrated yet another distinct determinant class in enteric bacteria. Of the different determinants, class B, represented by transposon (Tn) 10, is found most frequently among faecal coliforms and Haemophilus species (Levy, 1981; Marshall et al., 1983) while class A determinants, Tn 1721 and RP1, are more common among Pseudomonas and Aeromonas (Marshall et al., 1983).

There has been no demonstration of homology, either by an in vivo transformation assay or DNA hybridisation studies, between tetracycline

resistance determinants of Gram-negative and Gram-positive bacteria (Smith et al., 1981; Burdett et al., 1982a; Eccles and Chopra, 1984) or between determinants of different Gram-positive genera (Burdett et al., 1982a; Polak and Novick, 1982). Tetracycline resistance genes of Staph. aureus are commonly located on small, multi copy plasmids and resistance is expressed inducibly (Iordanescu et al., 1978; Schafferman et al., 1978). Likewise small plasmids encoding tetracycline resistance have been isolated from several species of Bacillus (Polak and Novick, 1982). In contrast, tetracycline resistance in the streptococci, although very common, is frequently chromosomally encoded (Burdett, 1980). A detailed account of streptococcal tetracycline determinants will be given in following sections.

The mechanism of tetracycline resistance has been investigated in detail in derivatives of E. coli, and it is accepted that resistance is due mainly to decreased accumulation of the drug (Foster, 1983). Levy and McMurray (1978a) showed that cells containing tetracycline resistance plasmids exhibited a different energy dependent uptake system from that of sensitive cells and furthermore, that class A to D determinants all encoded active efflux of tetracycline after induction of resistance (McMurray et al., 1980). Ball et al., (1980) found that resistant cells bound less tetracycline and demonstrated a rapid energy dependent efflux of the drug when the bacteria were transferred to drug free medium. The efflux system was shown to be saturable at levels of drug which did not saturate the influx system of sensitive cells, had different pH and magnesium ion requirements and was competitively inhibited by minocycline (McMurray et al., 1980, 1982; Levy, 1981). Therefore, it seems that the plasmid encoded efflux is not simply a reversal of endogenous host influx systems. It has been suggested that decreased accumulation of tetracycline is not sufficient explanation for the differences in sensitive and resistant strains and that protein synthesis is less sensitive in resistant strains of E. coli (Levy and McMurray, 1978b) and Staph. aureus

(Sompolinsky and Krausz, 1973). However, there is no direct evidence for reduced ribosomal sensitivity or for intracellular inactivation of tetracycline (Levy, 1981).

Molecular analyses and DNA sequence data have shown similar structural organisation of the genes involved in tetracycline resistance expression of class A, RP1 and Tn 1721 (Altenbuchner et al., 1983) class B, Tn 10 (Hillen and Schollmeier, 1983) and class C, pSC101 (Stuber and Bujard, 1981). There are at least two tetracycline inducible proteins specified by Tn 10 - a 23,500 dalton repressor protein encoded by the tetR gene and a 43,300 dalton membrane bound TET protein, which migrates with an apparent molecular weight of 36,000 in sodium dodecyl sulphate polyacrylamide gels, encoded by a structural gene composed of two intracistronic complementation groups, tetA and tetB, which represent different domains of the TET protein (Curiale et al., 1984). The repressor and structural genes are transcribed divergently from a common intercistronic regulatory region, which is negatively autoregulated by the repressor protein (Altenbuchner et al., 1983; Hillen and Schollmeier, 1983; Hillen et al., 1984). The deduced amino acid sequences of class A, B and C repressor proteins show significant homology clustered at the amino-terminal three quarters with sequence homology as high as 80% (Postle et al., 1984; Unger et al., 1984). Additionally, the amino terminal region shows significant homology with the DNA recognition regions of other DNA binding proteins such as cro and λ repressors of bacteriophage λ and the lac repressor protein (Postle et al., 1984). Functional TET repressor is a dimer which binds to the operators of TET in a stoichiometry of four repressors to one control region (Altschmied and Hillen, 1984) resulting in 15- to 60-fold and 6- to 15-fold repression of β-galactosidase in tetA-lacZ and tetR-lacZ gene fusions respectively (Bertrand et al., 1984). During induction of resistance, tetracycline binds to the repressor resulting in loss of repressor-operator DNA binding ability (Unger et al., 1984). The

repressor has a stronger binding affinity for tetracycline than for DNA and it is conceivable that one function of repressor could be to sequester drug until the active efflux system is operational (Hillen et al., 1984).

Among the Gram-positive genera, the inducible tet determinant of a Bacillus plasmid pAB124 has been cloned in E. coli where it expressed constitutive resistance by reduced accumulation as with class A to D determinants and was shown to encode a 32,000 dalton membrane located TET protein (Eccles and Chopra, 1984). The tet region was found by DNA-DNA hybridisation studies to be homologous to another Bacillus plasmid pBC16 but not to any representatives of class A to class D. The sequence of a small staphylococcal tetracycline resistant plasmid has been determined by Khan and Novick (1983). They have identified a structural gene encoding an inducible TET protein of molecular weight 35,000 daltons and identified very tentatively a regulatory gene encoding a possible repressor protein of 18,000 daltons. These genes appear to be transcribed unidirectionally and the authors suggest that induction is controlled by a translational attenuation mechanism dependent on the potential secondary structures of the RNA message.

Aminoglycoside-aminocyclitol antibiotics

Resistance to this group of antibiotics can arise from spontaneous mutations, affecting transport of the drugs or causing alterations in the ribosome target site, and drug inactivation. Additionally, as described in the previous section, anaerobic bacteria and facultative organisms grown anaerobically are intrinsically resistant to aminoglycosides due to the absence or low activity of an effective uptake system (Bryan et al., 1979; Bryan and Kwan, 1981). This probably also explains the intrinsic low level resistance of enterococci.

Naturally occurring mutations to aminoglycoside resistance are not isolated frequently in clinical situations. Important exceptions are strains

of P. aeruginosa described by Bryan (1975, 1976) which were found to be resistant to gentamicin and streptomycin due to defective drug transport systems resulting, at least in part, from reduced permeability caused by changes in lipopolysaccharides (Bryan et al., 1984). Ribosomal resistant mutants can, however, be readily selected in the laboratory and usually have alterations in one or more ribosomal proteins necessary for aminoglycoside binding (Gale et al., 1981). Natural isolates due to such mutations are rare but nevertheless have been described in clinical isolates of N. gonorrhoeae (Maness et al., 1974), streptococci (Farber et al., 1983; Eliopoulos et al., 1984), Staph. aureus (Lacy and Chopra, 1972) and P. aeruginosa (Tseng et al., 1972).

The vast majority of clinical isolates resistant to aminoglycosides produce enzymes which modify the drugs. An important feature of aminoglycoside modifying enzymes is that, in contrast to other inactivating enzymes, there is no gross inactivation of the antibiotics in the culture medium (Davies and Benveniste, 1974; Davies and Smith, 1978; Courvalin et al., 1980a). The enzymes, which are synthesised constitutively, are located possibly in the periplasmic space of Gram-negative organisms (Dickie et al., 1978; Davies and Smith, 1978) or in the cytoplasm of Gram-negative (Perlin and Lerner, 1981) and Gram-positive organisms where they may be loosely associated with the cytoplasmic membrane and therefore "strategically placed" to inactivate incoming antibiotic (Foster, 1983). The result of enzymic modification is the inability of the drug to bind to the ribosome target site and hence inhibit protein synthesis (Davies and Kagan, 1981). Concomitant with the failure to bind to ribosomes, is the lack of onset of the second energy-dependent phase of drug accumulation (EDP11) which has been implicated in the lethal action of aminoglycosides (Bryan and Kwan, 1983).

However, if the rate of transport of an antibiotic in EDP1 exceeds the rate of inactivation, some antibiotic will bind to ribosomes causing protein

synthesis inhibition and such strains therefore appear sensitive to the antibiotic although possessing a modifying enzyme. Thus, resistance is due to a balance between the rate of uptake and the rate of inactivation of the antibiotic (Courvalin and Carlier, 1981; Bryan and van den Elzen, 1977).

Most aminoglycoside modifying enzymes are specified by plasmids (Davies and Smith, 1978; Courvalin and Carlier, 1981) and by transposable elements (Davies and Kagan, 1981; Foster and Kleckner, 1980) although chromosomally determined enzymes have been described in strains of Staph. aureus (Kayser et al., 1981), Serratia marcescens (John et al., 1982) and P. aeruginosa (Okii et al., 1983). The enzymes can be divided into three classes depending on the reaction catalysed. These are 1. aminoglycoside phosphotransferases (APH) which phosphorylate a hydroxyl group at the expense of ATP, 2. aminoglycoside nucleotidyltransferases (AAD) which catalyse the transfer of the adenyl moiety of ATP to a hydroxyl group of the antibiotic and 3. aminoglycoside acetyltransferases (AAC) which catalyse the transfer of acetate from acetyl-CoA to an amino group on the antibiotic and they are further subdivided according to the particular site which they modify on the antibiotic molecule. Thus, AAC(6') acetylates the 6'-amino groups of aminohexose I of susceptible drugs. Additionally, an enzyme can be bifunctional, for example the enzyme encoded by a Staph. aureus plasmid which has APH2" and AAC6' activities (Ubukata et al., 1984). The substrate range of the enzymes can be broad and so, due to overlap, different activities can modify the same molecule. For example, kanamycin B can be modified by seven different enzymes - AAC(3), AAC(2'), APH(3'), AAD(4')(4"), AAC(6'), AAD(2") and APH(2"). Davies and Smith (1978), Courvalin and Carlier (1981) and Foster (1983) are among those who have extensively reviewed the aminoglycoside modifying enzymes.

Chloramphenicol

High level resistance to chloramphenicol due to chromosomal mutations has not been encountered (Gale et al., 1981). The major mechanism of resistance to the drug in Gram-negative and Gram-positive bacteria is acetylation and thus inactivation by chloramphenicol acetyltransferase (CAT) which is usually determined by a plasmid or transposon. This has been the subject of a recent detailed review by Shaw (1983). However, another mechanism which is plasmid encoded and inducible by sub-inhibitory concentrations of the antibiotic, sometimes accounts for resistance in Gram-negative strains. It does not involve drug inactivation or ribosomal resistance but is thought to be caused by a barrier to drug permeability in the cytoplasmic membrane (Gaffney et al., 1981; Dorman and Foster, 1982).

Organisms which produce CAT are widespread possibly reflecting the large number of soil organisms which are capable of producing chloramphenicol or its analogues (Shaw, 1983). The enzymes are tetramers of identical subunits which have molecular weights of 22,000 to 26,000 and can be classified into various types on the basis of electrophoretic mobility, kinetic data, susceptibility to inhibitors and reactivity with antisera (Foster, 1983).

Three types of plasmid specified CAT have been described in enteric bacteria (Gaffney et al., 1978). Type I has been the most extensively studied and is the most common type encountered, probably due to the fact that genes for CAT type I are located on a transposable element, Tn9 (Matthews et al., 1983; Shaw, 1983). The type I determinant has also been shown to confer fusidic acid resistance in fusidic acid sensitive mutants of E. coli due to sequestation of the drug by the CAT protein, thus preventing antibacterial activity (Proctor et al., 1983). All the CAT enzymes of Gram-negative bacteria, with the exception of that produced by Agrobacterium, are expressed constitutively. H. influenzae, H. parainfluenzae and Bacteroides fragilis produce plasmid encoded CATs similar to type II (Shaw et al., 1978;

Roberts et al., 1982), while B. ochraceus CAT resembles type I (Britz and Wilkinson, 1978; Shaw, 1983). Novel chromosomally determined CATs are produced by species of Flavobacterium and Agrobacterium (Zaidenzaig et al., 1979).

Among the Gram-positive bacteria, five types of CAT have been described - types A to D and a fifth CAT specified by pC194 (Sands and Shaw, 1973; Fitton and Shaw, 1979; Wilson et al., 1981) - and unlike the Gram-negative CATs, all are inducible. The CATs of different species of streptococci appear to be related to those of the staphylococci (Courvalin et al., 1978). CATs are also produced by Clostridium perfringens, Bacillus pumilus and Streptomyces species (Shaw, 1983).

The mechanism of inactivation of chloramphenicol appears to be the same for all CATs and indeed, comparison of primary amino acid sequences has shown conservation of certain amino acids in the proposed catalytic site (Shaw et al., 1979; Horinouchi and Weisblum, 1982a; Harwood et al., 1983). The first step in the acetylation process couples the breaking of the thiol-ester bond of acetyl-S-CoA with the formation of a 3-acetoxy derivative of chloramphenicol. This monoacetate undergoes non-enzymic intramolecular rearrangement to form the 1-acetoxy derivative which is then acetylated at the 3-hydroxyl position, again at the expense of acetyl-S-CoA, to form 1,3-diacetoxy-chloramphenicol (Shaw, 1967). Since none of the acetoxy derivatives of the drug binds to ribosomes or has antibiotic activity (Shaw and Unowsky, 1968), a metabolic consequence of the need for diacetylation can be a decrease in the growth rate and the level of resistance expressed when acetyl-S-CoA is limiting (Nordstrom et al., 1972).

Constitutive synthesis of type I CAT in E. coli has been shown to be subject to catabolite repression (Harwood and Smith, 1971). Studies of the cat gene in an in vitro transcription-translation system showed that both

cyclic AMP and catabolite activator protein (CAP) were necessary for maximal rate of CAT production required for high level resistance (de Crombrugghe et al., 1973). From analysis of the cat gene DNA sequence and DNase protection experiments, two cyclic AMP-CAP binding sites were identified (Le Grice et al., 1982). Only the site overlapping the cat promoter is involved in the regulation of transcription probably by interaction with RNA polymerase.

The regulation of inducible CAT synthesis in Gram-positive organisms has been studied using the small staphylococcal plasmid pC194 and the cloned chromosomal determinant (cat-86) of B. pumilus (Horinouchi and Weisblum, 1982a; Harwood et al., 1983), induction of which has been shown to be independent of the promoter used to activate the gene (Mongkolsuk et al., 1984). Identification of a 37-base pair inverted repeat sequence between the likely start of transcription and the translation initiation codon of pC194 led Horinouchi and Weisblum (1982a) and Shaw (1983) to propose that cat gene transcription was controlled autogenously by the CAT protein interacting with the "hairpin" loop formed by the inverted repeat, either as a repressor in the absence of or inducer in the presence of chloramphenicol. However, it was shown that the level of pC194 CAT specific mRNA was unchanged by induction (Byeon and Weisblum, 1984), although the opposite was true of cat-86 (Duvall et al., 1984), and study of expression by derivatives deleted in all or part of pC194 or cat-86 structural genes, excluded the possibility of the direct participation of CAT in its own regulation (Byeon and Weisblum, 1984; Mongkolsuk et al., 1984). Nevertheless, the importance of the inverted repeat sequences in regulation was shown by isolation of pC194 constitutive variants deleted in all or a functional part of the inverted repeat (Ambulos et al., 1984) and the demonstration, by lacZ gene fusions, that the region essential to induction was the 144-base pair segment between the promoter and structural cat-86 gene (Mongkolsuk et al., 1984) which also contains an inverted repeat sequence

capable of sequestering the ribosomal binding site in a stem-loop formation (Duvall et al., 1983).

Currently, it is thought that CAT synthesis is regulated by a mechanism similar to translational attenuation, although unlike other such mechanisms, alternative stem-loops are not possible (Duvall et al., 1983; 1984; Byeon and Weisblum, 1984). The mechanism by which ribosomes could block stem-loop formation or destabilise the structures are unknown. Comparison of the sequence of the stem-loop of pC194 mRNA with an inverted complementary repeat sequence found in 23S rRNA showed an exact match over a nine nucleotide sequence which included the paired region containing the ribosome binding site (Byeon and Weisblum, 1984). It was therefore proposed that as a result of binding of chloramphenicol to the 50S ribosome subunit, the nine nucleotide sequence of the 23S rRNA became accessible to bind to the nascent CAT mRNA during its synthesis thus freeing the ribosome binding site to act with 16S RNA in the 30S subunit. For synthesis of CAT, a free 50S subunit would have to replace that bound to chloramphenicol. Alternatively, the demonstrable sequence homology, upstream from the inverted repeats, with the extreme 3'-end of 16S rRNA could permit binding of the 30S ribosomal subunit with subsequent association of a chloramphenicol modified 50S subunit. The physical interaction of this "dead-end complex" at a precise location upstream of the inverted repeat could, during transcription, prevent stem-loop formation (Horinouchi and Weisblum, 1982a; Mongkolsuk et al., 1984).

Macrolide-lincosamide-streptogramin B antibiotics

Gram-negative organisms are generally intrinsically resistant to clinically achievable levels of MLS antibiotics, either because of impermeability of the outer membrane or because of the level of methylation of adenine residues in 23S ribosomal RNA (Costerton and Cheng, 1975; Tanaka and Weisblum, 1975).

One exception to this is the genus Bacteroides which is susceptible to and often treated with the lincosamide, clindamycin (Bartlett et al., 1972).

High level resistance of Gram-positive bacteria to the MLS group is usually specified by plasmids, particularly in the genera Staphylococcus and Streptococcus where the determinants may be located on a transposon - Tn551 of Staph. aureus plasmid pI258 (Novick et al., 1979) and Tn917 of S. faecalis plasmid pAD2 (Tomich et al., 1980). In addition, plasmid determined resistance has been reported in clinical isolates of Bacteroides (Welch et al., 1979; Rotimi et al., 1981), coryneform bacteria (Schiller et al., 1980) and clostridia (Brefort et al., 1977) while chromosomal resistance genes have been described in B. licheniformis (Docherty et al., 1981) and S. pneumoniae (Engel et al., 1980). On the basis of DNA-DNA hybridisation studies, Ounissi and Courvalin (1982) have described four classes of MLS resistance loci. Class A is the most frequently encountered and includes most of the streptococcal and many staphylococcal determinants. Class B is comprised of staphylococcal plasmids pE194 and pE5 while classes C and D are represented by the loci of B. licheniformis and Bacteroides respectively. However, comparison of the deduced amino acid sequences of the MLS determinant proteins representing class A (pAM77) and class B (pE194) showed that half the amino acids were identical in sequence indicating a common origin (Horinouchi and Weisblum, 1982b).

Resistance to MLS antibiotics is due to N^6,N^6-dimethylation of adenine in 23S ribosomal RNA (Graham and Weisblum, 1979; Ranzini and Dubin, 1983) probably resulting in a conformational change which prevents binding of the drugs (Shivakumar and Dubnau, 1981). The RNA methylase encoded by the ermC gene of staphylococcal plasmid pE194 is a 29,000 dalton protein which methylates 50S ribosomal subunits or 23S RNA but not 70S ribosomes (Shivakumar and Dubnau, 1981). A RNA methylase has also been identified as the product of the B. licheniformis MLS resistance locus (Docherty et al., 1981).

Expression of resistance can be constitutive or inducible although the differences in regulation do not correlate with differences in classes (Ounissi and Courvalin, 1982). The regulation of inducible resistance by pE194 has been intensively studied and the complete nucleotide sequence of this plasmid is known (Horinouchi and Weisblum, 1982c). Two open reading frames encoding the 29,000 dalton methylase preceded by a 2,700 dalton control or leader peptide have been found to be essential for expression of resistance. In this system, only erythromycin and the closely related oleandomycin can act as inducers and it has been shown that, as induction can occur in the absence of transcription, regulation must be posttranscriptional (Horinouchi and Weisblum, 1980; Shivakumar et al., 1980). Each open reading frame is preceeded by a ribosome binding site and associated with these sites are appropriately positioned translation initiation codons. Within the leader region and overlapping the 5'-end of the methylase structural gene is a series of six repeated sequences which have the potential to form various hairpin loop structures in the ermC message (Hahn et al., 1982). The translation attenuation model proposes that in the absence of drug, the conformation of the messenger RNA is such that only the leader peptide is translated and that the ribosome binding site and initiation codon for the methylase are masked by secondary structure. In the presence of sub-inhibitory concentrations of erythromycin, drug binds to ribosomes which begin to translate the leader peptide. However, nascent peptidyl-tRNA formation is blocked by the antibiotic at a critical length and the result of this ribosome stalling is thought to be a conformational change in the RNA message which exposes the methylase ribosome binding site and initiation codon thus allowing methylase translation (Horinouchi and Weisblum, 1980; 1982c; Hahn et al., 1982).

Studies of ermC-lacZ gene fusions cloned into E. coli have shown that ermC retains the ability to be induced in the new host (Kirsch and Lai, 1984). Partial deletion of the methylase structural gene, leaving the putative 5'-

attenuator resulted in an increase of β-galactosidase activity related to the increase in erythromycin concentration but when functional ermC was introduced, significantly less β-galactosidase was produced at comparable drug concentrations. This effect was thought to be due to methylation of ribosomes causing negative feedback inhibition.

GENE TRANSFER IN THE GENUS *STREPTOCOCCUS*

Transformation

Natural transformation systems have been described in streptococci, including group F (LeBlanc *et al.*, 1978b), *S. sanguis* (Ranhand, 1974; Davidson *et al.*, 1976; Westergren, 1982) and *S. mutans* (Perry and Kuramitsu, 1981; Perry *et al.*, 1983), as well as other bacterial genera such as *Bacillus* (Dubnau, 1976) and *Haemophilus* (Stuy, 1962; Sisco and Smith, 1979) but much of the present understanding of the process of transformation, however, has come from studies of *S. pneumoniae*.

The ability to take up extracellular DNA is not a permanent property but is dependent on a competent state acquired for a short period during the late logarithmic growth phase when the cells reach a particular density (Tomasz and Hotchkiss, 1964). Unlike the *E. coli* transformation system in which cells are made competent by $CaCl_2$ treatment and heat shock (Cosloy and Oishi, 1973), streptococcal competence is a natural process dependent on extracellular activators. The activators or competence factors are small basic proteins (Tomasz and Mosser, 1966; Leonard and Cole, 1972) which bind to cell membrane proteins and signal the onset of competence (Ziegler and Tomasz, 1970). Mutants defective in achieving spontaneous competence have been isolated by insertion-duplication mutagenesis using the erythromycin resistance gene of pAMβ1 and, although the exact nature of the defect is unknown, they probably fail to produce or produce inactive extracellular competence factor (Morrison *et al.*, 1984). In *S. sanguis*, the production of competence factor has been found to be dependent on the strain and the medium used and is inversely related to the presence of competence factor inactivator (Gaustad, 1983).

The competent state in *S. pneumoniae* has been shown to be associated with a change in the pattern of proteins synthesised by a culture with at least 16 new polypeptides being made (Morrison and Baker, 1979; Morrison *et al.*, 1982).

Other changes associated with competence, which may be secondary to the redirection of cellular metabolism, include appearance of a novel surface antigen, altered cell wall structure leading to the unmasking of an agglutinin, leakage of DNA and nucleases, increased autolysis and a requirement for choline in the cell wall (Lacks, 1977).

Following the development of competence, uptake of extracellular DNA is possible and a generally accepted model for uptake in S. pneumoniae has been proposed by Lacks (1977). Double-stranded DNA binds to specific sites on the surface of competent cells and is accompanied by random single strand breakage or nicking. A membrane located nuclease, the major endonuclease, is necessary for subsequent entry of donor DNA. The enzyme produces a double strand break opposite the nick and processive action of the endonuclease results in internal single-stranded DNA and an equivalent amount of extracellular oligonucleotides derived from the complementary strand. In this original model, the requirement for double-stranded DNA was absolute and it was suggested that entry of one strand resulted passively and processively from the alternate attachment and hydrolysis of the complementary strand by the nuclease. This DNA translocation did not however explain the essential requirements for calcium ions and an energy source such as glucose (Seto and Tomasz, 1974; 1976). Moreover, recent work has shown that single-stranded phage-plasmid hybrid molecules are capable of transforming S. pneumoniae, albeit with greatly reduced efficiency (Barany, 1982; Barany and Boeke, 1983). While not excluding the possibility that the mechanisms of uptake of single- and double-stranded DNA may be different, these authors suggest that membrane potential or calcium gradient generated by fermentation of glucose could provide the energy of DNA uptake. Recently,it has been shown in H. influenzae that the transformation frequency of homologous DNA increased with the proton motive force and DNA uptake could be driven with either the electrical potential or pH gradient components (Bremer et al., 1984).

On uptake by S. pneumoniae, donor DNA undergoes an eclipse of transforming activity due to its single-stranded nature. In this state before integration into the recipient chromosome, it is found to be associated with the major competence specific protein in a nuclease resistant eclipse complex (Morrison and Mannarelli, 1979; Vijayakumar and Morrison, 1983). Similar complexes have been described in S. sanguis (Raina and Ravin, 1978) and the protein component of these complexes is thought to have a role in transport, protection against nuclease digestion and, possibly, in recombination (Raina et al., 1979; Vijayakumar and Morrison, 1983). Integration of single-stranded donor DNA into the recipient chromosome can occur where there are regions of significant homology (Fox and Allen, 1964; Mejean and Claverys, 1984) but if no homology exists or if the segment is too short (Morrison and Guild, 1972), the DNA is usually degraded (Cato and Guild, 1968; Lacks et al., 1967). In S. pneumoniae, integration efficiency has been shown to be dependent on the markers or mutations carried by the donor DNA and is under the control of the Hex system which recognises and corrects base mismatches during donor-recipient heteroduplex formation (Claverys et al., 1982; 1983).

Transformation with heterologous plasmid DNA was first demonstrated in a streptococcal system by LeBlanc and Hassel (1976) when they successfully transformed the Challis strain of S. sanguis using the S. faecalis MLS resistance plasmid, pAMβ1 (but not using the tetracycline resistance plasmid pAMα1 which is unable to replicate in S. sanguis; Ranhand and LeBlanc, 1984). The β plasmid could be isolated from S. sanguis as a covalently closed molecule but velocity gradient analysis showed that the plasmid had undergone deletion. Subsequent studies of plasmid transformation of S. sanguis and S. pneumoniae showed that uptake occurred by a mechanism similar to chromosomal DNA and thus closed circular plasmid DNA is bound, randomly linearised by nucleases and enters the cell as a single strand (Barany and Tomasz, 1980; Saunders and Guild, 1981a,b; Behnke, 1981). Restoration of a circular plasmid requires

annealing of complementary strands originating from two different donor molecules and deletions are thought therefore to occur when annealing between a unit length molecule and a shorter complementary fragment results in long stretches of single-stranded DNA. Intrastrand annealing within the single-stranded regions at points of local homology would produce deletions (Behnke, 1981). The production of deletions has been exploited in order to obtain miniplasmid derivatives suitable as molecular cloning vehicles (Macrina et al., 1980; 1982; Behnke et al., 1982) and to localise biological functions on the physical maps of the parent plasmids (Malke, 1981; Behnke and Gilmore, 1981).

Plasmid transformation of B. subtilis has been shown to be due to multimeric forms of closed circular plasmids with monomeric forms being inactive (Canosi et al., 1978; Mottes et al., 1979). In contrast, S. sanguis and S. pneumoniae can be transformed with multimeric and monomeric forms (Barany and Tomasz, 1980; Saunders and Guild, 1981a; Macrina et al., 1981), the former transforming with first-order kinetics whilst the latter follow second-order kinetics, reflecting the need for two independently derived single strands to produce a closed circular molecule in the recipient. Furthermore, it has been shown that open circular and linear molecules are also active although with reduced efficiency (Saunders and Guild, 1981b; Behnke, 1981). Unique linear forms produced by restriction endonucleases are inactive due to a lack of overlapping homologous sequences which prevents circularisation but transforming activity is restored when linear molecules generated by different endonucleases are mixed.

The fact that transformation with monomeric plasmid DNA is a second-order process in S. sanguis and the low overall efficiency of plasmid establishment in both S. sanguis and S. pneumoniae has led to problems in 'shotgun' cloning of chromosomal fragments and construction of gene libraries (Macrina et al., 1981; Lacks et al., 1982). In S. sanguis, the likelihood of transformation either by multimers or monomers carrying the identical insert

is extremely low and annealing of only partly homologous molecules most likely leads to mispairings resulting in deletions (Behnke et al., 1982). To obviate the problem, a helper plasmid cloning system has been developed in which the recipient cell contains a resident homologous plasmid which participates in the recombinational "rescue" of chimeras with shared homology (Tobian and Macrina, 1982; Macrina et al., 1982; Malke and Holm, 1982). In S. pneumoniae the efficiency of transformation has been greatly increased by the use of recombinant plasmids carrying DNA segments homologous to the chromosome (Lacks et al., 1982; Lopez et al., 1982).

A polyethylene glycol induced transformation system has recently been described in group N S. lactis by Kondo and McKay (1982). Using protoplast recipients, they have been successful in transforming a lactose-negative strain to lactose fermenting ability with S. lactis plasmids ranging in size from 35 to 55kb. Moreover, transformation to erythromycin resistance has been demonstrated with the S. faecalis plasmid pAMβ1 and the S. sanguis cloning vector pGB301, the latter indicating the usefulness of the transformation system for cloning plasmid encoded genes in S. lactis (Kondo and McKay, 1982; 1984).

Conjugation

Conjugal transfer in streptococci was first reported by Tomura et al., (1973) with a haemolysin-bacteriocin determinant although direct evidence of plasmid involvement was not given. Reports by Jacob and coworkers (1974; 1975) followed, describing a strain of S. faecalis subspecies zymogenes which contained two plasmid species - pJH1 encoding multiple antibiotic resistance and pJH2 encoding haemolysin-bacteriocin production. Both plasmids were transmissable to a plasmid free recipient strain during mixed incubation in nutrient broth by a process which required viable donor cells and was resistant to DNase I treatment. In addition, no transfer was observed using

cell free filtrates of donor and no evidence of bacteriophage in the donor strain could be found. The authors therefore concluded that cell-to-cell contact was necessary for transfer and that such transfer occurred by conjugation.

Numerous conjugative plasmids in S. faecalis have now been described which encode a variety of functions including antibiotic resistance (Van Embden et al., 1977; Marder and Kayser, 1977; Horodniceanu et al., 1979a,b; Courvalin et al., 1980a), resistance to ultraviolet light (Frazier and Zimmerman, 1980; Miehl et al., 1980) and production of haemolysin and bacteriocin (Dunny and Clewell, 1975; Oliver et al., 1977; Borderon et al., 1982). In the industrially important group N lactic streptococci, transfer by conjugation of metabolic plasmids specifying protease production or ability to ferment lactose, as well as plasmids encoding bacteriocin production, has been demonstrated (McKay et al., 1980; Walsh and McKay, 1981; Neve et al., 1984). Conjugative transfer of antibiotic resistance has also been reported in streptococci of group A (Malke, 1979), group B (Horodniceanu et al., 1979a; Burdett, 1980) and groups C and G (Bougueleret et al., 1981). Certain of these plasmids, noteably those encoding MLS resistance, have been shown to have a broad host range and are capable of intergroup transfer (LeBlanc et al., 1978; Malke, 1979; Engel et al., 1980; Gasson and Davies, 1980) as well as intergeneric transfer to lactobacilli (Gibson et al., 1979) staphylococci (Engel et al., 1980; Schaberg et al., 1981; 1982) and bacilli (Landman et al., 1980). In addition, it has been shown that certain conjugative plasmids can mobilise non-conjugative plasmids (Dunny and Clewell, 1975; Tomich et al., 1979; Burdett, 1980; Smith et al., 1980) and chromosomal markers (Franke et al., 1978). Indeed, in order to circumvent the inability of S. faecalis to take up DNA by transformation, a technique has been developed in which a non-conjugative S. sanguis-E.coli shuttle vector is mobilised to an S. faecalis recipient via cointegrate formation with a conjugative plasmid (Smith and Clewell, 1984).

The mechanism by which conjugal transfer occurs in streptococci is poorly understood. Unlike the well documented role of pili in mating pair formation of the classical F plasmid conjugation system of E. coli (Clark and Warren, 1979; Bradley, 1981) no correlation between possession of fimbriae and a conjugative plasmid has been observed (Handley and Jacob, 1981). Krogstad and coworkers (1980) reported intercellular "connections" during conjugation in S. faecalis but it was not clear whether these connections were artefacts of the preparation or true examples of conjugal contacts (Clewell, 1981).

In order to detect transfer of the majority of conjugative streptococcal plasmids, it is necessary to carry out matings under conditions which enforce cell-to-cell contact. This is usually obtained by collecting a mixture of donor and recipient cells on a membrane filter which is subsequently incubated on nutrient agar. In S. faecalis, however, certain large plasmids (53 to 70 kilobase pairs; kb) have been described which transfer with high frequency in mixed broth cultures. These plasmids commonly encode haemolysin and bacteriocin production, although some antibiotic resistance plasmids have been reported (Clewell et al., 1980a; Dunny et al., 1981a,b), and have a narrow host range, in that transfer occurs only to S. faecalis recipients (Dunny et al., 1978; Clewell et al., 1980a; Clewell, 1981). A study of the physiological conditions affecting transfer has shown that recipient growth phase has no effect on transfer but maximum efficiency is obtained using early exponential phase donor cells in the ratio of 10^7 donors to 10^8 recipient cells per ml (Dunny et al., 1982a). Plasmid transfer in this unique system is characterised by the appearance of mating aggregates after 40 to 120 minutes growth in mixed culture (Dunny et al., 1978). It was subsequently shown that incubation of donor cells in the presence of a cell free filtrate of recipient cells induced auto-aggregation and that, whereas plasmid transfer normally required a 2 to 4 hour incubation period, high frequency transfer could be

obtained after just 10 minutes incubation with an induced donor culture (Dunny et al., 1978; 1979; 1981b). The substance responsible for the donor aggregation response was shown to be a soluble, protease sensitive, heat stable protein or peptide of molecular weight around 1000 daltons, excreted by potential recipient strains and referred to as a clump inducing agent (CIA) or sex pheromone (Dunny et al., 1978). Quantitation of CIA activity could be achieved by measuring the highest serial twofold dilution of culture filtrate which produced clumping of donor cells (Dunny et al., 1978) and the titre was shown to be variable usually between 4 and 64 depending on the plasmid carried by the donor and the donor strain itself (Dunny et al., 1981b; 1982a). The production of CIA by recipient strains closely followed cellular growth during log phase and reached a stable maximum as the cells entered stationary phase (Dunny et al., 1979) although in the case of the liquefaciens subspecies of S. faecalis, CIA activity rapidly decreased at stationary phase presumably due to the proteolytic activity of these strains. This was confirmed by the isolation of a protease-negative mutant which maintains the maximum level of CIA (Ike et al., 1983).

Further studies showed that recipient strains produced multiple CIAs, specific for a particular class of plasmid, and that acquisition of a given plasmid appeared to "shut off" production of the corresponding CIA only, while allowing the cell to become responsive to exogenous pheromone (Dunny et al., 1979; Clewell et al., 1980a). Recently it has been shown that the apparent shutting off of pheromone production actually reflects plasmid encoded modification of endogenous CIA (Ike et al., 1983). Plasmid containing isogenic derivatives of recipient cultures were found to produce a substance which inhibited specifically the action of CIA and comparison of the molecular weights of the CIA with the corresponding inhibitor indicated that the latter was the CIA which had undergone a chemical addition of 350 to 400 daltons. That the inhibitor was in fact a modified form of CIA and not a novel substance, was

confirmed by demonstration of activity regeneration on treatment with phosphodiesterase II and by the fact that a mutant, defective in CIA production, also failed to produce inhibitor. The inhibition of CIA by the modified CIA is thought to result from competition for a binding site or transport system on the donor cell surface.

In the model of aggregation proposed by Dunny and coworkers (1979) and Ike and Clewell (1984), the response of the donor cell to exogenous CIA is the activation of synthesis of an aggregation substance (AS), encoded either chromosomally, or more likely by the plasmid, which would recognise an as yet unidentified, chromosomally determined and constitutively expressed binding substance (BS) located on the cell surface of both donor and recipient cells. Regulation of AS synthesis is assumed to be under the control of a plasmid located gene which specifies a repressor while another plasmid locus is thought to be involved in the inactivation of chromosomally determined pheromone. Using Tn917 to generate insertion mutations in the haemolysin-bacteriocin plasmid pAD1, Ike and Clewell (1984) have investigated the molecular basis of the clumping response. Several types of mutation were obtained, some of which resulted in constitutive clumping i.e. constitutive synthesis of AS, and which were found by restriction endonuclease analysis to cluster in two regions separated by 1.7kb; *traA* (1.5kb), mutants of which had a "dry" colony morphology and *traB* (1.3kb), mutants of which had a normal colony morphology. Neither mutant type produced the active pheromone cAD1 but instead produced the corresponding inhibitor suggesting that no insertions were obtained in the putative pheromone inactivation determinant and it was postulated that *traA* and *traB* products may be involved in negative regulation of AS synthesis.

Studies have shown that induction of the aggregation response requires RNA and protein synthesis (Dunny *et al.*, 1978) and the presence of phosphate and divalent cations at physiological pH (Yagi *et al.*, 1983). The requirement for these ions may reflect stabilisation of binding by modulation of the

repulsive effects of the net negative charge on the bacterial surface. Antiserum raised against an induced donor strain carrying a conjugative plasmid has been shown to block aggregation by binding to a pheromone induced surface antigen (Kessler et al., 1982; Yagi et al., 1983). This novel antigen appears as a dense amorphous layer on the surface of induced cells when viewed by immunofluorescence and immunoperoxidase and it is considered to be the AS of the above model (Yagi et al., 1981; Kessler and Yagi, 1983). The AS is a protein with an apparent molecular weight of 78,000 (Kessler et al., 1982; Kessler and Yagi, 1983). Interestingly, specific antiserum prepared against induced cells harbouring one conjugative plasmid, readily cross-reacted with AS induced in strains harbouring different plasmids which make use of different pheromones. Thus, despite differences in pheromone specificity, the ASs produced by different strains have significant structural similarity (Yagi et al., 1983).

In addition to induction of AS, pheromones appear to elicit other functions related to conjugation. Investigating donor-donor matings between isogenic strains containing derivatives of the same conjugative plasmid - one with a tetracycline resistance transposon insert and the other with an erythromycin resistance transposon insert - Clewell and Brown (1980) demonstrated that plasmid transfer was enhanced primarily in the direction of induced to uninduced cells regardless of which donor was induced. This indicated that the pheromone, in addition to AS, induced an as yet uncharacterised preparation for plasmid transfer. Induction of both donors prior to mating resulted in reduced transfer frequency and was thought to be suggestive of induced surface exclusion functions. The authors proposed that CIA induction induces a polycistronic operon, which encodes several functions related to transfer, in a system analagous to that described for the tra operon of Gram-negative organisms (Clark and Warren, 1979).

Aggregation and associated high frequency transfer by conjugation has also been reported in strains of S. lactis (Gasson and Davies, 1980; Walsh and McKay, 1981). The transfer of plasmid DNA in group N strains occurs normally at low frequency and only when matings are carried out on membrane filters or the surface of agar (McKay et al., 1980). However, after transfer of a plasmid specifying ability to utilise lactose, Gasson and Davies (1980) reported that a large percentage of lactose utilising transconjugants exhibited aggregation and subsequent high frequency transfer of lactose genes in broth culture. The aggregation response was different from that of S. faecalis in that the original donor did not aggregate and no sex pheromone could be demonstrated. Further studies by Walsh and McKay (1980) showed that lactose metabolism (in the parental strain mediated by a 55kb plasmid), aggregation and high frequency conjugation were associated with the acquisition of a 104kb plasmid. Improved plasmid isolation techniques showed that the 104kb plasmid was also present in parental strains but at very low copy number (0.06 copies per cell) and restriction endonucleases analysis indicated that it was a cointegrate molecule composed of the 55kb lactose plasmid pSK08 and a co-resident 48.4kb plasmid pRS01 (Anderson and McKay, 1984). The recombinant molecule was generated as an intermediate in the transposition of a 0.8 to 1.0kb insertion element on pSK08 to pRS01. The aggregation response was found to be associated with an inversion element on pRS01 of 4.3kb and a contiguous region spanning a total of 23.1kb, aggregation being dependent on the orientation of the inversion element and probably also on gene dosage.

Conjugation in the absence of plasmids

The conjugal transfer of streptococcal antibiotic resistance determinants in the absence of extrachromosomal DNA was first reported by Shoemaker and coworkers (1980) in two clinical isolates of S. pneumoniae which had previously been found to be resistant to tetracycline and chloramphenicol (Miyamura et al.,

1977; Dang-Van et al., 1978). Analysis of transformation and physical data, obtained from velocity and dye-buoyancy experiments, showed that the cat and tet genes were contained in adjacent heterologous insertions (4 to 8kb and greater than 30kb respectively) in the chromosome (Shoemaker et al., 1979). The transfer of these genes was demonstrated at low frequency to pneumococcal but not S. faecalis recipients by a process which required cell-to-cell contact on membrane filters and which was DNase resistant (Shoemaker et al., 1980; Smith and Guild, 1980). The process thus resembled conjugation and was distinguished from transformation by a number of criteria - 1. the yield of transconjugants was unaffected by the Hex status of the recipient, 2. transfer was independent of the membrane endonuclease necessary for entry of transforming DNA, 3. transfer of point mutations to streptomycin and erythromycin resistance which efficiently transformed recipients was completely abolished by the presence of DNase, and 4. chloramphenicol resistance was never transferred alone although this was the most frequent event in transformation. No plasmid DNA could be detected in parental or transconjugant strains and introduction of a conjugative plasmid by transformation had little or no effect on the transfer of the chromosomal resistances.

Similar DNase resistant transfer from multiple resistant S. pneumoniae to pneumococcal and group D recipients has been described (Buu-hoi and Horodniceanu, 1980). Again no plasmids could be detected and the chromosomal location of resistance genes has been confirmed in one of the strains (Guild et al., 1981).

The possible mechanisms of transfer have been discussed by Guild et al., (1982). They have rejected the possibility of Hfr-like mobilisation since transconjugants could all retransfer resistances and no mobilisation of the chromosome could be detected. Excision of a plasmid intermediate also seems unlikely as the efficiency of transfer of insertions is not decreased when the recipient strain possessed an in vivo restriction system which was shown

to restrict conjugative transfer of plasmids. Instead, they postulate transfer of a single strand of the insert followed by integration at sites of flanking homology or an as yet obscure transposition process.

In S. faecalis, low frequency transfer on membrane filters of a chromosomally encoded tetracycline resistance determinant was described by Franke and Clewell (1980;1981). Transfer required viable donor cells and was DNase resistant. The data indicated that the determinant was located on a 15kb transposon, designated Tn916, which was capable of transposition to different conjugative haemolysin plasmids introduced into the host cell as well as transfer to a plasmid free recipient. Insertion into these plasmids caused, in some cases, inactivation and, in others, hyperexpression of haemolysin suggesting that the transposon could insert into multiple sites. By analysis of hybridisation profiles of transconjugants, it was subsequently shown that Tn916 could also insert into different sites in the recipient chromosome (Clewell, 1981; Gawron-Burke and Clewell, 1982). The fact that digested recipient chromosomal DNA from different transconjugants showed different patterns when hybridised to a Tn916 probe argued against the possibility that the determinant was located on a plasmid which had escaped physical detection. Both transposition and transfer were found to be independent of the host recombination system (Franke and Clewell, 1981; Clewell, 1981).

Certain tetracycline resistant transconjugants were isolated which exhibited increased transfer frequencies in secondary matings. Introduction of a haemolysin plasmid into these strains followed by analysis of the frequency of occurrence of the hyperhaemolytic phenotype during secondary matings showed that transposition was similarly increased, indicative of a common step(s) in Tn916 transposition and transfer (Gawron-Burke and Clewell, 1982). Studies involving the transfer of Tn916 located on a conjugative erythromycin resistance plasmid showed that the transposon was excised precisely and at high frequency in the recipient strain (Gawron-Burke and Clewell, 1982).

Excision resulted either in transconjugants in which the resistances were no longer linked, i.e. Tn916 integrated into the recipient chromosome, or in the loss of tetracycline resistance, and was possibly related to zygotic induction of Tn916 recombination enzymes. The model of Tn916 behaviour suggested by Gawron-Burke and Clewell (1982) proposed that spontaneous excision occurs at low frequency from the bacterial chromosome and that the element forms a circular molecule incapable of vegetative replication. Excision is accompanied by the induction of enzymes which allow the transposon to 1. re-insert into the chromosome either at the original or at a new site, 2. insert into a resident plasmid, 3. transfer into a recipient strain or 4. segregate. Conjugation therefore could be achieved by the same mechanism used by plasmids, with zygotic induction of enzymes to allow insertion into the recipient chromosome.

Recently, Nida and Cleary (1983) have described the conjugative transfer of Tn916 to a group A streptococcal isolate and showed that the transposon could insert in multiple sites of the chromosome and that some insertions resulted in inactivation of the determinant for production of the cytolytic toxin, streptolysin S. Tetracycline sensitive revertants regained the ability to produce streptolysin S, confirming the observation of Gawron-Burke and Clewell (1982) that excision is precise. Precise excision of Tn916 from a pBR322 derived vector in *E. coli* has also been demonstrated (Gawron-Burke and Clewell, 1984) although insertion of the transposon into the *E. coli* chromosome was rare. In contrast, introduction of chimeric DNA containing Tn916 into *S. sanguis* resulted in frequent transposition, sometimes to multiple sites, in the host chromosome. These results suggest the suitability of Tn916 for targeting the cloning genes of Gram-positive bacteria by insertional mutagenesis and, following cloning of the DNA sequence containing Tn916, functional integrity of the gene of interest could be regained by the precise excision of the transposon.

The extent of homology of tetracycline resistance determinants has been investigated using an in vivo transformation assay (Smith et al., 1981) based on the ability of tetracycline determinants from different sources to transform a tetracycline sensitive point mutation in the chromosomal insertion of an S. pneumoniae isolate. Two distinct groups emerged - those determinants which were unable to transform the mutant to tetracycline resistance and were of plasmid origin and those which transformed with high efficiency and were chromosomally encoded. Among the latter group were all the S. pneumoniae clinical isolates tested, Tn916 and three strains of group B including that which has since been shown to carry a transposon (Smith and Guild, 1982). These results lend support to the idea that some resistance genes of S. pneumoniae are located on conjugative transposons.

Transfer of antibiotic resistance has been reported in a number of other streptococcal groups including groups A, B, F, G (Horodniceanu et al., 1981) and D (S. faecium and S. bovis) and in viridans streptococci (Le Bouguenec and Horodniceanu, 1982; Horodniceanu et al., 1982a,c). The transfer has been shown to be at low frequency on membrane filters and resistant to the presence of DNase although plasmid free transfer has only been inferred by the lack of physical evidence for extrachromosomal DNA in most of these strains and their transconjugants. Recent investigations by Smith and Guild (1982) of one of the group B strains have indicated however that the resistance genes are carried on a chromosomal transposable element with properties similar to Tn916. In contrast, one group G strain (Horodniceanu et al., 1981) and a strain of S. mutans (LeBlanc et al., 1982) have been shown to contain conjugative elements integrated into the chromosome which are capable of autonomous replication on transfer to recipient cells. The absence of plasmid DNA in a donor strain does not therefore necessarily indicate that resistance determinants are located on the conjugative transposons of Franke and Clewell (1980; 1981).

Transduction

Bacteriophage mediated transfer of genetic traits such as antibiotic resistance and ability to produce haemolysin or to ferment lactose has been shown to occur in streptococcal groups A, C, G (Wannamaker, 1982) and N (McKay et al., 1973), and possibly in pneumococci (Parker et al., 1979) but has never been demonstrated in group B or D streptococci (Clewell, 1981). Among the group A S. pyogenes, production of the streptococcal exotoxin responsible for the characteristic rash of scarlet fever, is known to be associated with a specific group of temperate bacteriophage which have the ability to convert nontoxigenic strains to toxin production (Nida and Ferretti, 1982). The mechanism of this toxigenic phage conversion is however unclear as is the location (chromosomal or phage DNA) of the gene specifying the exotoxin (Ferretti et al., 1982).

The transfer of chromosomal streptomycin and erythromycin resistance determinants by the virulent transducing phage A25 has been demonstrated between group A (Leonard et al., 1968; Malke, 1970a) and between group C strains (Wannamaker et al., 1973), as well as intergroup transfer from group A to group G strains (Colon et al., 1972) and from group C to group A strains (Wannamaker et al., 1973). Transductional analysis of chromosomal antibiotic resistance loci in multiple resistant group A streptococci has been useful in establishing that there are three distinct linkage groups - spectinomycin, erythromycin, spiramycin; streptomycin, fusidic acid, bacitracin, kasugamycin; rifampicin, streptolydigan (Malke, 1972; Stuart and Ferretti, 1978). That transduction could be important in the spread of naturally occurring antibiotic resistance among streptococci was demonstrated by Malke (1975) using a group A isolate which was lysogenic for the transducing phage P13234mo and which carried an MLS resistance plasmid, ERL1. Phage mediated transfer of ERL1 was not only demonstrated between group A strains but was subsequently shown to occur between groups A and C, and groups A and G (Malke et al., 1975) and

phage A25 mediated transduction of ERL1 was reported from a group A strain to group G and back to group A (Skjold et al., 1979). In addition to antibiotic resistance, transduction of other markers, such as resistance to ultraviolet light (Malke, 1970b) and production of the cytolytic toxin, streptolysin S (Skjold et al., 1982), have been reported in group A streptococci. Furthermore, it has been shown that the production of M protein, which confers virulence to group A strains by virtue of its antiphagocytic properties, is positively controlled by the specific integration of a temperate bacteriophage (Spanier and Cleary, 1980; Cleary and Spanier, 1982).

Studies of intraspecies (S. lactis to S. lactis) and interspecies (S. cremoris to S. lactis) transduction in group N streptococci using temperate phage induced from donor strains have been profitable for identification of plasmids involved in lactose metabolism (Klaenhammer and MacKay, 1976; MacKay et al., 1976; Snook et al., 1981). Often, transduction of plasmid to lactose defective recipients has resulted in deletions due to a limitation in size of the DNA packaged in the phage head (McKay et al., 1973; Snook et al., 1981) and such deleted plasmids were transduced at elevated frequency in subsequent transduction experiments (Klaenhammer and McKay, 1976). Gasson (1982) has shown that the lactose plasmid of S. lactis 712 undergoes spontaneous deletion and has postulated that transducing phage merely select deleted plasmids of appropriate size which would account for the increased transduction frequency in strains harbouring already deleted plasmids. Occasionally, lactose-positive transductants have been isolated which contain larger molecular weight plasmids than the parent strain probably resulting from recombination between an incoming transduced lactose plasmid and a deleted lactose-negative derivative in the recipient. Transductants which have not acquired detectable plasmid DNA have also been described presumably due to the integration of lactose genes into the chromosome (McKay and Baldwin, 1979; Snook et al., 1981).

Although there have been several reports describing the bacteriophage of pneumococci (Porter and Guild, 1976; Bernheimer, 1979; Lopez et al., 1982), there has been little exploitation of phage mediated gene transfer in this group. A study by Porter and coworkers (1979) investigated the transfer of chromosomally located mutations to antibiotic resistance by a natural phage isolate. The process resembled generalised transduction in that unlinked markers of donor cell DNA were packaged in phage structures which adsorbed to recipient cells and, in this form, the DNA was resistant to the action of DNase. However, unlike the phage infection process, entry of donor markers did not require phage but instead was dependent on development of recipient cell competence using the endonuclease dependent pathway of transformation.

ANTIBIOTIC RESISTANCE GENES OF STREPTOCOCCI

Tetracycline resistance genes

Surveys of antibiotic resistance have shown that tetracycline resistance is commonly encountered in streptococci (Finland, 1979; Dixon and Lipinski, 1982) although, unlike the situation in Gram-negative bacteria, the majority of determinant loci do not appear to be located on plasmid molecules. An investigation of 30 group B clinical isolates found that only three strains possessed plasmids encoding tetracycline resistance (Burdett, 1980). Of these, two were small, nonconjugative, multicopy plasmids which could be mobilised by the introduction of a conjugative plasmid, and one was a large conjugative plasmid. The remaining strains had no detectable plasmid and were unable to transfer tetracycline resistance. A similar lack of plasmid located tetracycline resistance has been reported in other studies of group B streptococci (Horodniceanu *et al.*, 1979a) as well as oral streptococci (Hawley *et al.*, 1980), group D species *S. faecalis* (van Embden *et al.*, 1977) and *S. faecium* (Le Bouguenec and Horodniceanu, 1982) and groups A, B, F and G streptococci (Horodniceanu *et al.*, 1981).

The failure to detect and identify tetracycline resistance plasmids in many streptococcal strains has led to the conclusion that the determinants are chromosomally located although, as some have been shown to be transferable by conjugation on membrane filters, the possibility remains that the genes could be located on plasmids refractory to isolation. However the chromosomal location of certain tetracycline resistance determinants has been established by transformation studies as for the 30kb *tet* insertion of *S. pneumoniae* (Shoemaker et al., 1979), by studies of transposition with, for example, Tn916 of *S. faecalis* (Franke and Clewell, 1981) and the resistance determinants of the group B strain *S. agalactiae* B109 (Smith and Guild, 1982), and from cloning experiments involving an *S. mutans* tetracycline resistance determinant (Tobian and Macrina, 1982).

On the basis of location and transfer characteristics, streptococcal tetracycline resistance determinants can be divided into five groups. First are those determinants located on small, multicopy, non-conjugative plasmids which can be mobilised by other conjugative plasmids, examples of which are pMV158 and pMV163 of S. agalactiae (Burdett, 1980) and pAMα1 of S. faecalis strain DS5 (Clewell et al., 1974; Dunny and Clewell, 1975). The latter plasmid has been the subject of some interest since the resistance gene has the ability to undergo amplification in the presence of tetracycline, resulting in generation of tandem repeats (Clewell et al., 1975). The determinant was shown to be located on a 4.2kb segment flanked by homologous direct repeat sequences of 380 base pairs referred to as RS1 sequences (Yagi and Clewell, 1977) and it was postulated that amplification was due to recombination, either intra- or inter-molecular, between RS1 sequences (Clewell and Yagi, 1977). Concomitant with amplification was a decrease in plasmid copy number (Yagi and Clewell, 1976) suggesting a copy number control mechanism sensitive to the total plasmid mass. Amplification was found to be reversible and in addition tetracycline sensitive variants, having deleted the 4.2kb segment, could be obtained although neither amplification nor deletion occurred in a recombination deficient host (Yagi and Clewell, 1980). A recent report by Perkins and Youngman (1983) has shown that the 4.2kb segment of pAMα1, designated pAMα1Δ1, could function as an autonomous replicon in B. subtilis and confer tetracycline resistance. Further, it was shown by restriction mapping that pAMα1Δ1 was almost identical to the B. cereus tetracycline resistance plasmid, pBC16 and showed extensive homology with a Staph. aureus kanamycin resistance plasmid pUB110.

The second group of streptococcal tetracycline determinants are those located on large conjugative plasmids which can confer tetracycline resistance alone, for example the 53kb pCF10 of S. faecalis strain SF-7 (Dunny et al., 1981b) and the 45.5kb pMV120 of S. agalactiae (Burdett, 1980), or can also specify resistance to other antibiotics such as the 80.7kb plasmid pJH1 of

S. faecalis strain JH1 and the 115kb plasmid pJH5 of S. faecalis strain JH6 (Jacob and Hobbs, 1974; Courvalin et al., 1978). A study of the host range of conjugative tetracycline resistance plasmids by Horodniceanu and coworkers (1982b) showed that strains of groups D and B could act as recipients. Some plasmids such as pMV120 also transferred at low frequency to groups A, B, C, G and H recipients and another plasmid, p1P685, transferred into a Staph. aureus recipient. However, in these latter examples, no plasmid DNA could be detected in recipient strains suggesting that the tet genes had become integrated into the chromosome.

A third set of determinants are those, located on the host chromosome, which are incapable of transfer, such as those commonly encountered in strains of group B streptococci (Horodniceanu et al., 1979a; Burdett, 1980) and other streptococcal groups (Bougueleret et al., 1981; Horodniceanu et al., 1982c).

The fourth class are those determinants which again are chromosomally encoded and incapable of autonomous replication but which are transferable by a conjugation-like mechanism. Such loci are often linked to other antibiotic resistances with which they can co-transfer and have been described in groups A, B, F and G streptococci and S. pneumoniae (Shoemaker et al., 1979; Buu-hoi and Horodniceanu, 1980). Some, for example Tn916 of S. faecalis and the chromosomal insertion of S. agalactiae strain B109, which specifies tetracycline, chloramphenicol and erythromycin resistance, have been shown to be capable of transposition to multiple sites of other replicons (Gawron-Burke and Clewell, 1982; 1984; Smith and Guild, 1982). It remains uncertain how many other conjugative chromosomal determinants are located on transposable elements.

A possible fifth group is typified by the tetracycline resistance determinant of S. mutans strain DL5 described by LeBlanc et al. (1982). It appears to be a 12kb element, inserted into the host chromosome, which is

conjugative and, on transfer, capable of autonomous replication in S. faecalis and S. mutans recipient strains.

Strains may contain more than one type of determinant. For instance, S. faecalis strain JH1 harbours two conjugative plasmids, pJH1 conferring resistance to tetracycline, streptomycin, kanamycin, gentamicin and MLS antibiotics and pJH2 (58kb) encoding haemolysin and bacteriocin production (Jacob and Hobbs, 1974). In addition to the tetracycline resistance locus on pJH1, LeBlanc and Lee (1982) have described another tetracycline resistance determinant, integrated into the chromosome, which differed in both resistance level and regulation of expression. The pJH1 associated determinant specified constitutive resistance to 40µg tetracycline per ml. On transfer of tetracycline resistance, the majority of transconjugants were haemolytic, co-resistant to kanamycin, streptomycin and erythromycin and contained two plasmids which corresponded to pJH1 and pJH2 on agarose gels. A small proportion however harboured a single co-integrate plasmid of 138.7kb. In a subsequent study, transconjugants were obtained which were haemolytic and constitutively resistant to 40µg tetracycline per ml but susceptible to other pJH1 associated resistances. These strains were shown by restriction endonuclease analysis, to contain hybrid plasmids composed of pJH2 into which were inserted fragments originating from pJH1 (Banai and LeBlanc, 1983). Together with information obtained from study of spontaneous deletions of pJH1 determinants, the authors have constructed a physical and functional map of the plasmid.

The presence of a second tetracycline resistance determinant in strain JH1 was suspected from the observations that transconjugant strains containing pJH1 did not express a resistance level as high as the donor and that pJH1 cured derivatives of JH1 remained resistant to 80µg tetracycline per ml (LeBlanc and Lee, 1982). On mating in broth culture, one transconjugant

was obtained which was tetracycline resistant but susceptible to kanamycin, streptomycin and erythromycin and was haemolytic only when tetracycline was included in the blood agar. In contrast to transconjugants containing pJH1, tetracycline resistance in this strain was inducible. Restriction endonuclease analysis and DNA-DNA hybridisation studies revealed that the isolate contained plasmid pJH2 into which was inserted a 17.8kb fragment which shared no homology with pJH1 but was homologous with a fragment of chromosomal DNA from strain JH1 and its pJH1 cured derivative. The chromosomal locus may therefore represent another example of a resistance transposon like the conjugative Tn916, which is also capable of transposition into the haemolysin plasmid of its host.

Burdett and coworkers (1982a) have investigated the molecular relatedness of tetracycline determinants by DNA-DNA hybridisation studies using probes constructed from the small, non-conjugative plasmid pMV163 and 5kb chromosomal fragment from S. agalactiae strain B109. Both probes were shown to contain the sequences necessary for expression of tetracycline resistance when cloned in either orientation into E. coli. They have identified three distinct streptococcal loci on the basis of DNA hybridisation data. The tetL locus, carried by the non-conjugative plasmids pMV163, pMV158 and pAMα1 and by the conjugative plasmid pJH1, specifies constitutive resistance to tetracycline but not to the related antibiotics, minocycline and chelocardin. The tetM determinant located on strain B109 chromosomal DNA hybridised to chromosomal DNA fragments from several other S. agalactiae strains which have been found capable of tetracycline resistance transfer in the apparent absence of plasmids, S. pneumoniae and S. faecalis strain DS16 which contains Tn916. This probe also showed homology to plasmid pAM211 which carried Tn916 and a large (97kb) conjugative S. faecalis plasmid p1P614. Preliminary work reported by LeBlanc and Lee (1982) suggested homology with the chromosomal resistance locus of S. faecalis strain JH1 which, like that of strain B109, conferred inducible resistance to tetra-

cycline and minocycline. A chromosomal S. mutans tetM gene has been cloned on a 2.8kb fragment in E. coli where resistance was expressed constitutively, and minicell studies have identified two proteins (33,000 and 35,000 daltons), encoded by S. mutans DNA, one or both of which are involved in expression of tetracycline resistance (Tobian et al., 1984). The third class of determinant, tetN, was identified on the basis of complete lack of homology with either probe. Only one member of this class, the conjugative plasmid pMV120, has been described and has been shown to confer constitutive resistance to tetracycline and minocycline. No homology was seen on hybridisation of either probe to any of the four tetracycline determinants of E. coli, plasmid pT181 of Staph. aureus or plasmid pBC3106 from B. sphaericus. The results of the DNA hybridisation studies correlate well with the study by Smith et al. (1981) which investigated homology by the ability of various tet loci to transform a S. pneumoniae point mutation to tetracycline resistance (see section on transformation).

A study of the distribution of tet classes in streptococci (Burdett et al., 1982b) showed that of 31 tetracycline resistant,plasmid containing S. faecalis from human and animal sources, all reacted with the tetL probe and 30 also reacted with the tetM probe. Among 13 S. agalactiae strain tested, one failed to react with either probe, 2 contained tetL and tetM and the rest contained tetM determinants. This finding is in accordance with previous observations with group B strains in which plasmid located tet genes are rare (Burdett, 1980) but suggests that in S. faecalis, plasmid encoded tetracycline resistance is more common.

Preliminary work on the mechanism of tetracycline resistance in streptococci has indicated that tetL exhibits drug efflux typical of that observed with Gram-negative genes but that there is no difference in tetracycline accumulation in sensitive cells and those containing tetM or tetN suggesting a non-efflux resistance mechanism (Burdett, 1984).

Aminoglycoside resistance genes

Streptococci, in particular those of group D, are intrinsically resistant to low levels (< 250µg per ml) of aminoglycoside antibiotics probably due to inefficient transport of the drugs as a result of poor oxidative membrane energisation (Chopra and Ball, 1982). As in Gram-negative organisms and certain Gram-positive genera such as staphylococci, most streptococcal high level resistance to aminoglycosides (>2000µg per ml) is dependent on the constitutive production of drug modifying enzymes (Courvalin et al., 1978; 1980a) which detoxify the drugs on entry to the cell and prevent ribosome binding (Bryan and Kwan, 1983). Notable exceptions to this are the streptomycin resistant viridans streptococci described by Farber and coworkers (1983) and clinical enterococcal isolates studied by Eliopoulos et al. (1984). No streptomycin modifying enzymes could be demonstrated but it was found that polypeptide synthesis by ribosomes isolated from these strains was resistant to inhibition by the drug. Unlike enzymic inactivation, ribosomal resistance to streptomycin was probably not plasmid associated and in enterococci, was shown to confer considerably higher MIC values.

All three classes of modifying enzymes - phosphotransferases (APH), nucleotidyltransferases (AAD) and acetyltransferases (AAC) - have been identified in streptococci and comparison of parameters such as substrate range, molecular weight and isoelectric point have shown that enzymes with identical site specificity from staphylococci and streptococci are very similar but different from the corresponding enzymes of Gram-negative bacteria (Courvalin et al., 1980b; Courvalin and Carlier, 1981). In addition, certain enzyme subclasses have been described in the Gram-positive cocci which have not been detected in Gram-negative organisms. The enzymes described in streptococci therefore appear to represent a subset of the staphylococcal enzymes suggestive of a potential for exchange of these resistance determinants within the Gram-positive cocci (Carlier and Courvalin, 1982).

Among clinical isolates of S. faecalis, determinants encoding high level aminoglycoside resistance by enzymic modification have been shown to be transmissible (Marder and Kayser, 1977; Horodniceanu et al., 1979b; Murray et al., 1983) and in several studies have been associated with conjugative plasmids. Horodniceanu et al. (1979b) described several plasmids in strains of S. faecalis which conferred high level resistance to gentamicin, kanamycin and chloramphenicol. Physical characterisation of the plasmid pIP800 from one of these strains, showed that it was a 66.7kb conjugative plasmid and, from substrate profile studies, encoded two enzyme activities, APH (2") and AAC (6') (Courvalin et al., 1980a). On transfer to a group D recipient, one transconjugant was obtained which was sensitive to aminoglycosides and contained a plasmid with a 2.8kb deletion. Other S. faecalis and S. faecium strains have been isolated which contain conjugative plasmids specifying resistance to kanamycin, streptomycin, erythromycin and tetracycline (pJH1, 80.7kb; Jacob and Hobbs, 1974), kanamycin, streptomycin and erythromycin (pJH4, 39.4kb; Courvalin et al., 1978) and kanamycin, streptomycin, erythromycin, tetracycline and chloramphenicol (pJH5, 115kb; Courvalin et al., 1978). The plasmids were shown to encode the enzymes APH (3')-III and AAD (3") (9) and in hybridisation studies, a complementary RNA probe generated from an APH (3')-III encoding Staph. aureus plasmid was found to be homologous to DNA fragments of all three plasmids but not to aminoglycoside resistance plasmids of Gram-negative organisms (Courvalin et al., 1980b). The kanamycin and streptomycin resistance determinants of pJH1 have been localised to a 16.5kb fragment and although resistance to the antibiotics is mediated by the APH (3')-III and AAD (3") (9) enzymes respectively, natural segregation of the resistances has never been observed (Banai and LeBlanc, 1983). Streptomycin and kanamycin resistance genes of the non-conjugative S. faecalis plasmid pAD2 have however been cloned separately into S. sanguis and were located on 1.8kb and 2.3kb fragments respectively (Clewell et al., 1982a). The APH (3') (5")-III gene of pJH1 has also been cloned in E. coli on a 1.5kb fragment

and the sequence determined (Trieu-Cuot and Courvalin, 1983). The deduced gene product had a molecular weight of 29,200 daltons and comparison of the deduced amino acid sequence with those of Tn903 representing APH 3'5"-I and Tn5 representing APH 3'-II, both from Gram-negative bacteria, indicated a significant structural relationship despite the lack of DNA hybridisation. By extrapolation, the S. faecalis enzyme must also be related to that produced by Streptomyces fradiae (a neomycin producer) since it is also structurally similar to Tn903 and Tn5 encoded enzymes (Thompson and Gray, 1983) which suggests a common evolutionary origin for these resistance genes.

Transferable resistance to kanamycin and streptomycin has also been reported in a survey of S. faecium strains (Le Bouguenec and Horodniceanu, 1982). Donor strains were co-resistant to MLS antibiotics and tetracycline and some were also chloramphenicol resistant. Analysis of kanamycin resistant transconjugants revealed that in some cases, no plasmid DNA could be detected while in others, small plasmids (18-25kb) were isolated. Yet other transconjugants were obtained which showed co-transfer of one or more donor resistance markers. However, no firm evidence, such as the ability to retransfer, was given in this study to link resistance to plasmids and results could be interpreted as transfer of chromosomally encoded resistance with coincidental transfer of a cryptic plasmid. Transfer of high level resistance to streptomycin and kanamycin to group D recipients in the absence of detectable plasmid has been reported from strains of group A, B, G, D and viridans streptococci (Horodniceanu et al., 1982a). High level, chromosome borne resistance to kanamycin due to synthesis of an APH (3') (5")-IIIenzyme with an apparent molecular weight of 32,500 daltons has been reported in S. pneumoniae (Carlier and Courvalin, 1982; Collatz et al., 1984) and after cloning of the aminoglycoside and associated MLS resistance genes into E. coli it was found that the determinants were located on a 18.5kb element (designated Tn1545) capable of transposition.

The host range of aminoglycoside resistance plasmids from group D streptococci has been investigated using recipient strains representing groups A, B, C, D, G. H and S. pneumoniae as well as Staph. aureus and L. innocua (Horodniceanu et al., 1982b). In contrast to the broad host range displayed by MLS resistance plasmids (Buu-hoi et al., 1984), transfer of aminoglycoside resistance plasmids could be demonstrated only to group B and D recipients. In one instance, that of p1P1075 specifying gentamicin and kanamycin resistance, no plasmid DNA could be isolated after transfer to a group B recipient and retransfer from this recipient was not observed suggesting that the determinants for resistance had become inserted in the chromosome of the new host.

High level aminoglycoside resistance is of particular importance in therapy of serious streptococcal infections such as endocarditis when it is necessary to use synergistic combinations of aminoglycosides and β-lactam antibiotics. Studies using labelled streptomycin showed that antibiotics which affected cell wall synthesis such as penicillin and vancomycin, caused increased aminoglycoside uptake and that this was the basis for antibiotic synergism (Moellering and Weinberg, 1971). Strains which carry determinants for high level aminoglycoside resistance are therefore resistant also to antibiotic synergism (Calderwood et al., 1977; Krogstad and Moellering, 1982; Murray et al., 1983). Recently, a strain of S. faecalis has been isolated which is insensitive to synergism of all possible combinations of penicillin and commercially available aminoglycoside antibiotics (Combes et al., 1983) due to high level aminoglycoside resistance which was found to be transferable and conferred by four modifying enzymes. Thus, the strain possessed a single mechanism of resistance to streptomycin, probably AAD (6) but possibly AAD (3") (9), two mechanisms of resistance to gentamicin APH (2") and AAC (6') and three mechanisms of resistance to kanamycin , APH (2") APH (3') (5") -III and AAC (6').

Chloramphenicol resistance genes

Resistance to chloramphenicol has been reported in a number of streptococcal groups. The determinants are usually found in association with other resistance genes and are often located on plasmids. Examples of such plasmids in S. faecalis are pJH5, a large (115kb) conjugative plasmid which also encodes resistance to kanamycin, streptomycin, tetracycline and MLS antibiotics (Courvalin et al., 1978) the conjugative 39.4kb plasmid pFK14 encoding co-resistance to streptomycin and MLS antibiotics (Marder and Kayser, 1977) and p1P800 (Courvalin et al., 1980a) also designated p1P655 (Horodniceanu et al., 1979b) a 66.7kb conjugative plasmid also specifying resistance to gentamicin and kanamycin. Transfer of these plasmids has been demonstrated to group D recipients and p1P800 has also been shown to transfer to a group B recipient but to no other streptococcal groups (Horodniceanu et al., 1982b). Only one S. faecalis plasmid, p1P686 (68kb), resistant to chloramphenicol alone and capable of transfer only to group D recipients has been described (Horodniceanu et al., 1982b). Chloramphenicol resistance, possibly plasmid encoded, has also been found to be transferable from strains of S. faecium to S. faecalis (Le Bouguenec and Horodniceanu, 1982) always with co-transfer of other resistance determinants.

In a study of antibiotic resistant group B streptococci strains resistant to chloramphenicol only did not transfer the determinant (Horodniceanu et al., 1979a). Another strain B117, co-resistant to tetracycline, transferred both resistances but only when selection was done on tetracycline and only to a group B recipient. Further study demonstrated transfer of both resistance genes from this strain to group B, C and G receipients but no plasmid DNA could be detected (Horodniceanu et al., 1982c). Similar transfer of chloramphenicol in association with other resistance loci and in the absence of detectable plasmid DNA has been reported from strains of groups A and G to various streptococcal recipients (Horodniceanu

et al., 1981; 1982c). S. agalactiae B109, also originally described by Horodniceanu et al. (1979a) has subsequently been shown to transfer by conjugation its chromosomal chloramphenicol-tetracycline-erythromycin resistance insertion to groups A, B, C, D, G and H (Horodniceanu et al., 1982c) and the 60kb insertion has been shown to transpose to the haemolysin-bacteriocin plasmid pAD1 (Smith and Guild, 1982).

In addition to apparently chromosomally located determinants, chloramphenicol encoding plasmids have been described in group B strains. These plasmids p1P501, p1P612 and p1P635 also encode MLS resistance and have the typical broad host range and size (28-35kb) of other MLS plasmids (Horodniceanu et al., 1979a; 1982c; Buu-hoi et al., 1984). The chloramphenicol resistance gene of p1P501 has been shown to be expressed in Staph. aureus (Schaberg et al., 1982) and species of Pediococcus (Gonzalez and Kunka, 1983) as well as many streptococcal groups and from cloning experiments into S. sanguis, the determinant has been localised to a 6.3kb fragment of the plasmid (Evans and Macrina, 1983). MLS plasmid associated chloramphenicol resistance has also been reported in a group A (Horodniceanu et al., 1982c) and a group G strain (Bougueleret et al., 1981), the former being non-conjugative and the latter capable of transfer to group D recipients only. Comparison of the restriction endonuclease patterns of these and other chloramphenicol-MLS plasmids with plasmids encoding MLS resistance only, showed that they were very similar but chloramphenicol encoding plasmids contained a 6.2kb fragment not found in MLS plasmids (Horodniceanu et al., 1982c).

In strains of S. pneumoniae, initial reports suggested that chloramphenicol resistance could be plasmid encoded (Miyamura et al., 1977; Dang-Van et al., 1978) but transformation analysis and co-sedimentation studies by Shoemaker and coworkers (1979) demonstrated that the determinant formed part of a heterologous chromosomal insertion. The chloramphenicol resistance

gene was located on a 4 to 8kb fragment and closely associated with a large insertion (30kb) encoding tetracycline resistance. Both determinants were capable of conjugative transfer on membrane filters to S. pneumoniae but not S. faecalis recipients (Shoemaker et al., 1980). Chloramphenicol resistant transconjugants were always co-resistant to tetracycline but the reverse was not true, suggesting that the insertion encoding chloramphenicol lacked the necessary genetic information for self-transfer.

As in other genera, the mechanism of chloramphenicol resistance in streptococci, regardless of the location of the determinants, has been shown to be di-acetylation of the molecule via 1- and 3-monoacetylated intermediates catalysed by the enzyme chloramphenicol acetyltransferase (CAT; Shaw, 1967). Like the CAT enzymes of staphylococci, but in contrast to those of enterobacteria, the streptococcal CATs are inducible by sub-inhibitory concentrations of chloramphenicol and by its biologically inactive analogue 3-deoxychloramphenicol (Courvalin et al., 1978; Robins-Brown et al., 1979). A comparison of CATs produced by a group A strain, S. pneumoniae and S. faecalis showed that the enzymes were identical in respect of molecular weight (75,000-80,000 daltons) pH optimum and heat stability (Miyamura et al., 1977) and as in other bacterial CATs, the streptococcal enzymes have been found to be homomeric, composed of four subunits of approximately 22,000 daltons (Dang-Van et al., 1978; Zaidenzaig et al., 1979). Immunological studies have shown that the enzymes produced by group B and D strains cross-react with anti-sera to staphylococcal CAT but not with anti-serum to E. coli enzymes (Courvalin et al., 1978; Zaidenzaig et al., 1979) and the close relationship of staphylococcal type D CAT to that specified by S. faecalis plasmid pJH6 was confirmed by demonstration of identical electrophoretic mobility (Courvalin et al., 1978). On the basis of biological, physical and immunological properties therefore, the enzymes produced by staphylococci and streptococci appear to be closely related although the extent of DNA sequence

homology of the determinants has not yet been investigated.

Macrolide-lincosamide-streptogramin B resistance determinants

Although only one mechanism of MLS resistance is known, that of dimethylation of adenine residues in 23S RNA (Weisblum, 1975), the regulation of resistance expression in streptococci can be of three types. Among the first streptococcal plasmids to be described, the S. faecalis plasmid pAMβ1 exhibits constitutive resistance (Yagi et al., 1975). Constitutive expression of MLS resistance has also been described in group A (Malke, 1974; Dixon and Lipinski, 1982), group B (Horodniceanu et al., 1979a; Dixon and Lipinski, 1982) and S. pneumoniae (Carlier and Courvalin, 1982). An inducible mode of resistance has been demonstrated in strains of group A (Malke, 1974; Yagi et al., 1975) group B (Horodniceanu et al., 1979a), group D (Corb and Murray, 1977), group H (Yagi et al., 1978) and S. pneumoniae (Weisblum et al., 1979) in which the maximum level of expression of MLS resistance is observed only after prior exposure to sub-inhibitory concentrations of the inducer erythromycin or lincomycin. Common in group A strains, and also encountered in groups B, C and G, is the zonal pattern of resistance to lincomycin (Dixon and Lipinski, 1982) which is characterised by growth in sub-inhibitory concentrations of lincomycin (below 0.05μg per ml) and between 50 to 200μg per ml but not in intermediate concentrations. A study by Malke and coworkers (1981b) of group A plasmid encoded zonal resistance showed that pre-growth in sub-inhibitory concentrations of erythromycin not only induced erythromycin resistance but also induced growth in intermediate concentrations of lincomycin while low concentrations of lincomycin failed to induce erythromycin resistance or growth in intermediate lincomycin concentrations. Single step mutations giving rise to either constitutive erythromycin resistance or constitutive lincomycin resistance could be obtained but generalised constitutive resistance required sequential two step mutations.

Conjugative MLS resistance plasmids have been reported in many groups of streptococci including group A (Clewell and Franke, 1974; Malke, 1979), group B (Horodniceanu et al., 1976; 1979a; Hershfield, 1979), groups C and G (Bougueleret et al., 1981) and group D (Clewell et al., 1974; El-Solh et al., 1978; Engel et al., 1980). The plasmids are similar in size, 25 to 30kb and, in contrast to most other streptococcal plasmids, exhibit an extraordinarily broad host range. One such plasmid, pAMβ1, was originally identified and characterised in S. faecalis strain DS5 which also harbours pAMα1 encoding tetracycline resistance and a conjugative haemolysin-bacteriocin plasmid, pAMγ1 (Clewell et al., 1974). It was originally thought that pAMβ1 was non-transmissable as the presence of pAMγ1 or a related plasmid, pAD1, in the donor strain inhibits the transfer of the MLS plasmid (Clewell, 1981). However, by transforming pAMβ1 into a group F strain, LeBlanc and coworkers (1978a) demonstrated transfer of the plasmid on membrane filters to S. mutans, S. sanguis and S. salivarius recipients. Burdett (1980) reported transfer of pAMβ1 and other related plasmids by conjugative on membrane filters from S. faecalis to group B and D recipients and indeed used the ability of these "sex factors" to mobilise non-conjugative plasmids in her analysis of the location of tetracycline resistance determinants of group B isolates.

Several studies of the host range of streptococcal MLS resistance plasmids have been made. Malke (1979) reported conjugal transfer of group A and B plasmids to group A, D and H recipients, and Gasson and Davies (1980) demonstrated transfer of pAMβ1 between the lactic streptococci. In addition to intra- and inter-specific transfer, intergeneric transfer of MLS plasmids has been demonstrated to lactobacilli (Gibson et al., 1979), Staph. aureus (Engel et al., 1980; Schaberg et al., 1982), Listeria (Perez-Diaz et al., 1982), B. thuringiensis (Lereclus et al., 1983) and Pediococcus (Gonzalez and Kunka, 1983) with subsequent autonomous replication in all new hosts. Mobilisation of non-conjugative plasmids was also reported during matings

of S. faecalis with Staph. aureus (Schaberg et al., 1982), S. faecalis with S. pneumoniae (Smith et al., 1980) and matings between strains of B. thuringiensis (Lereclus et al., 1983). A recent comprehensive report has been published by Buu-hoi et al., (1984) investigating the transfer of MLS plasmids, originally described in group B, C, D and G strains, from group D donors to ten different streptococcal recipients representative of groups A, B, C, D, G, H and S. pneumoniae as well as Staph. aureus and Listeria innocua. In accordance with previous studies (Malke, 1979; Engel et al., 1980; Gonzalez and Kunka, 1983), host ranges of the different plasmids were similar although not identical and the frequency of transfer to different recipients was variable indicating the importance of the recipient. Plasmid stability was also variable in S. sanguis, S. pneumoniae, Staph. aureus and L. innocua hosts. Similar observations of plasmid maintenance were reported by Engel et al., (1980) in a Staph. aureus host and LeBlanc et al., (1978a) reported an inability to isolate MLS plasmid DNA from a S. salivarius host indicating either a rapid degradation after extraction from the strain or integration into the chromosome.

With respect to structure, heteroduplex analysis of several MLS resistance plasmids from group A strains has revealed that there can be considerable similarities (Boitsov et al., 1979; Behnke et al., 1980). The plasmids were composed in part of two "unique sequences" one of which carried the resistance determinant while the remainder of the molecules, as much as 92% in some cases, was present as long inverted repeat sequences. Physical mapping of spontaneous deletions obtained on transformation of S. sanguis located the replication functions on these repeat sequences but no function could be assigned to the remainder of the inverted repeats (Behnke et al., 1980; Malke, 1981).

Due to their clinical importance and potential as genetic tools for recombinant DNA technology, the broad host range plasmids have received

considerable attention in terms of their molecular arrangement. Malke (1981) by analysing natural deletion derivatives obtained by transformation of S. sanguis with a group A plasmid localised the gene encoding erythromycin and lincomycin resistance to a 1.8kb fragment and identified the region of DNA involved in plasmid replication. The possession of several unique restriction endonuclease sites have made these derivatives (pSM7, pSM8, pSM9, pSM10) attractive as cloning vehicles (Malke et al., 1981; Malke and Holm, 1982) and in addition by constructing a chimeric plasmid with pBR322, Malke and Holm (1981) have demonstrated expression of MLS resistance in E. coli. Other approaches to generating streptococcal cloning vehicles (pVA680, pVA736, pVA738) have been successfully attempted by insertion of the erythromycin resistance gene originating from pAMβ1 into a small cryptic plasmid from S. ferus (Macrina et al., 1980; 1982). A third family of cloning vectors (pGB301 and derivatives) has been obtained by spontaneous deletion of the group B plasmid p1P501 which encodes MLS and chloramphenicol resistance (Horodniceanu et al., 1976) and restriction endonuclease data have localised the resistance determinants, copy number control functions and replication region (Behnke and Gilmore, 1981). pGB301 and its derivatives have been shown in B. subtilis mini cells to produce three proteins associated with erythromycin resistance, chloramphenicol resistance and a one likely to be associated with plasmid replication or maintenance respectively (Behnke et al., 1982).

Recently, detailed physical maps of S. faecalis MLS resistance plasmid pAMβ1 (LeBlanc and Lee, 1984) and S. agalactiae MLS and chloramphenicol resistance plasmid p1P501 (Evans and Macrina, 1983) have been constructed. Analysis of deletion derivatives of pAMβ1 located the MLS determinant on a 1.1kb fragment and the replication functions on a 2.95kb fragment (LeBlanc and Lee, 1984). The latter functions contained in a 5kb fragment were ligated to E. coli plasmid pACKC1 to obtain an E. coli-S. sanguis shuttle vector which could express the E. coli kanamycin resistance gene of pACKC1 in S. sanguis and a cloned chromosomal streptomycin resistance locus from

S. mutans, in E. coli. Evans and Macrina (1983) also used deletion analysis as well as cloning data to locate resistance determinants and replication functions of p1P501. In addition, both groups have identified regions involved in conjugation but neither has yet been successful in cloning these functions.

Determinants conferring inducible resistance to MLS antibiotics have been identified on a transposon, Tn917. Originally associated with the 25.9kb non-conjugative plasmid pAD2 which also encodes streptomycin and kanamycin resistance (Tomich et al., 1979), the 5.1kb element was found to transpose to a conjugative haemolysin-bacteriocin plasmid pAD1 also harboured by S. faecalis strain DS16. Additionally, the transposon was shown to insert into pAMγ1 and pAMα1 of S. faecalis strain DS5 (Tomich et al., 1978; 1980) and into multiple sites of pAD1 (Clewell et al., 1982a). Heteroduplex analysis has shown that the resistance determinant is flanked by homologous inverted repeat sequences of approximately 0.28kb (Tomich et al., 1980) and is structurally related to a family of transposons, typified by Tn3, which have been isolated from a wide diversity of genera (Heffron, 1983). Indeed, comparison of physical maps and heteroduplex studies have revealed extensive homology between Tn917 and the staphylococcal transposon Tn551 while DNA sequence determination has shown significant homology between the terminal inverted repeats of Tn917, Tn551 and the Gram-negative Tn3 (Perkins and Youngman, 1984). Functional similarity between Tn917 and Tn551 or Tn3 has also been demonstrated by the identification of a 5-base pair duplication on insertion of the transposon.

Exposure of DS16 cells to sub-inhibitory concentrations of erythromycin was shown, not only to induce MLS resistance, but also to increase the frequency of transposition and it was thought that the stable co-integrates of pAD1 and pAD2 formed in recipient cells after induction represented intermediates in the transposition process which subsequently failed to resolve in the

new host. However, this model of the transposition process would require two copies of Tn917 to be present in the co-integrated structure and this was shown not to be the case (Clewell et al., 1982a). The involvement of transient co-integrate intermediates containing two copies of Tn917 nevertheless remains a possibility. Both induction of resistance and transposition enhancement have been found to be sensitive to protein inhibition by chloramphenicol although the isolation of mutants with normal inducible resistance phenotypes but lacking enhanced transposition responses, suggested that the two functions were under separate control (Clewell et al., 1982a). Tn917 has been shown to undergo transposition in B. subtilis and to insert into multiple chromosomal sites indicating its value as an insertional mutagen (Youngman et al., 1983).

Preliminary work has suggested that other MLS resistance transposons may occur in streptococci. Le Bouguenec and Horodniceanu (1982) postulated that transposition could account for the difference in size of plasmids isolated from parental and transconjugants strains of S. faecium. Banai and LeBlanc (1983) described insertion of the S. faecalis MLS determinant of pJH1 into the co-resident plasmid pJH2, the size of the insertion being similar to Tn917. They have subsequently shown that the 5.1kb segment could insert into at least four different sites on pJH2 and was homologous to Tn917 by the criteria of DNA hybridisation and comparison of endonuclease AvaI restriction patterns (Banai and LeBlanc, 1984). Like Tn917, the segment designated Tn3871 expressed inducible MLS resistance. Characterisation of the conjugative chromosomal insert of S. agalactiae B109 which encodes resistance to chloramphenicol, tetracycline and MLS antibiotics has shown that the element is capable of transposition to multiple sites of the plasmid pAD1 (Smith and Guild, 1982) and results suggested that the MLS determinant was capable of independent transposition. Interestingly, the transfer of plasmid p1P501 into a strain in which B109 insertion was already present has

been shown to be inhibited and in strains containing both p1P501 and the B109 insertion element, the plasmid and insertion were found to be incompatible, suggesting a relationship of surface exclusion and incompatibility genes (Horodniceanu et al., 1981).

Several studies have demonstrated the relationship of MLS plasmids isolated from different clinical strains suggesting a common origin for these determinants. S. faecalis pAMβ1 and S. pyogenes pAC1 encoding constitutive and inducible MLS resistance respectively, were shown by DNA-DNA hybridisation experiments to be 95% homologous (Yagi et al., 1975) and similar results were obtained on examination of homology between plasmids originating from groups D and B (El-Solh et al., 1978). Comparison of the restriction endonuclease profiles of plasmids from group A, B and D isolates (Hershfield, 1979) and from groups B, C and G strains (Bougueleret et al., 1981; Horodniceanu et al., 1981) showed a number of digestion fragments in common. Using complementary RNA probes prepared from pAMβ1, pAM77 (a 6.8kb, non-conjugative plasmid from S. sanguis encoding inducible MLS resistance; Yagi et al., 1978) and p1258 (a 27kb Staph. aureus plasmid containing the constitutive MLS resistance gene of Tn551; Novick et al., 1979b), Weisblum and coworkers (1979) demonstrated sequence homology in heterologous hybridisations with target DNA prepared from the above templates, S. pyogenes pAC1 and chromosomal DNA of MLS resistant S. pneumoniae. Gilmore et al. (1982) investigated the relationship between MLS resistance loci and replication function sequences of plasmids obtained from groups A, B, D and H streptococci, Staph. aureus and B. fragilis as well as chromosomal DNA of Lactobacillus casei and Streptomyces erythreus, an erythromycin producer. The DNA probes were a 1.7kb fragment containing MLS determinants and a 1.5kb fragment containing the replication origin and copy number control region of the group A plasmid pSM19035 (Behnke and Ferretti, 1980). Hybridisation of the resistance probe was seen with all streptococcal plasmids including

pAD2, which carries Tn917, and with Staph. aureus p1258 (Tn551) but not with Staph. aureus pE194, B. fragilis p1P410 or chromosomal DNA of L. casei or S. erythreus. The replication origin-copy number probe hybridised with all plasmids giving positive MLS resistance homology except for pAD2, p1258 and pAM77. Lack of homology of pAD2 and p1258 probably reflected the transposon location of the determinants and suggested that the resistance gene of pAM77, an MLS plasmid which is unusual in respect of its small size, may have originated as a transposable element. On the basis of DNA-DNA hybridisation studies, Ounissi and Courvalin (1982) have identified four classes of MLS resistance determinants: class A includes all the streptococcal determinants examined as well as Staph. aureus Tn554; class B is comprised of Staph. aureus plasmids pE194 and pE5; class C contains the chromosomally specified locus of B. licheniformis; class D is typified by B. fragilis p1P410.

Although lack of sequence homology, as judged by DNA hybridisation experiments, has been observed, Horinouchi and Weisblum (1982b) have shown that there can be nevertheless considerable similarities in the structural genes encoding MLS resistance. Comparison of the nucleotide sequences of the structural genes of pAM77 and pE194, revealed that approximately half the nucleotides were identical while analysis of the deduced amino acid sequences showed striking conservation. Indeed, where there were differences in amino acid sequence, the alternative corresponding residues were frequently structurally related.

BETA-HAEMOLYSIN AND BACTERIOCIN GENES OF GROUP D STREPTOCOCCI

The ability to produce haemolysins and bacteriocins is widely distributed among Gram-negative and Gram-positive bacteria and indeed, production of β-haemolysin has been used as a major criterion in the classification of pyogenic streptococci and is the criterion for the sub-classification of group D S. faecalis subsp. zymogenes. All the determinants for production of β-haemolysin in S. faecalis subsp. zymogenes, which have so far been examined, are carried by plasmids of similar size, 50 to 60kb (Clewell, 1981; Borderon et al., 1982). The production of haemolysin in these strains is always associated with bacteriocin production and it has been shown that the two activities are attributable to the same molecule (Brock and Davie, 1963; Dunny and Clewell, 1975; Yagi et al., 1983).

Evidence for the plasmid linkage of haemolysin-bacteriocin was first reported by Tomura et al., (1973) who described both the transfer of these traits to S. faecalis recipients by a mechanism which was probably conjugation and the loss of the traits on irradiation with the ultraviolet light. Subsequently Jacob and coworkers (1975) identified, in strains of S. faecalis subsp. zymogenes, plasmids which encoded haemolysin-bacteriocin production and which were transmissible by conjugation in broth culture to a plasmid free recipient. Plasmids pJH2 and pJH3 were shown to be of similar size (57.5kb), present as a single copy and in addition to haemolysin-bacteriocin, encoded resistance to the corresponding bacteriocin. On studying the loss of the pJH2 associated haemolysin trait, some strains could be isolated which were non-haemolytic and non-bacteriocinogenic but retained bacteriocin resistance and pJH2. This observation was presumably due to a point mutation or small deletion in the haemolysin-bacteriocin determinant of pJH2. S. faecalis haemolysin has been shown to be composed of an activator A and a catalytic molecule L (Granato and Jackson, 1971a,b) and, on cross-streaking of the strains, combinations which resulted in restoration of haemolytic activity

were observed suggesting that mutations had occurred producing either defective A or L.

Reports describing the haemolysin-bacteriocin plasmid pAMγ1 of S. faecalis strain DS5 (Clewell et al., 1974) not only showed that the plasmid was capable of transfer by conjugation in broth culture but that it could also mobilise the non-conjugative, co-resident tetracycline resistance plasmid pAMα1 (Dunny and Clewell, 1975). However, certain discrepancies in the phenotypes of derivatives were observed which led to the suggestion that there were in fact two bacteriocin activities encoded by pAMγ1. It has been shown since that in fact strain DS5 contains three plasmids - pAMγ1 (60kb) which encodes haemolysin-bacteriocin activity and bacteriocin resistance, pAMγ2 (52.8kb) which encodes a second bacteriocin and bacteriocin resistance, and pAMγ3 (45.8kb) the presence of which reduces the production of the pheromone cPD1 (Clewell et al., 1982b; LeBlanc et al., 1983). All three plasmids have been shown to be capable of mobilising pAMα1.

Similar conjugative plasmids encoding haemolysin-bacteriocin, including pAD1 (Clewell et al., 1982a) pOB1 (Oliver et al., 1977), pX-14 (Frazier and Zimmermann, 1977) and pPD5 (Yagi et al., 1983) have been described in other strains of S. faecalis although in some cases, notably pOB1 and pPD5, there has originally been confusion as to the activities encoded by these plasmids because it has subsequently been shown that their hosts in fact contained two plasmids, one of which encoded haemolysin-bacteriocin activity and the other which encoded a different bacteriocin. In the case of strain 39-5 which harbours pPD5, the co-resident bacteriocin plasmid pPD1 was found to be almost the same size and often co-transferred (Yagi et al., 1983).

A characteristic of the transfer of all the S. faecalis haemolysin-bacteriocin plasmids (with the possible exception of pPD5), the bacteriocin plasmids pPD1, pAMγ2 (but not pOB2; Oliver et al., 1977) and pAMγ3 is the use of the pheromone dependent aggregation system which allows high frequency

transfer in broth culture to recipient S. faecalis (Clewell, 1981). A study of the host range of such plasmids found that no transfer was observed to S. faecium, S. durans, S. bovis, E. coli or L. innocua recipients (Borderon et al., 1982). As described previously, acquisition of these plasmids results in the production of a proteinaceous aggregation substance on the cell surface in response to the corresponding pheromone of S. faecalis recipient cells. The location of the genes for aggregation substance is not yet known but it has been shown that aggregation substances produced in response to different pheromones are immunologically related (Kessler and Yagi, 1983; Yagi et al., 1983).

Digestion of pAMγ1 and pAD1 with the restriction endonuclease EcoR1 resulted in the generation of identical fragments indicating the close structural relationship of these haemolysin-bacteriocin plasmids isolated from different sources (Clewell et al., 1982b). Moreover the two plasmids were shown to share the same pheromone system and both exhibited the property of inhibiting the transfer of the MLS plasmid pAMβ1. Similarly, restriction endonuclease profiles of haemolysin-bacteriocin plasmids isolated from a group of six S. faecalis strains were identical (Borderon et al., 1982) but comparison of the physical maps of pAD1 and the bacteriocin plasmid pPD1 showed no similarity (Yagi et al., 1983). Using Tn916 and Tn917 as insertional mutagens, the haemolysin-bacteriocin determinant has been located on the physical map of pAD1 (Clewell et al., 1982a) and has been used as a probe to investigate homology between haemolysin and bacteriocin genes from several sources (LeBlanc et al., 1983). DNA-DNA hybridisation studies indicated that the haemolysin-bacteriocin loci of different S. faecalis plasmids are very closely related and, although restriction endonuclease profiles of pAD1 and pAMγ1 were quite different from pJH2, pPD5 and pOB1, there was considerable DNA sequence homology suggestive of a common origin. There was no hybridisation observed between the cloned pAD1 haemolysin-bacteriocin determinant and the bacteriocin plasmids,

pAMγ2 and pPD1, or pAMγ3 and likewise no homology with total cell DNA isolated from S. pyogenes, S. agalactiae, S. anginosus and S. sanguis all of which produce chromosomally determined β-haemolysins.

Other species of bacteriocinogenic group D streptococci are commonly encountered (Tomura et al., 1973) but there is little information on the nature of the genes for bacteriocin production in strains other than S. faecalis. In S. faecium, Keness and coworkers (1978) reported that bacteriocin determinants were located on small 3.6 to 5.8kb plasmids but Le Bouguenec and Horodniceanu (1982), although they described transferable bacteriocin production, could not relate this phenotype with the acquisition of particular plasmids. In two strains of S. faecium, however, bacteriocin production and resistance to bacteriocin was correlated with transfer of a 5.3kb plasmid and a 3.6kb or a 7.1kb plasmid respectively (Kramer et al., 1983; Reichelt et al., 1984).

OBJECT OF RESEARCH

In group D streptococci, it is known that antibiotic resistance, as well as haemolysin and bacteriocin production, is often encoded by genetic determinants located on plasmids, many of which are capable of self transfer by conjugation or which can be mobilised by other conjugative plasmids. Alternatively, antibiotic resistance may be specified by genes situated in the chromosome, some of which may be capable of transposition to other replicons and of transfer by conjugation in the absence of plasmid DNA.

Although numerous enterococcal strains which display multiple antibiotic resistance phenotypes have been described, detailed studies of the entire plasmid complement of such strains and the location of the resistance genes have been limited to only three Streptococcus faecalis strains, namely DS5 (Clewell et al., 1974; 1982b), DS16 (Clewell et al., 1982a; Franke and Clewell, 1981), and JH1 (Jacob and Hobbs, 1974; Banai and LeBlanc, 1983; 1984a,b). Group D streptococcal strains in this study were chosen on the basis that they had shown resistance to two or more antibiotics in a previous investigation (Blankson, 1981). The principle aims of the research were 1. to extend the information on the phenotypes and ascertain the mode of resistance expression, 2. to investigate the nature of the resistance genes with respect to transfer characteristics and mitotic stability, 3. to determine if the strains contained plasmids and whether antibiotic resistance was specified by these plasmids or by the chromosome and 4. if possible, to compare and contrast the plasmid contents of these strains with those of the well characterised strains.

MATERIALS AND METHODS

BACTERIAL STRAINS

Source

Table 1 lists the bacterial strains used in this study. The group D streptococci under investigation were isolated and identified previously as part of the work towards a Ph.D. degree by M. Blankson (1981). Strains K46, K55, K60, K87 and K88 were isolated from clinical specimens obtained from Victoria Hospital, Kirkcaldy and were identified as *S. faecium* (K46), *S. faecalis* (K55), *S. faecalis* subsp. *liquefaciens* (K60, K88) and *S. faecalis* subsp. *zymogenes* (K87).
Strains SB69 and SB94 were isolated from water sampled below a sewer outfall and identified as *S. faecium* and *S. faecalis* respectively.

The well characterised strain DS5 was included in the study as an example of a known plasmid containing group D *Streptococcus*. Originally reported as having only three plasmid species (Clewell *et al.*, 1974), it has recently been shown to possess five plasmids which encode haemolysis and bacteriocin production, resistance to ultraviolet light and pheromone response (pAMγ1; 60.0kb), bacteriocin production and pheromone response (pAMγ2; 52.8kb), pheromone response (pAMγ3; 45.8kb), resistance to MLS antibiotics (pAMβ1; 26.5kb) and tetracycline resistance (pAMα1; 9.1kb) (Clewell *et al.*, 1982b; LeBlanc *et al.*, 1983).

Strains JH2-1, JH2-2 and JH2-17 were generously provided by Dr. A.E. Jacob, University of Manchester. They are mutant derivatives of *S. faecalis* strain JH2 which has been shown to be plasmid free (Jacob and Hobbs, 1974) and therefore suitable as a recipient in mating experiments. Strain JH2-1 has chromosomally encoded resistance to fusidic acid, strain JH2-2 to fusidic acid and rifampicin. Strain JH2-17 is resistant to rifampicin and is a thymine auxotroph.

Table 1. Group D streptococcal strains

Strain designation	Species and subspecies	Source
K46	*S. faecium*	clinical specimen
K55	*S. faecalis*	clinical specimen
K60	*S. faecalis* subsp. *liquefaciens*	clinical specimen
K87	*S. faecalis* subsp. *zymogenes*	clinical specimen
K88	*S. faecalis* subsp. *liquefaciens*	clinical specimen
SB69	*S. faecium*	sewer outfall
SB94	*S. faecalis*	sewer outfall
DS5	*S. faecalis* subsp. *zymogenes*	clinical specimen (Clewell *et al.*, 1974; Dunny and Clewell, 1975)
JH2 derivatives	*S. faecalis*	clinical specimen (Jacob and Hobbs, 1974; A.E. Jacob, personal communication)

Growth and maintenance

The solid medium used for routine growth of organisms was Diagnostic Sensitivity Test agar (DST; Oxoid). Cultures were streaked on to DST agar, grown aerobically at 37°C for 36h and stored for up to three weeks at 4°C. These cultures were used as a working stock and the phenotype was checked prior to use in an experiment. For long term maintenance, bacterial strains were inoculated into Luria broth containing 0.5% (w/v) agar, incubated at 37°C for 48h and stored at 4°C. Luria broth consisted of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, adjusted to pH 7.5 with sodium hydroxide. In parallel, cultures were grown in Luria broth to which was added an equal volume of an aqueous solution of glycerol-dimethyl sulphoxide, each at 6% (v/v), and after mixing, these cultures were stored at -70°C. To sub-culture from this frozen stock, a small quantity of material was scraped from the surface and streaked on to DST agar.

All media used for the growth of JH2-17 or its derivatives were supplemented with 2μg ml^{-1} thymine.

Detection of haemolysin production

Cultures were grown aerobically at 37°C for 24 to 48h on DST agar supplemented with 6% (v/v) defibrinated horse blood (Gibco). β-haemolysis was easily recognised after incubation as a clear zone around the colonies, and while α-haemolysis was more difficult to see, "greening" of the blood became apparent after cultures had been kept at room temperature for up to 72h.

Detection of bacteriocin production

The method described by Dunny and Clewell (1975) with minor modifications was used to detect bacteriocinogenic strains. The indicator strain was grown

overnight at 37°C in Brain Heart Infusion broth (BHI; Oxoid) and 25µl added to 4ml liquefied 0.75% (w/v) agar at 50°C which was poured over a DST agar plate. When the overlay had solidified, colonies of the organisms under test were stab inoculated into the agar. Approximately 22 bacterial strains could be tested on each agar plate. After incubation at 37°C overnight, zones in which the growth of indicator organisms was inhibited could be seen around bacteriocin producers.

DETERMINATION OF ANTIBIOTIC RESISTANCE PHENOTYPE

Antibiotics and preparation of antibiotic agar

The antibiotics used are listed in Table 2. All antibiotic solutions were prepared freshly and the use of sterile diluents and containers obviated sterilisation. Solutions which required ethanol, dimethyl sulphoxide or 0.5M Tris pH 8.0 were prepared by dissolving the antibiotic in a minimal amount of solvent and adding sterile distilled water to give the correct volume. Where the technical information on specific activity of the antibiotics was available, the weight of dry antibiotic was adjusted accordingly, but if there was no such information, the specific activity was taken to be 1.

For the preparation of solid antibiotic medium, antibiotic solutions were made 25 or 100 times the concentration required and diluted to the correct concentration in sterile liquefied nutrient agar at 50°C which, after thorough mixing, was poured into 9cm plastic petri dishes and allowed to solidify. The medium was dried before use in a circulating hot air oven at 50°C for 5 to 10 min. If the antibiotic medium was not required for immediate use, plates were stored at 4°C for one week, in the case of ampicillin and penicillin, or up to four weeks for other antibiotics.

Table 2. Antibiotics

Antibiotic	Manufacturer	Specific activity ($\mu g\ mg^{-1}$)*	Solvent
erythromycin	Sigma	NA	ethanol
lincomycin hydrochloride	Sigma	953.4	distilled water
pristinamycin IA	Specia	NA	ethanol
tetracycline	Sigma	NA	ethanol
chloramphenicol	Sigma	NA	ethanol
benzyl penicillin sodium salt	Glaxo	1000.0	distilled water
ampicillin	Sigma	NA	distilled water
streptomycin sulphate	Sigma	NA	0.5M Tris pH 8.0
kanamycin sulphate	Sigma	750.0	0.5M Tris pH 8.0
neomycin sulphate	Sigma	NA	0.5M Tris pH 8.0
gentamicin sulphate	Sigma	615.0	0.5M Tris pH 8.0
spectinomycin dihydrochloride	Sigma	NA	0.5M Tris pH 8.0
sodium fusidate	Leo Laboratories	NA	distilled water
rifampicin	Sigma	NA	dimethyl sulphoxide

* NA = information not available

Determination of minimal inhibitory concentrations

The minimal inhibitory concentrations (MICs) of antibiotics were determined in duplicate using an antibiotic agar dilution method. Serial two-fold dilutions of antibiotic solutions were made in sterile distilled water at 25 times the concentration required and antibiotic agar plates of each dilution were prepared as above. Bacterial strains were grown on DST agar at 37°C for 36h and five isolated colonies of each strain were suspended in 3ml sterile 0.9% (w/v) sodium chloride. A loopful of each suspension was placed on the dilutions of antibiotic in DST agar and the agar allowed to dry at room temperature before incubating at 37°C for 24h. Included on all plates were the strains JH2-2 which is known to be susceptible to all antibiotics but fusidic acid and rifampicin, and DS5 which is resistant to tetracycline and MLS antibiotics. The MIC was taken as the lowest concentration ($\mu g\ ml^{-1}$) of antibiotic which inhibited the growth of three or more colonies.

Expression of antibiotic resistance

In order to determine whether expression of resistance was inducible or constitutive, the method described by Yagi et al (1975) was used. Antibiotics tested were erythromycin, tetracycline and chloramphenicol. Bacterial strains were grown in BHI broth overnight and 10ml of these cultures were used to inoculate fresh 40ml aliquots of BHI broth. Growth of the strains at 37°C in an orbital incubator shaker at 150 rpm was monitored at 30 min intervals by measuring the absorbance at 610nm. When the absorbance reached 0.15 to 0.20 at the start of exponential growth, the cultures were divided into three equal volumes, one of which received inducer.

Concentrations of antibiotics used as inducers were erythromycin 0.05$\mu g\ ml^{-1}$, tetracycline 0.1$\mu g\ ml^{-1}$ and chloramphenicol 5.0$\mu g\ ml^{-1}$. Growth was continued to an absorbance of 0.45 – 0.50 (mid-exponential phase) when one

portion which did not contain inducer and the portion to which inducer had been added, were challenged with the appropriate antibiotic. Concentrations of antibiotics used as challenge were erythromycin 100μg ml^{-1}, tetracycline 20μg ml^{-1} and chloramphenicol 20μg ml^{-1}. All three portions of each culture were incubated as above until the stationary phase of growth was reached.

Test for beta-lactamase activity

Penicillinase activity was tested using Intralactam (Mast Laboratories) and Beta-test (Medical Wire) strips according to the manufacturer's recommendations. Strips were moistened with sterile distilled water and culture, taken from DST agar, was applied to the strip using a bacteriological loop. If, after a period of up to 10 min, a yellow colour appeared, the test was positive. Cephalosporinase activity was tested using the synthetic chromogenic cephalosporin nitrocefin (Glaxo). An aliquot (0.05ml) of an overnight culture in BHI broth was added to an equal volume of a 0.5mg ml^{-1} aqueous solution of nitrocefin. After incubation at 37°C for up to 30 min, a positive result was recorded when the solution changed from a straw colour to red. In all tests, JH2-2 and β-lactamase producing Staph. aureus and E. coli containing RP4 were included as negative and positive controls respectively.

TRANSFER EXPERIMENTS

Broth mating procedure

Donor and recipient strains were grown overnight at 37°C in Oxoid Nutrient Broth No. 2 supplemented with 0.2% (w/v) glucose and 0.1M Tris with pH adjusted to 7.8 using 5M HCl (N2GT) which had been sterilised at 10psi for 10 min. An aliquot (1ml) was inoculated into 20ml fresh N2GT in 100ml Ehrlenmeyer flasks which were placed in an orbital incubator shaker at 37°C and cultures grown for 1.5 to 2h shaking at 200 rpm to give approximately 5×10^8 colony forming units (cfu) ml^{-1}. The procedure described by Dunny and coworkers (1979) was used. Mating mixtures consisted of 0.05ml donor culture and 0.5ml recipient culture mixed in 4.5ml fresh N2GT broth i.e. a donor: recipient ratio of 1:10 and a total of approximately 5×10^7 cfu ml^{-1}. The actual ratio and concentration of bacteria was checked by performing viable counts on cultures added to mating mixtures. Serial 10-fold dilutions of culture were made in sterile 0.9% (w/v) sodium chloride and one drop from a Pasteur pipette (average volume 25µl) of dilutions 10^{-6} to 10^{-4} was placed in duplicate on DST agar. After allowing the agar to dry at room temperature plates were incubated for 36h at 37°C and the cfu ml^{-1} estimated.

Mating mixtures were incubated without shaking for 4h at 37°C, with cultures of donor and recipient strains alone treated in the same fashion. After incubation all cultures were mixed vigorously and the number of viable donor and recipient bacteria estimated by viable counting as before using appropriate selective agar - selection of donors was with DST agar containing 25µg ml^{-1} erythromycin except with strain SB69 where 20µg ml^{-1} tetracycline was used, and for recipients, with DST agar containing 50µg ml^{-1} fusidic acid or 100µg ml^{-1} rifampicin depending on the recipient strain. In fact, the number of donor cfu should be estimated for each drug under test as the donor viable count at the end of mating will in part consist

of these recipients which have received the resistance and hence be dependent on the transmissibility. Estimating the donor viable count on only one antibiotic therefore has the effect of underestimating transfer frequency of those markers where the actual frequency is less than the selected marker and vice versa when the transfer frequency is greater.

Filter mating procedure

Bacterial strains were grown by the same procedure as that described for broth matings, although latterly BHI broth was used as nutrient medium. As above, the viable count of cultures was estimated before mixing 0.5ml donor and 4.5ml recipient to give a ratio of one donor to ten recipients and a total count of approximately 5×10^8 cfu ml^{-1}. Immediately after mixing, 1ml of the mating mixture was filtered on to a 25mm diameter 0.45µm filter (Sartorius, type SM113) which was placed on DST agar and incubated at 37°C for 18h. After incubation, the cells on the filter were re-suspended in 1ml nutrient medium by vigorous agitation using a vortex mixer and the number of viable donor and recipient bacteria estimated on appropriate selective solid medium. Selection for donors and recipients was as above.

Selection of transconjugants

Transconjugants were selected on the basis of their simultaneous resistance to both a donor antibiotic resistance trait and a recipient antibiotic resistance trait. Concentrations of antibiotics in µg ml^{-1} used for selection were : erythromycin 25; lincomycin 100; tetracycline 20; streptomycin, kanamycin and neomycin 1000; chloramphenicol 20; ampicillin and penicillin 10; fusidic acid 50; rifampicin 100. Transfer of haemolysin activity could not be selected directly but was assessed on DST agar containing 6% (v/v) defibrinated horse blood and fusidic acid or rifampicin.

Fusidic acid was used in preference to rifampicin in blood agar as the colour of the latter antibiotic interfered with haemolysin detection, particularly if the transfer frequency was low and the background of non-haemolytic colonies consequently high, a situation which results in almost imperceptible haemolytic zones.

After incubation of mating mixtures and thorough mixing, duplicate 0.1ml aliquots were spread using a sterile glass spreader, on well dried DST agar plates containing selective antibiotics. When necessary, aliquots of serial 10-fold dilutions prepared for viable counts were also spread in order to achieve a suitable number of single colonies. All agar plates were allowed to dry at room temperature before incubating at 37°C for 48 to 72h. Transconjugant colonies which grew on selective plates were counted and the number of transconjugants per ml estimated. From this count and that of the donor cells at the end of mating, the frequency of transconjugants per donor was calculated.

Determination of transconjugant phenotype

In order to determine the phenotype of transconjugant colonies, master plates were prepared on DST agar by conventional "picking and patching". To avoid bias, all single colonies on a selective plate or all single colonies of a section of a selective plate were taken for preparation of the master plate. Patches of donor and recipient strains were also included on master plates which were incubated at 37°C for 36h and replicated, using sterile velvets, on to DST agar plates containing single antibiotics or 6% horse blood, representing non-selected donor and recipient markers. Antibiotic concentrations were the same as those used for transconjugant selection. As many as six replicas were made from each master, the last replica being a DST agar plate without antibiotic to check the efficiency of replication. Replica plates were incubated at 37°C for 36 to 48h.

Preparation and assay of clump inducing agent (CIA)

The procedure of Dunny and coworkers (1979) was used to assay CIA activity. CIA producing strains were grown in N2GT broth at 37°C in an orbital incubator shaker at 150 rpm to late exponential phase at an absorbance of 0.8 at 610nm. Cultures were centrifuged for 10 min in a Sorvall SA-600 rotor at 8000 rpm, the supernatant decanted and filtered through a 25mm diameter 0.45µm filter (Millipore) and finally, the filtered supernatant was autoclaved at 15 psi for 20 min before storage at 4°C.

Response to CIA was assayed in 'U-shaped' microtiter trays (Sterilin). Autoclaved filtrate was diluted in fresh N2GT to give serial two-fold dilutions in 50µl volumes. Strains to be tested for response to CIA were grown in N2GT at 37°C overnight, diluted to an absorbance of 0.5 at 660nm and 50µl added to each dilution. The range of final dilutions of CIA was 1/2 to 1/256 with a well containing N2GT and responder cells alone as a control. Microtiter trays were incubated at 37°C for 90 to 120 min on a rotating table (Rotatest shaker, Luckham) before examining wells for clumping of responder cells. The CIA titre was taken as the reciprocal of the highest dilution in which clumping occurred.

ELIMINATION OF PLASMID DNA

Loss of plasmid DNA during storage

Bacterial strains were stored at room temperature in Luria broth containing 0.5% (w/v) agar for 10 to 12 months. BHI broth supplemented with 0.5% (w/v) yeast extract (Difco), 0.2% glucose and 0.1 volume 1M Tris-HCl, pH 8.0 (BYGT; Dunny et al., 1981b)sterilised at 15 psi for 15 min in 20ml amounts , was inoculated with 0.2ml of these cultures and incubated at 37°C in an orbital incubator shaker at 200 rpm. Overnight incubation was necessary to obtain visible growth of the organisms. Serial 10-fold

dilutions in 0.9% (w/v) sterile sodium chloride were made and 0.1ml of dilutions 10^{-6} to 10^{-4} was spread on dried BHI agar (BHI broth containing 1.5% (w/v) Oxoid agar No. 1) which was incubated at 37°C for 48h.

Master plates were prepared by picking and patching, as described previously, on to fresh BHI agar. Approximately 200 single colonies of each strain was taken. Master plates were incubated at 37°C for 36h before replicating using sterile velvets on to BHI agar containing an appropriate antibiotic or horse blood. The concentrations of antibiotics and blood used were the same as for analysis of transconjugant phenotypes and included on master plates were patches of JH2-1, as susceptible control, and the parental strain. Up to six replica plates were made from each master, the last being BHI agar without antibiotic. After incubation at 37°C for 48h, growth of patches on antibiotic and blood agar was compared with that on the BHI agar replica and where no growth occurred on antibiotic or where there was no haemolysis on blood, it was taken as evidence of loss or 'curing' of plasmid DNA.

Treatment with novobiocin

Novobiocin (Sigma) is an antibiotic which inhibits the B subunit of DNA gyrase and which has been shown to effect plasmid elimination possibly by a selective action on plasmid associated gyrase (McHugh and Swartz, 1977; Hooper et al., 1984). The most effective concentration of drug was shown to be that which inhibited multiplication or caused minimal killing (McHugh and Swartz, 1977).

Novobiocin was dissolved in a minimal amount of ethanol and sterile distilled water added to give a concentration of 2mg ml^{-1}. The antibiotic was diluted in sterile BHI broth to 100µg ml^{-1} followed by serial two-fold dilutions in 1ml sterile BHI broth to give a series of dilutions from 50 to 0.19µg ml^{-1} novobiocin in a total of 1ml broth. A tube con-

taining 1ml BHI broth without antibiotic was included in each series. Bacterial strains were grown overnight at 37°C in 5ml BHI broth, diluted 1 in 120 in fresh sterile BHI broth and 1 drop from a sterile Pasteur pipette (average 25µl) was added to each of the dilutions of novobiocin and the antibiotic free control, giving an estimated initial bacterial count of approximately 10^5 cfu ml^{-1}. After incubation overnight at 37°C, the cultures were agitated and examined for growth. The MIC for novobiocin was the reciprocal of the lowest dilution in which no growth was visible and the dilution at half MIC concentration, i.e. the lowest dilution with visible growth, was tested for cured derivatives. Serial 10-fold dilutions were made in sterile 0.9% (w/v) sodium chloride and duplicate 0.1ml aliquots of dilution 10^{-5} to 10^{-2} were spread on dried BHI agar. After incubation at 37°C for 36h, master plates were prepared and replicated as above.

PREPARATION OF PLASMID DNA

Vapnek method

This method of plasmid preparation (Vapnek et al., 1976) is commonly used for isolation of plasmid DNA from Gram-negative bacteria and an attempt was made, with minor modifications, to prepare plasmid DNA from strains of group D streptococci. Strains were grown in 30ml Luria broth in 250ml flasks at 37°C in an orbital incubator shaker at speed 200 rpm until the absorbance at 610nm was 0.8. Cells were pelleted by centrifugation for 10 min at 8000 rpm in a Sorvall SA-600 rotor resuspended in 2ml 10% (w/v) sucrose in 0.05M Tris-HCl pH 8.0 and treated with 0.8ml 0.2M EDTA, pH 9.0 to inhibit nucleases by chelating magnesium ion cofactors, and 0.4ml egg white lysozyme 5mg ml^{-1} (Sigma; specific activity 38 300 Units mg^{-1} protein) in 0.25M Tris-HCl, pH 8.0 to degrade the cell wall. After incubation for 5 min on ice, 0.3ml 2% (w/v) sarkosyl was added dispersed immediately by gentle agitation and the mixture was incubated a further 10 min at 37°C to

achieve lysis. The cell debris was removed by centrifugation for 1h at 18000 rpm in a Sorvall SA-600 rotor and the supernatant transferred to corex tubes. Deproteinisation of the sample was accomplished by extraction with redistilled phenol saturated with 10mM Tris-HCl, pH 8.0. An equal volume of phenol was added and mixed by inversion of the tube several times. To separate the aqueous and phenol phases, the tubes were spun for 5 min at 5000 rpm, the aqueous phase carefully removed avoiding the precipitate at the interface and the DNA precipitated by addition of 2 volumes of ethanol pre-cooled to $-20^{o}C$. After mixing by inversion, samples were incubated at $-20^{o}C$ overnight and the DNA collected by centrifugation for 10 min at 10000 rpm followed by thorough draining and resuspension in 0.2ml TE buffer (50mM Tris-HCl,5mM EDTA, pH 8.0). If necessary, samples were stored at $4^{o}C$.

Eckhardt method

Plasmid DNA preparation by this method involves the gentle lysis of cells directly in the slots of an agarose gel and was developed to detect plasmids of low copy number within a wide size range of 3 to 230kb from Gram-negative and Gram-positive bacteria(Eckhardt, 1978). The procedure requires the following solutions:

Lysozyme solution - lysozyme 75000 Units ml^{-1}
ribonuclease 1 0.3 Units ml^{-1} (Sigma)
disodium EDTA 0.05M
sodium chloride 0.1M
Ficoll 400,000 20% (w/v)
bromophenol blue 0.05% (w/v)
in TBE (89mM Tris base, 12.5mM
disodium EDTA, 8.9mM boric acid,
pH 8.2)

Ribonuclease 1 was first dissolved in 0.4M sodium acetate buffer, pH 4.0 to 10mg ml^{-1}, heated for 2 min at 98°C and diluted to the appropriate concentration.

SDS solution - sodium dodecyl sulphate (SDS) 2% (w/v)
Ficoll 400,000 10% (w/v)
in TBE

Overlay solution - SDS 0.2% (w/v)
Ficoll 400,000 5% (w/v)
in TBE

A 1% (w/v) vertical agarose gel (preparation described in following section) was prepared. Lysozyme solution (30µl) was placed in each well and 1 to 3 single colonies from 36h DST agar cultures were suspended in the solution so that it became slightly turbid. Cells were incubated in the lysozyme solution for 40 min, 60µl SDS solution layered on top and the two layers gently mixed with a toothpick moved side to side two or three times. Incomplete mixing was necessary to produce only partial lysis at this stage, lysis being completed by migration of SDS through the bottom layer during electrophoresis (Eckhardt, 1978). Finally 200µl overlay solution was carefully layered without disturbing the layers below and the slots sealed with molten agarose. Electrophoresis was for 60 min at 2mA and thereafter at 40mA and the bands of nucleic acid visualised as described in the following section.

Mutanolysin method

This method was developed by Monsen et al (1983) specifically for preparation of DNA from streptococci and uses mutanolysin isolated from Streptomyces globisporus (Sigma; specific activity 3585 Units mg^{-1} protein) in combination with the non-ionic detergent Triton X-100 and high concentrations of sodium chloride to effect lysis.

Bacterial strains were grown to mid-exponential phase (A_{610} 0.4) in 100ml BYGT broth supplemented with 20mM threonine which facilitates lysis possibly by weakening cross-linking of the peptidoglycan (Chassy, 1976). Cells were harvested by centrifugation for 10 min at 7000 rpm in a Sorvall SA-600 rotor, washed in 20ml 10mM sodium phosphate buffer, pH 8.0. To this suspension was added 50µl 0.5M disodium EDTA, pH 8.0, 125µl 20% (v/v) Triton X-100, 100µl 5.8 mg ml^{-1} mutanolysin and 0.5g solid sodium chloride to give a final concentration of 1.62M. The suspension was gently mixed until the salt was dissolved, incubated 5 to 60 min at 37°C until complete visual lysis was obtained, and incubated for 30 min at 4°C. The presence of sodium chloride directly enhances mutanolysin lysis possibly by destabilising streptococcal peptidoglycan (Monsen et al., 1983) in addition to causing preferential precipitation of linear (chromosomal) DNA. The lysate was spun at 4°C for 20 min at 15000 rpm and the supernatant poured into a corex tube. At this point, the published method continued with two serial caesium chloride-ethidium bromide buoyant density centrifugations, phenol extractions and ethanol precipitation. In this study, the centrifugation steps were omitted and the samples extracted twice as described above by addition of an equal volume of redistilled phenol saturated with 3% (w/v) sodium chloride, followed by mixing and separation of phases by centrifugation at room temperature for 5 min at 2500 rpm in a Sorvall SA-600 rotor. The aqueous phase was extracted twice with chloroform to remove the phenol and the DNA precipitated by addition of 0.5ml 3M sodium acetate and 2 volumes cold 95% (v/v) ethanol. Samples were incubated at -20°C overnight or until needed when they were spun for 20 min at 12000 rpm. After thorough draining and drying, the DNA pellet was resuspended in 300µl TE buffer (10mM Tris-HCl, 1mM disodium EDTA, pH 8.0).

Preparation of large plasmid molecules

This method was described by Crosa and Falkow (1981) as suitable for preparation of plasmid DNA up to 530kb from Gram-negative and Gram-positive bacteria. Unlike the other methods, it does not include enzyme treatment of the cells but relies on SDS alone to produce lysis. As with the following methods, covalently closed circular (ccc) plasmid DNA is enriched and separated from the majority of contaminating chromosomal DNA by raising the pH to 12.4, resulting in denaturation and strand separation of nicked or linear DNA - cccDNA is resistant to strand separation in alkaline conditions. On neutralisation, the chromosomal DNA forms an insoluble mesh, production of which is greatly facilitated by addition of sodium chloride to a 1M concentration. During centrifugation to remove chromosomal DNA, cccDNA remains in solution until addition of alcohol.

Bacterial strains were grown in 10ml BYGT broth at 37°C in an orbital incubator shaker at 200 rpm for 1.5 to 2h and harvested by centrifugation for 10 min at 7000 rpm in a Sorvall SA-600 rotor. Cells were resuspended in 1ml TE buffer (50mM Tris-HCl, 5mM disodium EDTA, pH 8.0), transferred to a 1.5ml eppendorf tube and pelleted by centrifugation for 5 min in an MSE microfuge. After resuspension in 40µl TE buffer, 0.6ml freshly prepared lysis buffer was added and gently mixed by inversion several times. Lysis buffer consisted of 4% (w/v) SDS in TE at pH 12.40, the pH of which was determined as accurately as possible since a pH of greater than 12.50 will irreversibly denature plasmid DNA. The sample was incubated with lysis buffer for 20 min at 37°C and neutralised by addition of 30µl 2M Tris-HCl pH 7.0, which was gently mixed by slowly inverting the tube until there was a noticeable change in viscosity. Chromosomal DNA was preferentially precipitated by addition of 240µl 5M sodium chloride and incubation for 1 to 4h at 4°C. The sample was centrifuged for 10 min in an MSE microfuge, the supernatant carefully poured into a new tube and 550µl cold isopropanol

added. After mixing, the sample was incubated at -20°C for 30 min, spun for 3 min in the microfuge and thoroughly drained. Drying of precipitated DNA was completed under vacuum and the precipitate resuspended in 30µl sterile TES (50mM Tris-HCl, 5mM disodium EDTA, 50mM sodium chloride, pH 8.0). If not used immediately, samples were stored at 4°C.

Dunny method

Preparation of plasmid DNA by this method was described by Dunny and coworkers (1981b) for use with group D streptococci and was carried out with minor modifications necessitated mainly by differences in available equipment.

Cultures were grown at 37°C in 25ml BYGT supplemented with 20mM threonine in an orbital incubator shaker at 200 rpm for 1.5 to 2h to give a mid-exponential phase culture. Cells were harvested as above, washed with 5ml TE buffer (50mM Tris-HCl, 5mM disodium EDTA, pH 8.0) and resuspended in 0.1ml TE buffer. To this suspension 25µl 40mg ml^{-1} lysozyme in TE buffer was added, mixed thoroughly and incubated for 20 min at 37°C. In order to lyse the cells and denature nicked or linear DNA, 2.4ml lysis buffer consisting of 1% (w/v) SDS in TE at pH 12.45 was added slowly, mixed gently by rotation and incubated for 20 min at 37°C. To neutralise, 0.4ml 2M Tris-HCl,pH 7.0 was added slowly with gentle but thorough mixing by inversion of the tube and chromosomal DNA was precipitated by addition of 0.3ml 5M sodium chloride followed by incubation at 4°C for 2 to 4h. Precipitate was removed by centrifugation for 20 min at 15000 rpm (Sorvall SA-600 rotor) and the supernatant was poured into a corex tube. At this stage, the RNA in the samples was sometimes removed by degradation with 150µl 2mg ml^{-1} ribonuclease 1 at 37°C for 30 min. The enzyme had been boiled for 10 min to remove contaminating DNases and stored frozen. The samples were deproteinised by extraction twice with an equal volume of

redistilled phenol saturated with 3% (w/v) sodium chloride as described above. In addition to extraction of proteins, particularly nucleases, it has been shown that single-stranded DNA, for example chromosomal DNA denatured after treatment with alkali, is preferentially entrapped in the precipitate at the interface between the phenol and aqueous layers and that 3% sodium chloride is the optimum concentration for this removal of denatured DNA (Currier and Nester, 1976). Residual phenol was removed by extraction with chloroform: isoamyl alcohol 24:1 (v/v). Throughout the extraction procedure wide bore Pasteur pipettes were used to transfer the aqueous phase to fresh tubes in order to minimise shearing. The DNA was precipitated by addition of 460µl 3M sodium acetate and 2 volumes cold 95% (v/v) ethanol followed by incubation at -20°C overnight or until the samples were required. After spinning for 20 min at 12000 rpm (Sorvall SA-600 rotor) and thorough draining, the DNA was resuspended in 100 to 200µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0).

Large scale preparation

The preparation of plasmid DNA on a large scale was carried out using a method modified from that described by Birnboim and Doly (1979) for isolation of plasmid DNA from Gram-negative bacteria. As with the previous method, chromosomal DNA denatured with alkali was removed by precipitation in high salt. The following solutions were used :

Solution I	sucrose	50mM
	Tris	25mM
	disodium EDTA	10mM
	pH 8.0	
Solution II	sodium hydroxide	0.2M
	SDS	1% (w/v)

Prepared by diluting stock solutions of 2M sodium hydroxide and 10% (w/v) SDS in distilled water.

Solution III 3M potassium acetate, pH 4.8.

The salt was dissolved in a minimal amount of distilled water, adjusted to the correct pH with glacial acetic acid and finally to the correct volume with distilled water.

Bacterial strains were grown in 1000ml BYGT in an orbital incubator shaker at 100 rpm for 5h. Cells were collected by centrifugation for 10 min at 7000 rpm in a Sorvall G5-3 rotor at 4°C, washed with 50ml 50mM Tris-HCl, pH 8.0 and resuspended in 50ml Solution I. To this suspension was added 250mg lysozyme which was dissolved and the suspension incubated at 37°C for 30 min or at 4°C for 30 min with Gram-negative strains. Proteinase K (10mg; Sigma) could be added at this stage and incubated for 60 min at 37°C. Lysis of the cells was completed by addition of 80ml fresh Solution II which was added slowly and mixed thoroughly followed by incubation for 10 min at 37°C. Incubation for Gram-negative organisms was for 5 min on ice. Chromosomal DNA was precipitated by addition of 40ml cooled Solution III which was thoroughly mixed and the sample incubated on ice for 15 min. Precipitate was removed by centrifugation for 5 min at 7000 rpm, the supernatant filtered through two layers of gauze and the volume measured. Isopropanol at -20°C was added at 0.6 volume (approximately 108ml), mixed and the sample spun immediately for 5 min at 7000 rpm. The supernatant was discarded and the precipitate thoroughly dried.

The precipitate was dissolved in 5ml TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) in preparation for caesium chloride-ethidium bromide density gradient centrifugation. More ethidium bromide intercalates into linear and open circular DNA than cccDNA hence the former types of DNA are less dense and form bands above cccDNA during equilibrium centrifugation. These bands can be visualised with ultraviolet light due to the fluorescence of the intercalated ethidium bromide.

The volume of samples dissolved in TE buffer was carefully measured, made up to 6.7ml with TE buffer and the sample added to 7.2g caesium chloride. This was dissolved before adding 0.7ml 3mg ml^{-1} ethidium bromide (Sigma). The mixture was poured into a 10ml polypropylene centrifuge tube (MSE) and the air expelled by addition of paraffin oil. Carefully balanced tubes were placed in an MSE 10 x 10ml rotor and spun at 20°C for 40h at 40,000 rpm. Bands were visualised using a 302nm ultraviolet lamp and the bands collected using a Pasteur pipette. The ethidium bromide was extracted several times by addition and mixing of butan-1-ol until the sample appeared colourless and the aqueous phase was dialysed at 4°C against 4 x 1000ml TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) overnight to remove the caesium chloride. If necessary, the DNA could be concentrated by precipitation with 2 volumes of ethanol as in previous methods. Samples were stored at 4°C.

AGAROSE GEL ELECTROPHORESIS

Preparation of agarose gels

Unless stated otherwise, the buffer used throughout for the preparation and electrophoresis of gels was TBE (89mM Tris, 2.5mM disodium EDTA, 8.9mM boric acid, pH 8.1). Electrophoretic grade agarose (BRL) was dissolved in running buffer by boiling to give gels between 0.35 and 0.8% (w/v). The apparatus used for horizontal gels was manufactured by BRL and was of two sizes. The smaller model H4, allowed gels of dimension 14cm x 11cm with a total gel volume of 100ml or 120ml giving a thickness of 7 to 8mm with 14 wells of 4mm x 2mm while gels of the larger model HO were 25cm x 20cm with a total volume of 200ml or 300ml resulting in a thickness of 4mm or 6mm. The slot former of the larger apparatus produced 20 wells of dimensions 6.4mm x 1mm or 6.4mm x 3mm. Molten agarose was cooled to 60°C before pouring into the plexiglass trays, the ends of which

had been sealed with tape. When the gel had solidified, the tape and slot former was removed and the gel submerged in running buffer.

Vertical gels were prepared by pouring molten agarose over a plug which had been previously poured and solidified and which consisted of 1.0g cyanogum, 0.1g ammonium persulphate and 40µl N,N,N',N'-tetramethyl-ethylenediamine in 10ml TBE. The slot former was positioned to provide sample wells and removed after the agarose had solidified.

Sample preparation, loading and electrophoresis

Aliquots of DNA samples were adjusted to the same volume by addition of buffer and prepared for loading on to the gels by addition and mixing of 0.1 volume of dye solution. The dye solution consisted of 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue in 50% (v/v) glycerol, or latterly 0.05% bromophenol blue in 30% (w/v) sucrose.

Before loading the samples, wells were filled with running buffer ensuring that there were no air bubbles present. After carefully loading the samples avoiding any spillage, the horizontal gel apparatus was attached to a power supply and a maximum current applied, usually 80mA for the small gel and 120mA for the large gel, until the dye had migrated into the gel in order to minimise the risk of loss of DNA due to diffusion out of the well. Electrophoresis was carried out at 1.5 to 2V per cm until the bromophenol blue neared the bottom of the gel. Electrophoresis conditions for vertical gels have been described previously.

Determination of plasmid sizes

Following electrophoresis, the agarose gel was placed in a solution of 0.5mg ml^{-1} ethidium bromide for 20 to 30 min, washed with tap water and the DNA visualised by transillumination (Model C-61 transilluminator, U.V. Products, U.S.A.) using an ultraviolet lamp (302nm). Photographs of the gels were taken with a Polaroid Land camera through a Kodak Wrattan

No. 22A yellow filter using Polaroid type 57 or type 667 film for instant photographs and Ilford FP4 film with a 4 to 8 min exposure. The mobility of DNA bands was measured in all cases from photographs of approximately the same size as the original gel.

The plasmids shown in Table 3 of known size were used for the construction of standard curves from which to extrapolate the size of unknown samples, since this is inversely proportional to migration rate. At low voltage gradients, a plot of size against the reciprocal of the mobility (m) gave a straight line while at higher voltage gradients, size against 1/m-mo where mo is a constant calculated from standard values was plotted as described by Southern (1979).

The standards used for the determination of length of linear DNA were, HindIII restriction endonuclease fragments of bacteriophage λ obtained from BRL and stored at -20°C. Standard curves were constructed as for cccDNA with fragment sizes 23.13kb, 9.42kb, 6.68kb, 4.36kb, 2.32kb and 2.03kb.

RESTRICTION ENDONUCLEASE DIGESTION

Two restriction enzymes, EcoRI and PstI from BRL were used to digest DNA samples obtained from bacterial strains by the method of Dunny (1981b). As instructed by the manufacturer, the reactions were carried out in buffers which were stored at -20°C at 10 times concentration. Final concentrations in the reaction mixtures are shown in Table 4.

Reaction mixtures consisted of :

40µl sample
5µl 10 x reaction buffer
4µl distilled water
1µl restriction endonuclease

Table 3. DNA of known size used in construction of standard curves

cccDNA species	Size (kb)	Source or Reference
pGC9114	65.90	C. Drainas, University of Ioannina, Greece.
RP4	51.50	Plasmid Reference Center, Stanford University Medical School.
Sa	37.88	Plasmid Reference Center
pAJ50	15.30	Jimenez and Davies (1980)
pBR325 containing Neurospora HindIII glutamate dehydrogenase fragment	13.94	J.R.S. Fincham, University of Edinburgh
pDB248	10.10	Beach and Nurse (1981)
pBR325 containing Neurospora crassa BamHI glutamate dehydrogenase fragment	7.88	J.R.S. Fincham, University of Edinburgh
pHC79	6.44	Hohn and Collins (1980)
pBR322	4.36	Rosenberg and Queen (1981)

Table 4. Restriction endonucleases EcoRI and PstI

Restriction endonuclease	Recognition sequence*	Specific activity	Reaction buffer
EcoRI	5'-G↓AATTC-3' 3'-CTTAA↑G-5'	10 units μl^{-1}	Tris-HCl 100mM, pH 7.5 Magnesium chloride 10mM Sodium chloride 50mM
PstI	5'-CTGCA↓G-3' 3'-G↑ACGTC-3'	9 units μl^{-1}	Tris-HCl 50mM, pH 8.0 Magnesium chloride 10mM Sodium chloride 50mM

* Arrow indicates cleavage site.

Mixtures containing EcoRI were incubated at 37°C for 2h and the reaction stopped by incubation for 10 min at 65°C followed by gentle cooling at 37°C for 10 min and storage on ice until preparation for agarose gels as described previously. Mixtures containing PstI were incubated at 30°C for 2h followed by treatment as for EcoRI. Given that digestions were carried out for 2h with excess enzyme, it was expected that digestion would be complete.

RESULTS

PHENOTYPE OF BACTERIAL STRAINS

Minimal inhibitory concentrations

The minimal inhibitory concentrations (MICs) of 12 antibiotics are shown in Table 5. Results obtained for JH2-2 were the same as those previously published results (Jacob and Hobbs, 1974) or were within a two-fold variation, such small differences probably being due to a different method of MIC determination. For the antibiotics erythromycin, lincomycin and the aminoglycoside-aminocyclitol group, strains were considered to be resistant if the MIC was $\geq$ 1000μg ml^{-1}, for tetracycline $\geq$ 64μg ml^{-1}, for pristinamycin > 40μg ml^{-1}, for benzylpenicillin and ampicillin 10μg ml^{-1}, and for chloramphenicol > 40μg ml^{-1}. These values for enterococcal resistance were a consensus based on previous studies (Taola et al., 1969; Clewell et al., 1974; Jacob and Hobbs, 1974; Van Embden et al., 1977; Courvalin et al., 1978; Bayne et al., 1983; Combes et al., 1983). Strain DS5 was, as expected, resistant to erythromycin, lincomycin, pristinamycin (MLS antibiotics) and tetracycline only.

All isolates in this study were resistant to tetracycline with MIC values up to 800 times the value of the susceptible strain JH2-2. Likewise all strains were insensitive to MLS antibiotics except strain K46 which was resistant to erythromycin and lincomycin but susceptible to pristinamycin, and strain SB69 which was susceptible to all three MLS antibiotics. Of the aminoglycoside-aminocyclitol antibiotics tested, all strains were susceptible to gentamicin (MIC < 64μg ml^{-1}) and and spectinomycin (MIC < 40μg ml^{-1}) and strains K55, K88, and SB69 were susceptible to kanamycin (MIC < 320μg ml^{-1}) and neomycin (MIC $\leq$ 160μg ml^{-1}). All the strains were however resistant to streptomycin, strains K60, K87, SB94 and K46 being in addition resistant to kanamycin and neomycin. Strain K88 was the only isolate which was resistant to chloramphenicol and the two S. faecium strains K46 and SB69 were the only strains which showed resistance to penicillin and ampicillin.

Table 5. Minimal inhibitory concentration of twelve antibiotics for strains of group D streptococci

Antibiotic	MIC ($\mu g\ ml^{-1}$) for strain								
	JH2-2	DS5	K55	K60	K87	K88	SB94	K46	SB69
Erythromycin	0.25(0.125)	>1000	>1000	>1000	>1000	>1000	>1000	1000	0.5
Lincomycin	80	>1280	>1280	>1280	>1280	>1280	>1280	1280	20
Pristinamycin	5	>40	>40	>40	>40	>40	>40	2.5	2.5
Tetracycline	ND (0.625)	150	64	512	512	512	512	64	64
Streptomycin	40(40)	40	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Kanamycin	80(40)	40	80	>1280	>1280	40	>1280	>1280	320
Neomycin	40(30)	80	80	>1280	>1280	80	>1280	>1280	160
Gentamicin	ND (20)	ND	32	16	16	16	16	64	16
Spectinomycin	40	40	40	40	40	40	40	40	20
Penicillin	2	2	2	2	2	2	2	40	40
Ampicillin	1	1	1	1	2	2	1	10	10
Chloramphenicol	5	10	10	10	10	80	10	20	5

ND = Value not determined. Values in parenthesis are those published by Jacob and Hobbs, 1974.

Production of haemolysin and bacteriocin

On nutrient medium containing horse blood, only strains DS5 and K87 were β-haemolytic and strain SB69 alone was α-haemolytic.

When bacteriocin production was tested using strain JH2-1 as indicator organism, strains DS5 and K87 were unequivocally bacteriocinogenic with zones of clearing around stab inocula of between 2 and 5mm diameter, while strain SB94 appeared to be weakly bacteriocinogenic producing inhibition of JH2-1 within a 1mm diameter zone. With strain K87I0 as indicator organism, stab inocula of strains DS5 and SB94 were surrounded by clear zones of 2 to 4mm diameter. In one experiment as expected, strain K87 produced no bacteriocin against K87I0 but surprisingly in another experiment appeared to produce a very weak bacteriocin. In this case, the zone of clearing was barely discernable but was plainly different when compared with growth of the indicator organism around the known non-producer JH2-1. None of the other strains produced detectable bacteriocin against JH2-1 or K87I0.

Mode of expression of erythromycin resistance

Figure 1 shows the results of a typical experiment to determine whether erythromycin resistance was inducible or constitutive. Growth, measured as absorbance at 610nm, was plotted against time for cultures grown in the presence or absence of a concentration of drug below that which inhibits growth of susceptible cells, followed by challenge with a drug concentration many times higher. Only the data for strains DS5 which is known to display constitutive resistance (Yagi et al., 1975), and K55 are shown, as all the strains produced similar results.

At the start of the logarithmic growth phase, the culture was divided equally and the inducer, erythromycin, added to one aliquot to give 0.05μg ml^{-1}. There was no detectable inhibition of growth by this concentration of antibiotic. After further growth, challenge of two aliquots in mid-

Figure 1a. Effect on growth of addition of 100µg ml^{-1} erythromycin to cultures of DS5 grown in the presence or absence of 0.05µg ml^{-1} erythromycin.

At 80 min, the culture was divided into three equal aliquots and inducer (0.05µg ml^{-1} erythromycin) was added to one aliquot. At 140 min, the induced culture and one other aliquot (uninduced) were challenged with addition of 100µg ml^{-1} erythromycin.

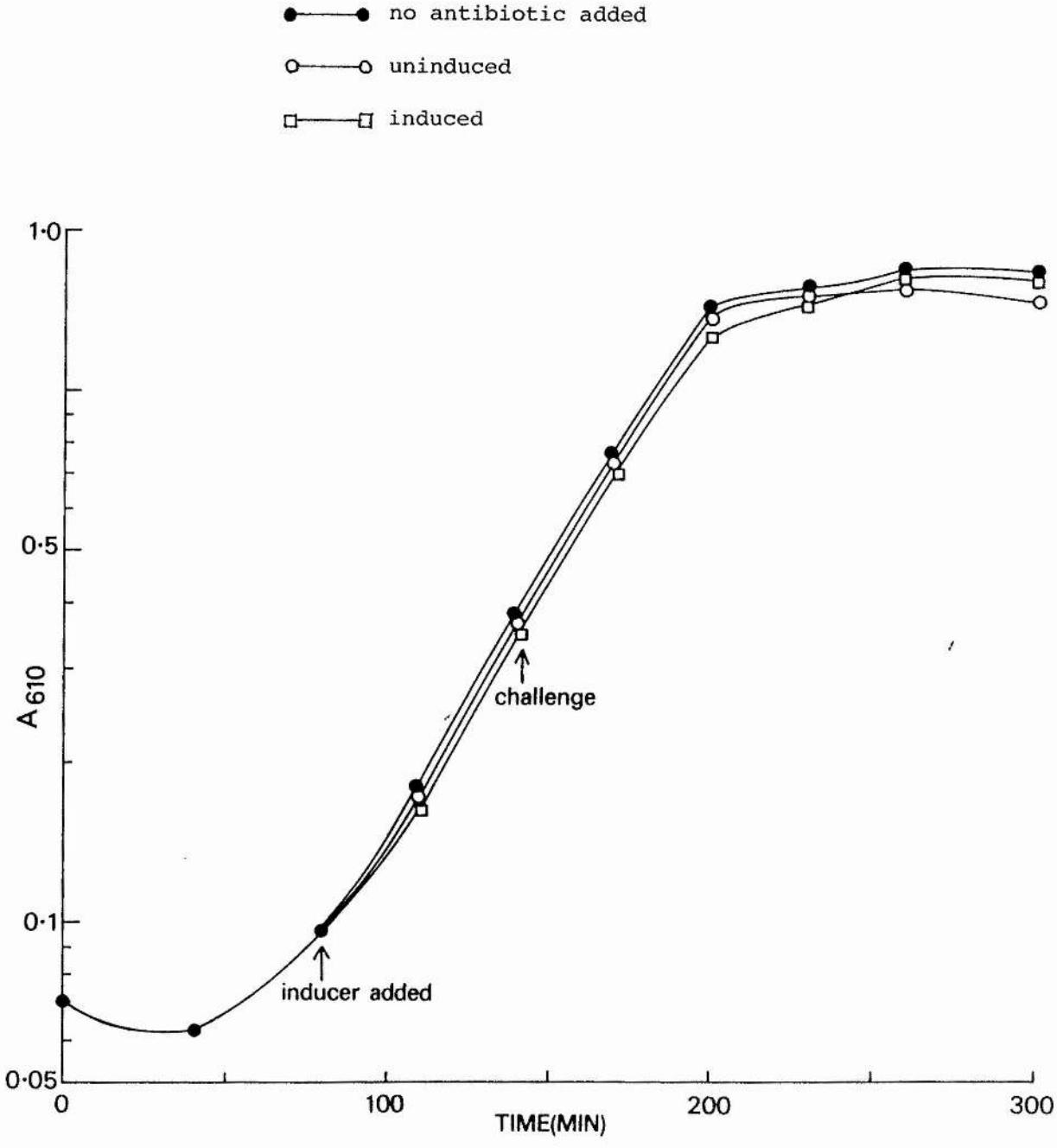
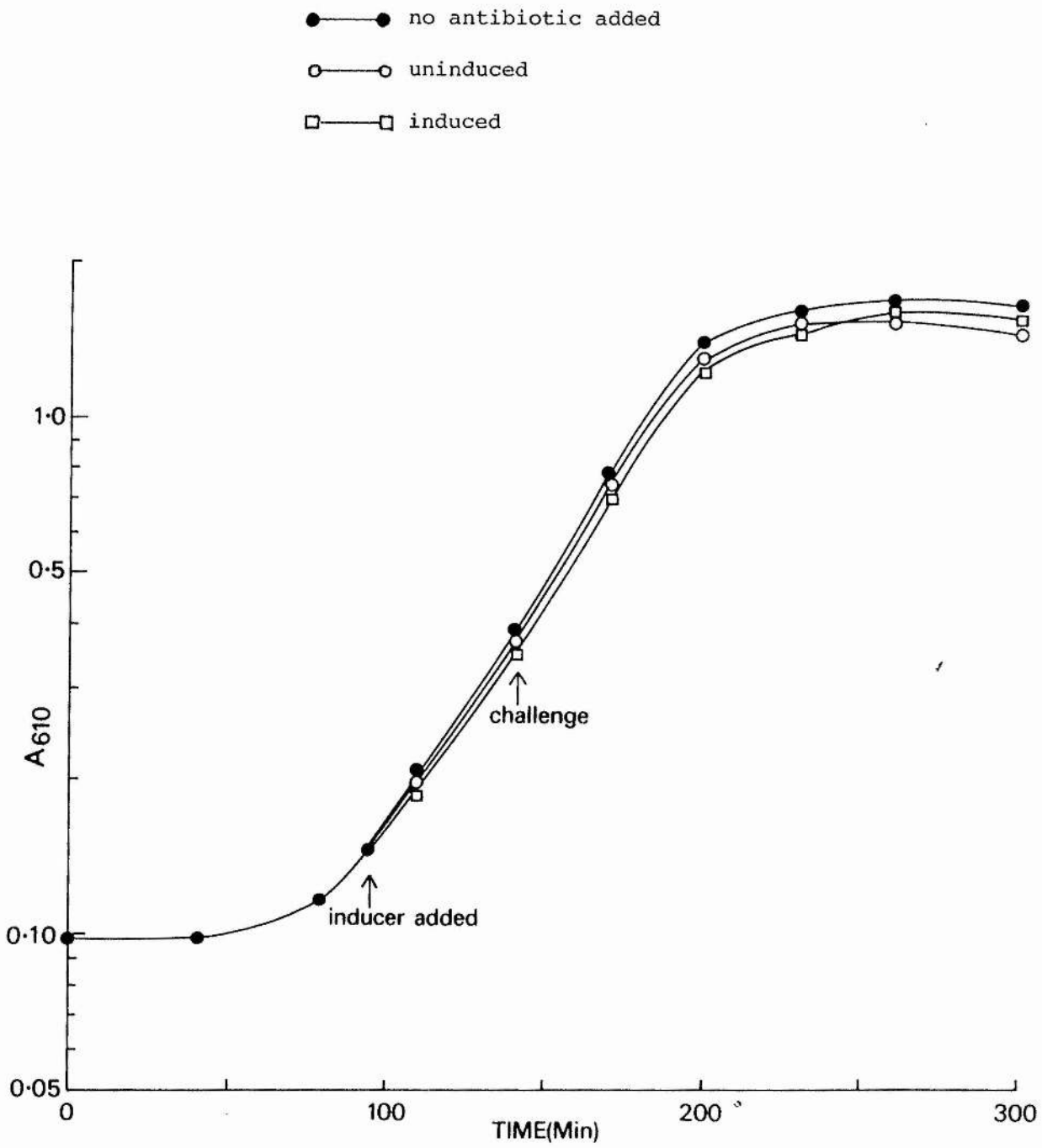


Figure 1b. Effect on growth of addition of 100µg ml^{-1} erythromycin to cultures of K55 grown in the presence or absence of 0.05µg ml^{-1} erythromycin.

At 95 min, the culture was divided into three equal aliquots and inducer (0.05µg ml^{-1} erythromycin) was added to one aliquot. At 140 min, the induced culture and one other aliquot (uninduced) were challenged with the addition of 100µg ml^{-1} erythromycin.



logarithmic phase, with 100μg ml^{-1} erythromycin did not cause any effect on the rate of growth of induced or uninduced culture nor was there any difference in growth rate between those aliquots challenged and the control which had no antibiotic added. Had resistance been inducible, it would have been expected that under these conditions, challenge of uninduced culture would have resulted in inhibition of growth followed by an increase in growth rate after a lag period while challenge of induced culture would have had no effect (Yagi et al., 1975).

Mode of expression of tetracycline resistance

Figure 2 shows the effect of challenge with 20μg ml^{-1} tetracycline of strains DS5 and K55 grown in the presence or absence of an inducing concentration (0.1μg ml^{-1}) of tetracycline. On addition of 20μg ml^{-1} tetracycline, there was no interruption of growth in either culture nor was there any difference between those cultures challenged and the control with no antibiotic added. The other strains gave similar results to DS5 and K55 the former of which is known to express tetracycline resistance constitutively (Burdett, 1980).

Mode of expression of chloramphenicol resistance

The effect of challenge with 20μg ml^{-1} chloramphenicol of strain K88 grown in the presence or absence of 5μg ml^{-1} chloramphenicol is shown in Figure 3. Addition of the challenge concentration of drug has no effect on the culture previously exposed to the inducing concentration of 5μg ml^{-1} chloramphenicol. In contrast, challenge of the culture which was not induced by prior growth in 5μg ml^{-1} drug resulted in a marked decrease in growth rate followed by a gradual resumption of normal growth after approximately 90 min.

Figure 2a. Effect on growth of addition of 20µg ml^{-1} tetracycline to cultures of DS5 grown in the presence or absence of 0.1µg ml^{-1} tetracycline.

At 30 min, the culture was divided into three equal aliquots and inducer (0.1µg ml^{-1} tetracycline) was added to one aliquot. At 90 min, the induced culture and one other aliquot (uninduced) were challenged with addition of 20µg ml^{-1} tetracycline.

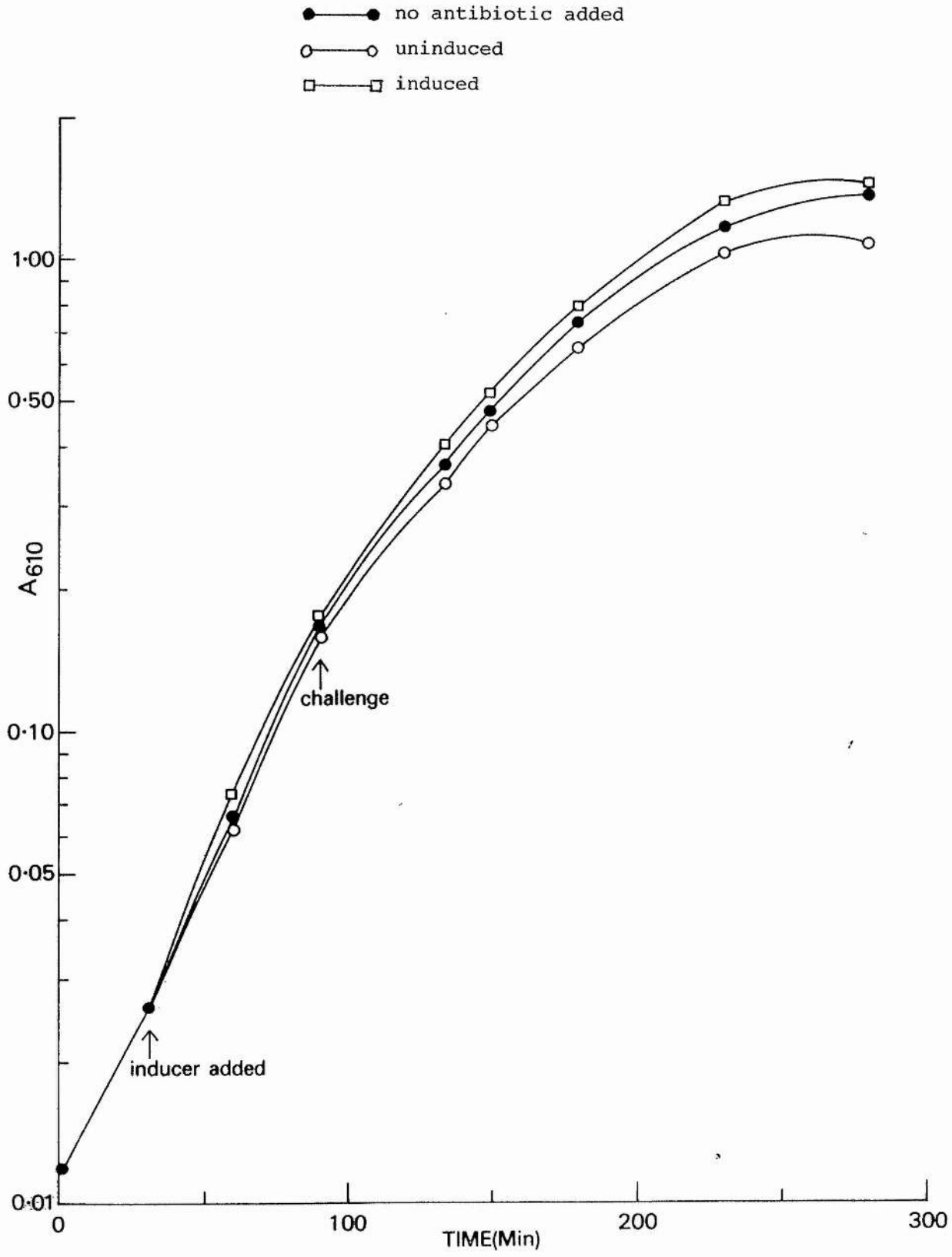


Figure 2b. Effect on growth of addition of 20µg ml^{-1} tetracycline to cultures of K55 grown in the presence or absence of 0.1µg ml^{-1} tetracycline.

At 30 min, the culture was divided into three equal aliquots and inducer (0.1µg ml^{-1} tetracycline) was added to one aliquot. At 90 min, the induced culture and one other aliquot (uninduced) were challenged with addition of 20µg ml^{-1} tetracycline.

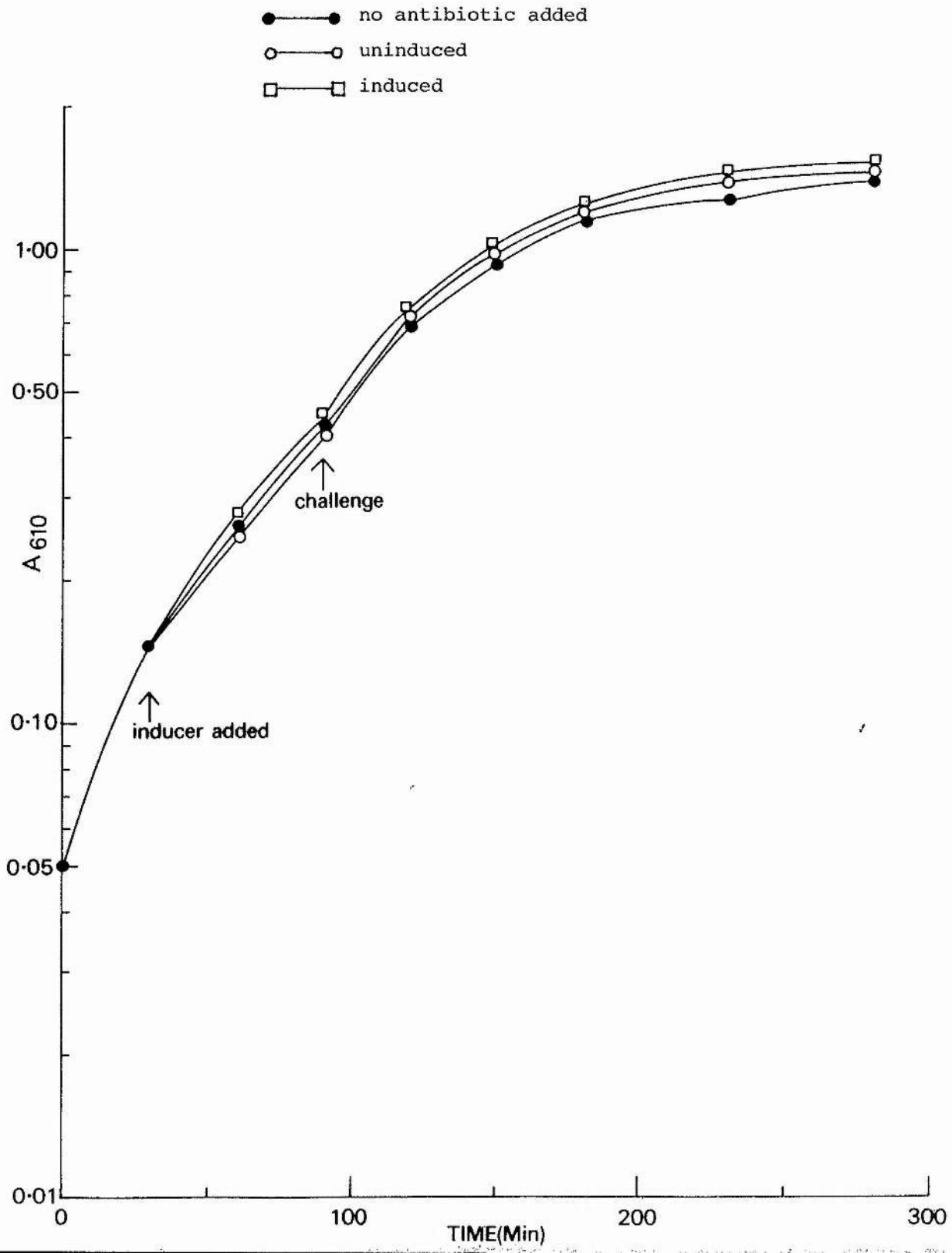
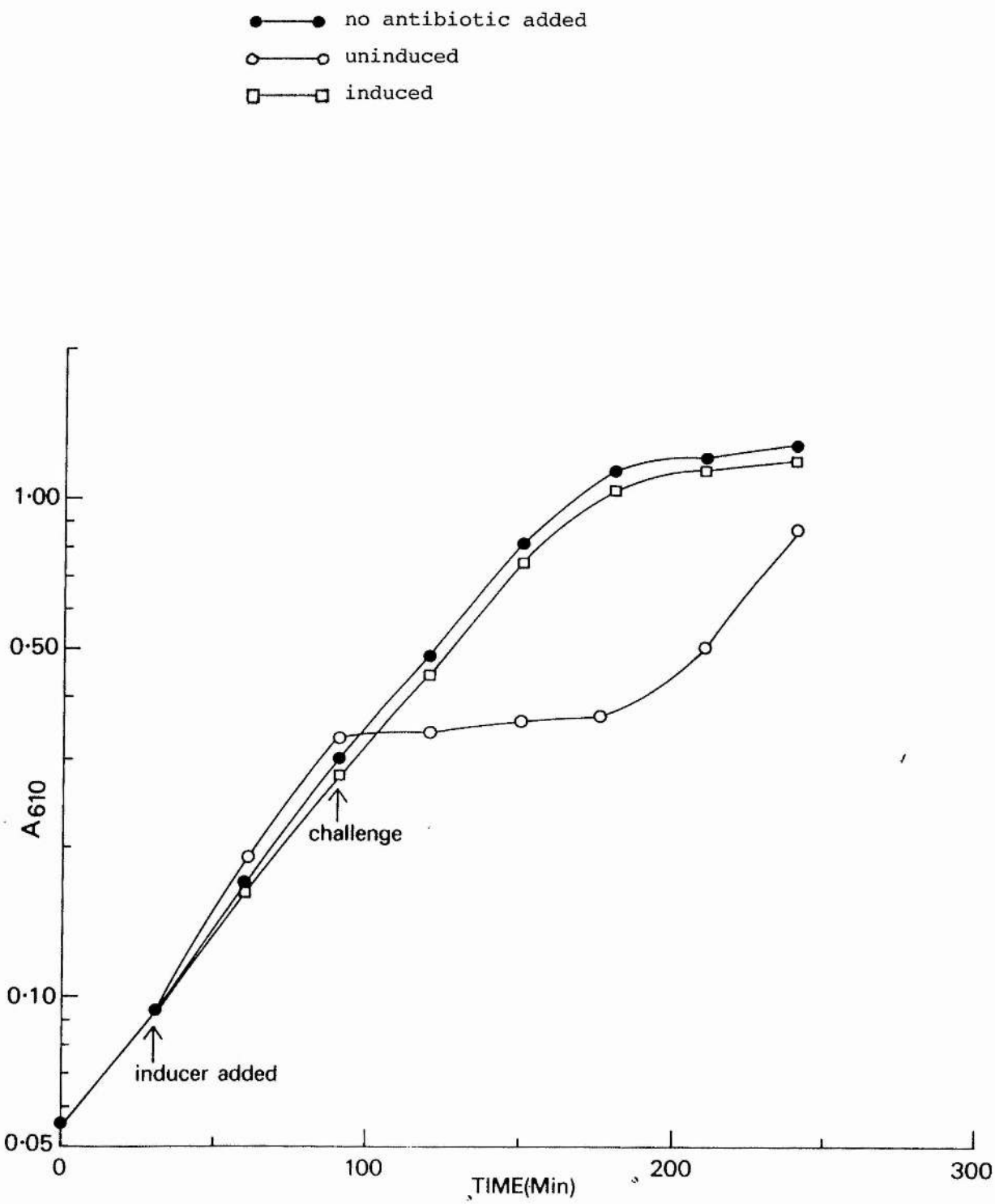


Figure 3. Effect on growth of addition of 20µg ml^{-1} chloramphenicol to cultures of K88 grown in the presence or absence of 5µg ml^{-1} chloramphenicol.

At 30 min, the culture was divided into three equal aliquots and inducer (5µg ml^{-1} chloramphenicol) was added to one aliquot. At 90 min, the induced culture and one other aliquot (uninduced) were challenged with addition of 20µg ml^{-1} chloramphenicol.



Beta-lactamase activity

Strains K46 and SB69 which were found to be resistant to penicillin and ampicillin were tested for the production of β-lactamase. On Beta-test and Intralactam strips, only the penicillinase producing E. coli control produced a change in colour from purple to yellow. As the strips are known to be ineffective in detecting β-lactamase activity of Gram-positive Staph. aureus, all strains were also tested using nitrocefin. With this substrate, the β-lactamase producing controls immediately turned the solution from straw coloured to red but none of the streptococci produced any change even after incubation at 37°C for 30 min.

Phenotype of bacterial strains

Table 6 was compiled from the information above and shows the relevant phenotypes of the strains in this study. All the strains expressed antibiotic resistance constitutively with the exception of inducible chloramphenicol resistance of strain K88. The strains showed multiple antibiotic resistance to between 4 and 8 drugs and in addition some were haemolytic or produced bacteriocin. No two strains had exactly the same phenotype.

TRANSFER EXPERIMENTS

Conjugation in broth culture

The transfer frequencies of phenotypic markers expressed as transconjugant colonies per donor colony forming unit after 4h broth culture matings are shown in Table 7. Throughout the description and discussion of results, values greater than 1×10^{-3} will be referred to as high frequency transfer, values between 1×10^{-6} and 9×10^{-4} as intermediate frequency transfer, and values less than 9×10^{-7} as low frequency transfer.

Table 6. Phenotype of bacterial strains used as donors in mating experiments

Strain	Antibiotic resistance phenotype	Other relevant phenotypic traits
DS5	Em Lm Pr Tc	Hly, Bcn
K55	Em Lm Pr Tc Sm	-
K60	Em Lm Pr Tc Sm Km Nm	-
K87	Em Lm Pr Tc Sm Km Nm	Hly, Bcn
K88	Em Lm Pr Tc Sm Cm(i)	-
SB94	Em Lm Pr Tc Sm Km Nm	Bcn
SB69	Ap Pn Sm Tc	αHly
K46	Em Lm Tc Sm Km Nm Ap Pn	-

Em, erythromycin; Lm, lincomycin; Pr, pristinamycin; Tc, tetracycline; Sm, streptomycin; Km, kanamycin; Nm, neomycin; Cm, chloramphenicol; Ap, ampicillin; Pn, penicillin ; Hly, β-haemolytic; Bcn, bacteriocinogenic; αHly, α-haemolytic.

Inducible nature of resistance shown by (i). All other resistances constitutive.

Results calculated for transfer of DS5 markers (Table 7a) in experiments 1, 2, 4 and 5 are in broad agreement with published observations (Dunny and Clewell, 1975), haemolysin activity being transferred at high frequency (10^{-3} to 10^{-2}), tetracycline resistance transferred at intermediate or low frequency (5×10^{-8} to 10^{-6}) and erythromycin resistance transfer undetected. During these experiments, the recipient viable count decreased by a factor of 10 to 50 from an initial count of approximately 5×10^{7} cfu ml^{-1} in experiments 1, 2 and 4 and 3×10^{8} cfu ml^{-1} in experiment 5, due to the production of bacteriocin by DS5 which is active against JH2 derivatives. Experiment 3 was atypical in that recipient viable count did not decrease during incubation. Concomitantly, there was a 100-fold increase in the frequency of transfer of haemolytic activity, a 10-fold increase in tetracycline resistance transfer and transfer of erythromycin resistance occurred at an intermediate frequency of 1.6×10^{-6}. This latter result is particularly unusual as transfer of the MLS plasmid, pAMβ1, is normally inhibited by pAMγ1 (Clewell, 1981).

Transfer of erythromycin resistances from K55 to JH2-2 occurred at intermediate frequency in all experiments and at low frequency with JH2-17 as recipient (Table 7b). Tetracycline resistance was also transferable in experiments 1, 4 and 5 at low frequency and experiment 2 at intermediate frequency but no transfer occurred in experiments 3 or 6. In the latter experiment, the transfer frequency of erythromycin was also atypically low indicating inefficient transfer in this case, but there was no obvious reason for not obtaining tetracycline resistant transconjugants in experiment 3. Transfer of streptomycin resistance was very unpredictable with experiments 1, 2, 3 and 5 yielding no streptomycin resistant transconjugants and low frequency transfer in experiments 4 and 6.

Transfer of erythromycin resistance from K60 to JH2-2 and JH2-17 (Table 7c) was always in the high frequency range although there was considerable

Table 7 a. Transfer frequencies of DS5 markers per donor colony forming unit after 4 h mating in broth culture

Expt. No.	Recipient	Final viable count (cfu ml^{-1})		Transfer frequencies per donor of selected markers		
		Donor	Recipient	Em or Lm	Tc	Hly
1	JH2-2	1.38×10^{8}	6.00×10^{6}	NO	6.24×10^{-7}	9.53×10^{-3}
2	JH2-2	4.60×10^{7}	1.60×10^{6}	NO	1.67×10^{-6}	4.34×10^{-3}
3	JH2-2	4.20×10^{7}	4.00×10^{8}	1.60×10^{-6}	1.70×10^{-5}	2.50×10^{-1}
4	JH2-2	1.60×10^{8}	3.60×10^{6}	NO	1.00×10^{-6}	3.10×10^{-3}
5	JH2-17	1.72×10^{9}	5.60×10^{7}	NO	4.60×10^{-8}	1.50×10^{-3}

NO =no transconjugant colonies obtained.

Table 7 b. Transfer frequencies of K55 markers per donor colony forming unit after 4 h mating in broth culture

Expt. no.	Final viable count (cfu ml^{-1})			Transfer frequencies per donor of selected markers		
	Recipient	Donor	Recipient	Em or Lm	Tc	Sm
1	JH2-2	1.00×10^{8}	1.28×10^{9}	5.85×10^{-6}	5.50×10^{-7}	NO
2	JH2-2	6.00×10^{7}	1.30×10^{9}	2.53×10^{-5}	6.00×10^{-6}	NO
3	JH2-2	6.20×10^{7}	3.20×10^{8}	3.20×10^{-5}	NO	NO
4	JH2-2	4.00×10^{7}	7.20×10^{8}	4.60×10^{-5}	3.80×10^{-7}	6.20×10^{-7}
5	JH2-2	1.70×10^{8}	3.60×10^{8}	1.60×10^{-5}	1.50×10^{-7}	NO
6	JH2-17	2.02×10^{9}	3.00×10^{9}	2.20×10^{-8}	NO	7.40×10^{-9}

NO = no transconjugant colonies obtained.

Table 7 c. Transfer frequencies of K60 markers per donor colony forming unit after 4 h mating in broth culture

		Final viable count (cfu ml^{-1})		Transfer frequencies per donor of selected markers				
Expt.no.	Recipient	Donor	Recipient	Em or Lm	Tc	Sm	Km	Nm
1	JH2-2	1.62×10^{7}	1.48×10^{9}	8.33×10^{-2}	6.05×10^{-3}	6.05×10^{-3}	1.60×10^{-2}	3.09×10^{-2}
2	JH2-2	1.60×10^{7}	1.12×10^{9}	4.15×10^{-1}	7.50×10^{-3}	1.10×10^{-2}	1.60×10^{-2}	4.00×10^{-2}
3	JH2-2	1.90×10^{8}	6.40×10^{8}	2.90×10^{-2}	3.20×10^{-5}	1.80×10^{-4}	3.50×10^{-4}	6.80×10^{-4}
4	JH2-17	3.80×10^{8}	2.88×10^{9}	1.30×10^{-3}	6.60×10^{-5}	2.10×10^{-4}	3.30×10^{-4}	3.95×10^{-4}

variation (1×10^{-3} to 5×10^{-1}). In experiments 1 and 2, tetracycline resistance was transferred at high frequency but in experiments 3 and 4 at intermediate frequency and this trend in the latter two experiments, was continued with transfer of streptomycin, kanamycin and neomycin indicating that the overall efficiency of transfer in experiments 3 and 4 was less. Generally the frequency of streptomycin resistance transfer was less than that of kanamycin resistance which was less than that of neomycin resistance but the values for transfer frequencies of aminoglycoside resistances were closer to each other than to the values for transfer of tetracycline or erythromycin resistances.

During broth matings with K87 as donor in Table 7d, only haemolysin activity was repeatedly transferred with high frequency to JH2-2 and intermediate frequency to JH2-17. The latter value however could be an underestimate due to the difficulty described in Materials and Methods in visualising β-haemolysis in blood agar containing rifampicin. In experiments 1 and 3 no transfer of antibiotic resistance was observed and, as occurred in DS5 matings, the recipient viable count decreased during incubation probably due to the action of donor bacteriocin. In experiment 2 however, transfer of all antibiotic resistances occurred at similar intermediate frequencies and in experiment 4, erythromycin and tetracycline resistances were transferred at low frequency. The recipient viable count during both these experiments did not show the expected decrease.

Resistance transfer from the K88 donor shown in Table 7e was very inconsistent and always at low frequency. Only tetracycline resistance was transferred in all experiments, erythromycin resistance transferred in four experiments, chloramphenicol resistance in three and streptomycin resistance in two. There did not appear to be any relationship between transfer frequencies of the different antibiotic resistance markers.

Table 7d. Transfer frequencies of K87 markers per donor colony forming unit after 4h mating in broth culture

Expt. No.	Recipient	Final viable count (cfu ml^{-1})		Transfer frequencies per donor of selected markers					
		Donor	Recipient	Em or Lm	Tc	Sm	Km	Nm	Hly
1	JH2-2	7.80×10^{7}	4.60×10^{5}	NO	NO	NO	NO	NO	1.67×10^{-3}
2	JH2-2	4.20×10^{7}	1.34×10^{9}	1.45×10^{-5}	1.70×10^{-5}	1.80×10^{-5}	3.70×10^{-5}	2.80×10^{-5}	4.80×10^{-2}
3	JH2-2	2.30×10^{7}	$<10^{5}$	NO	NO	NO	NO	NO	1.30×10^{-3}
4	JH2-17	2.68×10^{8}	1.92×10^{8}	5.60×10^{-6}	1.70×10^{-6}	1.90×10^{-8}	3.8×10^{-8}	3.8×10^{-8}	5.60×10^{-5}

NO = no transconjugant colonies obtained

Table 7e. Transfer frequencies of K88 markers per donor colony forming unit after 4h mating in broth culture

Expt. No.	Recipient	Final viable count (cfu ml^{-1})		Transfer frequencies per donor of selected markers			
		Donor	Recipient	Em or Lm	Tc	Sm	Cm
1	JH2-2	9.00×10^{7}	1.62×10^{9}	1.10×10^{-7}	2.20×10^{-7}	1.10×10^{-7}	2.20×10^{-7}
2	JH2-2	1.28×10^{8}	1.28×10^{9}	1.17×10^{-7}	3.90×10^{-8}	NO	3.90×10^{-8}
3	JH2-2	4.00×10^{8}	1.98×10^{9}	NO	5.00×10^{-8}	NO	NO
4	JH2-2	4.60×10^{8}	3.60×10^{7}	2.20×10^{-8}	1.10×10^{-8}	NO	NO
5	JH2-17	7.20×10^{8}	8.00×10^{9}	6.20×10^{-8}	8.30×10^{-8}	1.40×10^{-7}	2.20×10^{-7}

NO = no transconjugant colonies obtained

The resistance markers of strain SB94 (Table 7f) all transferred at intermediate frequency to JH2-2 (experiments 1 and 2) with the frequency of transfer of erythromycin resistance consistently higher than other markers and the frequencies of aminoglycosides resistance transfer almost identical. Mating with JH2-17 (experiment 3) resulted in high frequency transfer of erythromycin and aminoglycosides resistances and intermediate frequency transfer of tetracycline resistance, the same general trends being observed as with the JH2-2 recipient. Although the donor was found to produce bacteriocin against a JH2 derivative, the recipient viable counts do not decrease and instead give evidence of growth as is usual for matings with bacteriocin non-producers. Possibly as SB94 was only weakly bacteriocinogenic against JH2-1, the rate of killing of recipient was less than the growth rate or less than the rate of acquisition of bacteriocin resistance.

Matings of the S. faecium strains K46 and SB69 with JH2-2 were attempted in three separate experiments. In no case was transfer of any of the donor markers detected in broth matings.

Conjugation on membrane filters

Table 8 shows the results of individual experiments involving the mating of group D streptococcal isolates with JH2 recipient strains. The transfer frequencies are expressed as the number of transconjugant colony forming units per donor colony forming unit after 18h mating on membrane filters. Definitions of high, intermediate and low frequencies are the same as those given in the previous section.

The transfer of β-haemolytic activity from DS5 shown in Table 8a was demonstrated on membrane filters at high frequency within the same 10-fold range as in broth matings, while tetracycline resistance was transferred at intermediate frequencies, somewhat higher than those obtained in mixed broth culture. In striking contrast to broth matings, erythromycin

Table 7f. Transfer frequencies of SB94 markers per donor colony forming unit after 4h mating in broth culture

Expt. No.	Recipient	Final viable count (cfu ml^{-1})		Transfer frequencies per donor of selected markers				
		Donor	Recipient	Em or Lm	Tc	Sm	Km	Nm
1	Jh2-2	5.26×10^{8}	6.20×10^{8}	1.25×10^{-4}	3.60×10^{-5}	1.70×10^{-5}	2.70×10^{-5}	2.70×10^{-5}
2	JH2-2	5.00×10^{8}	9.60×10^{8}	1.00×10^{-4}	2.00×10^{-6}	2.80×10^{-5}	3.00×10^{-5}	3.00×10^{-5}
3	JH2-17	7.00×10^{8}	3.54×10^{9}	1.10×10^{-3}	4.70×10^{-4}	2.00×10^{-3}	2.00×10^{-3}	2.00×10^{-3}

Table 8a. Transfer frequencies of DS5 markers per donor colony forming unit after 18h mating on membrane filters

Expt. No.	Recipient	Final viable count (cfu ml^{-1})		Transfer frequency per donor of selected markers		
		Donor	Recipient	Em or Lm	Tc	Hly
1	JH2-2	1.40×10^{8}	4.80×10^{8}	4.55×10^{-5}	1.10×10^{-4}	8.60×10^{-3}
2	JH2-2	2.80×10^{8}	3.80×10^{8}	5.70×10^{-6}	7.10×10^{-4}	1.40×10^{-3}
3	JH2-2	1.06×10^{9}	4.80×10^{8}	4.70×10^{-7}	4.70×10^{-5}	2.30×10^{-3}
4	JH2-2	3.00×10^{8}	1.70×10^{8}	5.70×10^{-7}	2.00×10^{-5}	2.70×10^{-2}
5	JH2-17	5.24×10^{9}	1.12×10^{8}	3.80×10^{-5}	ND	ND

ND = not determined

resistance transfer occurred reproducibly at low or intermediate frequency and again, in contrast to broth mating experiments number 1, 2, 4 and 5, there was no decrease in the viable count of the recipient strain after 18h incubation on filters with the bacteriocinogenic donor. It has been suggested by LeBlanc and Lee (1982) that this latter effect occurs either because there is less bacteriocin produced under these conditions or because the bacteriocin may be effectively diluted by diffusion into the supporting solid medium.

In all experiments in Table 8b, erythromycin resistance was transmissible from K55 at intermediate frequency and in the same frequency range as that obtained during broth matings and likewise, transfer of tetracycline resistance was at the low to intermediate frequency seen during broth matings but occurred more reproducibly on filters. Streptomycin resistant transconjugants were isolated in five of the seven experiments at frequencies which varied 100-fold from low to intermediate but there was no correlation between efficient transfer of erythromycin and tetracycline resistances and transfer of streptomycin resistance with the possible exception of experiment 4, in which transfer of all resistances was slightly higher than usual.

The frequency of erythromycin resistance transfer from K60 (Table 8c) was very high and three of four experiments reached a value of 1.0, i.e. all the recipients received erythromycin resistance, representing an increase in frequency of 10- to 1000-fold over the frequencies obtained in broth matings. Tetracycline resistance transferred also at high frequency on filters and, although values were 10- to 1000-fold less than erythromycin, there was also a trend towards higher frequencies in filter matings than in broth matings. The aminoglycosides resistance transfer was observed at high frequency in the same range as tetracycline resistance. The frequency values for the aminoglycosides were very similar with a maximum

Table 8b. Transfer frequencies of K55 markers per donor colony forming unit after 18h mating on membrane filters

Expt. No.	Recipient	Final viable count (cfu ml^{-1})		Transfer frequency per donor of selected markers		
		Donor	Recipient	Em or Lm	Tc	Sm
1	JH2-2	1.30×10^{9}	7.00×10^{8}	2.31×10^{-5}	6.15×10^{-7}	NO
2	JH2-2	2.60×10^{8}	4.40×10^{8}	1.85×10^{-5}	2.30×10^{-7}	1.30×10^{-6}
3	JH2-2	3.00×10^{8}	4.60×10^{8}	3.00×10^{-6}	7.30×10^{-7}	3.30×10^{-8}
4	JH2-2	4.00×10^{8}	2.70×10^{8}	6.80×10^{-5}	1.10×10^{-6}	1.30×10^{-6}
5	JH2-2	7.80×10^{8}	6.80×10^{8}	1.50×10^{-5}	9.70×10^{-7}	1.30×10^{-8}
6	JH2-2	1.40×10^{8}	1.40×10^{8}	7.50×10^{-5}	5.70×10^{-7}	NO
7	JH2-17	6.40×10^{8}	9.20×10^{8}	1.19×10^{-6}	6.78×10^{-7}	ND

NO = no transconjugant colonies observed

ND = not determined

Table 8c. Transfer frequencies of K60 markers per donor colony forming unit after 18h mating on membrane filters

Expt. No.	Recipient	Final viable count (cfu ml^{-1})		Transfer frequency per donor of selected markers				
		Donor	Recipient	Em or Lm	Tc	Sm	Km	Nm
1	JH2-2	4.10×10^{8}	7.40×10^{8}	1.0	1.75×10^{-2}	3.50×10^{-2}	3.50×10^{-2}	3.10×10^{-2}
2	JH2-2	2.10×10^{8}	7.60×10^{8}	8.60×10^{-1}	1.05×10^{-2}	1.60×10^{-2}	1.70×10^{-2}	1.40×10^{-2}
3	JH2-2	9.40×10^{7}	8.00×10^{8}	1.0	3.20×10^{-3}	6.00×10^{-2}	2.50×10^{-2}	2.80×10^{-2}
4	JH2-2	1.10×10^{8}	6.80×10^{8}	1.0	ND	3.20×10^{-2}	2.40×10^{-2}	5.20×10^{-2}

ND = not determined

of a 2.5-fold difference within experiments and a 4.3-fold difference between experiments.

Mating of K87 with JH2 derivatives on membrane filters shown in Table 8d resulted in high frequency transfer of erythromycin resistance in five experiments and intermediate frequency transfer in one experiment with a variation of 1000-fold between experiments. Tetracycline resistance transferred at high frequency in two experiments, intermediate frequency in three experiments but in experiment 4 was unexpectedly low and therefore below the level detectable in the dilutions used. The frequency values for tetracycline resistance transfer varied 100-fold between experiments and were always at least 10-fold less than the corresponding values for erythromycin resistance transfer. Transfer frequencies for aminoglycoside resistance were high in three experiments and intermediate in three experiments. In all experiments, however, the frequencies were very similar for the individual aminoglycosides, the maximum variation being a 6-fold difference in experiment 5, and similar to the transfer frequencies for tetracycline resistance with the exception of experiment 4. Transfer of β-haemolytic activity was high in all experiments except experiment 4 where transfer frequency was below the level detectable. In the absence of positive selection for haemolytic transconjugants, this level was dependent on the recipient viable count as, if there was confluent growth on the blood agar, β-haemolysis of a small number of colony forming units was almost impossible to detect owing to the restriction on growth of individual units at high cell density on agar. In all but experiment 4, the frequencies of haemolysin transfer were close to those of erythromycin resistance transfer and higher than those for tetracycline and aminoglycosides resistance transfer. Generally, the transfer of markers was more reproducible on membrane filters and frequencies were higher, with the exception of β-haemolysin transfer.

Table 8d. Transfer frequencies of K87 markers per donor colony forming unit after 18h mating on membrane filters

Expt. No.	Recipient	Final viable count (cfu ml^{-1}) Donor	Recipient	Transfer frequency per donor of selected markers Em or Lm	Tc	Sm	Km	Nm	Hly
1	JH2-2	7.80×10^{7}	4.00×10^{8}	2.50×10^{-4}	7.7×10^{-5}	1.50×10^{-4}	1.50×10^{-4}	1.50×10^{-4}	5.10×10^{-3}
2	JH2-2	5.60×10^{8}	4.60×10^{8}	1.40×10^{-3}	1.10×10^{-4}	5.80×10^{-5}	1.30×10^{-4}	1.40×10^{-4}	3.60×10^{-3}
3	JH2-2	9.80×10^{7}	5.60×10^{8}	1.00×10^{-2}	4.00×10^{-3}	3.50×10^{-3}	5.60×10^{-3}	4.50×10^{-3}	5.10×10^{-2}
4	JH2-2	3.60×10^{8}	7.00×10^{8}	1.90×10^{-3}	$<2.78 \times 10^{-6}$	1.90×10^{-4}	1.50×10^{-4}	3.00×10^{-4}	$<2.78 \times 10^{-4}$
5	JH2-2	2.00×10^{7}	8.20×10^{8}	1.20×10^{-1}	5.00×10^{-4}	3.80×10^{-3}	2.60×10^{-2}	1.40×10^{-2}	4.30×10^{-1}
6	JH2-17	1.32×10^{8}	7.00×10^{8}	3.40×10^{-2}	1.50×10^{-3}	3.64×10^{-3}	3.48×10^{-3}	3.99×10^{-3}	1.51×10^{-2}

This may have reflected the fact that, as with DS5 matings on filters, the viable count of recipient in K87 matings did not decrease during mating. Similarly, antibiotic resistance transfer from K87 was observed only in broth matings when there was no decrease in recipient viable count.

In filter matings involving K88 donors (Table 8e), erythromycin resistance transferred at intermediate frequency (two experiments) or low frequency (four experiments). Tetracycline resistance transfer was observed at intermediate frequency in three experiments and at low frequency in all experiments. Transfer frequencies of chloramphenicol resistance were intermediate in two experiments and low in four experiments. There were however, no striking differences in the transfer frequency values of the different antibiotics within or between experiments, the maximum variation being 150-fold. The transfer of erythromycin and tetracycline resistance determinants was possibly more efficient on filters than in broth culture but the major contrast between the two mating methods was the reproducibility of the results obtained with filter matings in which all the antibiotic resistance determinants transferred to JH2 recipients.

Transfer of erythromycin resistance during filter matings with SB94 occurred at high frequency with a 40-fold difference between the highest and lowest values (Table 8f). Tetracycline resistance was transmissable at high frequency in four experiments, intermediate frequency in two experiments but in experiment 5 at a frequency below the level of detection in the dilutions tested for the presence of transconjugants. In all experiments, neomycin and kanamycin resistances transferred at high and very similar frequencies. In five experiments, the transfer frequency of streptomycin resistance was almost identical to the corresponding values for kanamycin and neomycin but in experiment 5 was 60-fold less. Generally, transfer on filters appeared to be slightly more efficient for

Table 8e. Transfer frequencies of K88 markers per donor colony forming unit after 18h mating on membrane filters

Expt. No.	Recipient	Final viable count (cfu ml^{-1})		Transfer frequency per donor of selected markers			
		Donor	Recipient	Em or Lm	Tc	Sm	Cm
1	JH2-2	6.80×10^{8}	6.00×10^{9}	2.94×10^{-7}	5.00×10^{-7}	2.35×10^{-7}	3.53×10^{-7}
2	JH2-2	2.82×10^{8}	1.72×10^{9}	8.15×10^{-7}	3.60×10^{-6}	4.60×10^{-7}	1.03×10^{-6}
3	JH2-2	9.40×10^{8}	3.90×10^{9}	2.60×10^{-6}	1.50×10^{-6}	2.30×10^{-7}	2.80×10^{-6}
4	JH2-2	1.60×10^{9}	2.10×10^{9}	1.00×10^{-7}	1.80×10^{-7}	7.50×10^{-8}	2.50×10^{-8}
5	JH2-2	2.50×10^{8}	9.60×10^{8}	1.10×10^{-6}	$<8.00 \times 10^{-8}$	5.20×10^{-7}	1.60×10^{-7}
6	JH2-17	6.60×10^{8}	1.72×10^{9}	6.06×10^{-7}	1.00×10^{-6}	8.10×10^{-7}	8.33×10^{-7}

Table 8f. Transfer frequencies of SB94 markers per donor colony forming unit after 18h mating on membrane filters.

Expt. No.	Recipient	Final viable count (cfu ml^{-1})		Transfer frequency per donor of selected markers				
		Donor	Recipient	Em or Lm	Tc	Sm	Km	Nm
1	JH2-2	2.36×10^{8}	2.58×10^{9}	2.50×10^{-3}	2.10×10^{-3}	2.10×10^{-3}	1.80×10^{-3}	2.40×10^{-3}
2	JH2-2	6.40×10^{9}	1.90×10^{9}	2.50×10^{-3}	1.90×10^{-3}	1.25×10^{-3}	1.25×10^{-3}	1.25×10^{-3}
3	JH2-2	1.48×10^{9}	1.64×10^{9}	3.04×10^{-2}	8.10×10^{-3}	5.10×10^{-3}	5.20×10^{-3}	4.90×10^{-3}
4	JH2-2	5.40×10^{8}	7.60×10^{8}	3.00×10^{-2}	1.90×10^{-4}	3.50×10^{-3}	5.40×10^{-3}	1.40×10^{-3}
5	JH2-2	9.60×10^{7}	2.80×10^{8}	1.10×10^{-1}	1.04×10^{-6}	7.30×10^{-4}	3.10×10^{-2}	4.60×10^{-2}
6	JH2-17	1.80×10^{9}	1.60×10^{9}	ND	1.10×10^{-2}	ND	ND	ND

ND = not determined

all resistances than than transfer in broth culture.

In contrast to the situation in broth matings, two filter mating experiments shown in Table 8g involving SB69 donors resulted in low frequency transfer of tetracycline resistance. None of the other donor markers was transferred nor was any transfer of donor markers from strain K46 detected in three filter mating experiments.

Mutation of strains to antibiotic resistance

In order to determine whether mutation of either donor or recipient strains to antibiotic resistance could interfere with the determination of transfer frequency, cultures of donor strains and of JH2-2 were tested on three occasions by spreading duplicate 0.1ml aliquots on to agar containing the appropriate antibiotics at concentrations used for selection of transconjugants. The frequency of mutation of donor strains to fusidic acid or rifampicin resistance was less than 10^{-8}. The frequency of mutation of JH2-2 to erythromycin, lincomycin, tetracycline, neomycin and chloramphenicol resistances was less than 10^{-9}, to streptomycin 2.35×10^{-9} and kanamycin 4.92×10^{-9}. On one further occasion, the mutation frequency of JH2-2 to tetracycline resistance was estimated by growing a culture in 500ml BHI broth to late logarithmic phase, concentrating the cells 100 times by centrifugation followed by resuspension in 5ml broth and spreading aliquots (0.1ml) on eight tetracycline agar plates. After 5 days incubation, no colonies were seen, the mutation frequency of JH2-2 to tetracycline resistance therefore being less than 8.22×10^{-12}.

Transconjugant phenotypes

Transconjugant colonies selected on each antibiotic were replicated on to the non-selected donor and recipient markers. Transconjugants obtained from matings with JH2-17 recipients were tested for thymine

Table 8g. Transfer frequencies of SB69 markers per donor colony forming unit after 18h mating on membrane filters

Expt. No.	Recipient	Final viable count (cfu ml^{-1})		Transfer frequency per donor of selected markers				
		Donor	Recipient	Tc	Sm	Ap	Pn	αHly
1	JH2-2	1.50×10^{9}	3.60×10^{9}	6.70×10^{-8}	NO	NO	NO	NO
2	JH2-2	9.50×10^{8}	3.90×10^{9}	9.47×10^{-8}	NO	NO	NO	NO
3	JH2-2	7.30×10^{8}	1.25×10^{9}	NO	NO	NO	NO	NO
4	JH2-2	2.00×10^{8}	3.50×10^{9}	NO	NO	NO	NO	NO

NO = no transconjugant colonies obtained

requirement by replicating on to DST agar without supplement which sustained only poor growth of JH2-17. Table 9 shows the phenotypes of transconjugants, obtained from broth and filter matings, as percentages of the total number of colony forming units tested for each antibiotic used in selection. In addition to the resistances shown, all transconjugants from matings with JH2-2 recipients were also resistant to fusidic acid and rifampicin and all transconjugants from matings with JH2-17 recipients were resistant to rifampicin and required thymine for normal growth. The percentages of different phenotypes obtained using erythromycin or lincomycin for selection were always the same regardless of the donor strain and so the results were combined. Thus erythromycin resistance is here synonymous with MLS resistance. Likewise, results obtained using streptomycin, kanamycin or neomycin for selection were also combined.

Selection with erythromycin resulted in transconjugants after only one broth mating with DS5 donors but such transconjugants were readily isolated after filter matings. All those obtained after broth mating (Table 9a) were co-resistant to tetracycline, with 16.67% also haemolytic. In contrast, 75% of those obtained after filter matings were susceptible to tetracycline and non-haemolytic, the remainder being co-resistant to tetracycline (9.24%) and haemolytic (15.76%). Almost half (43.16%) the transconjugants, selected after mating in broth cultures using tetracycline were tetracycline resistant only while the remainder were also β-haemolytic. After filter matings, the percentage of transconjugants resistant to tetracycline only was twice that obtained after broth matings and again the remainder were also β-haemolytic transconjugants obtained after either broth or filter matings were susceptible to erythromycin and tetracycline.

The majority of erythromycin selected transconjugants from matings with K55 donors shown in Table 9b were co-resistant to streptomycin with the percentage slightly higher after filter matings (90.36%) than broth matings

Table 9a. Phenotypes of transconjugants obtained after matings in broth culture and on membrane filters with strain DS5 donors

Selective antibiotic	Number of transconjugants tested		Phenotype observed	Percentage	
	Broth	Filter		Broth	Filter
Em or Lm	6*	184	Em	0	75.00
			Em Tc	83.33	9.24
			Em Tc Hly	16.67	15.76
Tc	95	167	Tc	43.16	86.83
			Tc Hly	56.84	13.17
Fus + 6% Horse blood	59	71	Hly	100.00	100.00

* Obtained in broth mating experiment 3 only

Table 9b. Phenotypes of transconjugants obtained after matings in broth culture and on membrane filters with strain K55 donors

Selective antibiotic	Number of transconjugants tested		Phenotype observed	Percentage	
	Broth	Filter		Broth	Filter
Em or Lm	282	320	Em	26.24	9.64
			Em Sm	73.76	90.36
Tc	55	126	Tc	100.00	100.00
Sm	8	73	Sm	62.50	2.74
			Em Sm	37.50	97.26

(73.76%), the remaining transconjugants being resistant to erythromycin only. All tetracycline resistant transconjugants were susceptible to erythromycin and streptomycin. The total number of streptomycin resistant transconjugants obtained from broth matings was small with 62.5% being streptomycin resistant only and the rest co-resistant to erythromycin. From filter matings however, where the number of streptomycin resistant transconjugants tested was much higher and hence the results more accurate, the great majority were in addition resistant to erythromycin and only 2.74% were resistant to streptomycin alone.

Transconjugants selected on erythromycin from matings involving K60 (Table 9c) were primarily resistant to erythromycin only, with just 1.5% co-resistant to streptomycin, kanamycin and neomycin and 4.89% resistant to all the donor markers after broth matings, and a very low percentage (0.93%) resistant to all donor markers after filter matings. Selection with tetracycline yielded transconjugants the majority of which were resistant to all donor markers although in broth matings a small percentage were tetracycline resistant only (1.42%) or jointly resistant to erythromycin (4.25%), while in filter matings, 4.11% were resistant to erythromycin also. None of the transconjugants selected on streptomycin, kanamycin or neomycin was resistant to these antibiotics alone. Most were resistant to all the donor markers but a small percentage (1.2% in broth matings and 0.76% in filter matings) were susceptible to tetracycline.

Table 9d shows that the percentage of each phenotype obtained from matings involving K87 donors was very dependent on the method of mating. For example, of the transconjugants selected with erythromycin after broth matings, 90.87% were also β-haemolytic while only 4.84% displayed this phenotype after filter matings. The most common phenotype observed after filter matings was that resistant to erythromycin only (58.13%), followed by the phenotype showing resistance to all the donor markers (22.14%).

Table 9c. Phenotypes of transconjugants obtained after matings in broth culture and on membrane filters with strain K60 donors

Selective antibiotic	Number of transconjugants tested		Phenotype observed	Percentage	
	Broth	Filter		Broth	Filter
Em or Lm	266	215	Em	93.61	99.07
			Em Sm Km Nm	1.50	0
			Em Tc Sm Km Nm	4.89	0.93
Tc	141	73	Tc	1.42	0
			Em Tc	4.25	4.11
			Em Tc Sm Km Nm	94.33	95.89
Sm Km or Nm	251	265	Em Sm Km Nm	1.20	0.76
			Em Tc Sm Km Nm	98.80	99.24

Table 9d. Phenotypes of transconjugants obtained after matings in broth culture and on membrane filters with strain K87 donors

Selective antibiotic	Number of transconjugants tested		Phenotype observed	Percentage	
	Broth	Filter		Broth	Filter
Em or Lm	219	289	Em	1.37	58.13
			Em Hly	90.87	4.84
			Em Tc	0.91	10.03
			Em Sm Km Nm	0	4.84
			Em Tc Hly	4.66	0
			Em Tc Sm Km Nm	2.28	22.14
Tc	106	175	Tc	8.49	2.28
			Em Tc	2.83	19.43
			Tc Hly	2.83	0
			Em Tc Hly	0.94	14.86
			Em Tc Sm Km Nm	84.90	63.43
Sm Km or Nm	208	493	Em Sm Km Nm	0.48	29.61
			Em Sm Km Nm Hly	0	0.61
			Em Tc Sm Km Nm	99.52	69.78
Fus + 6% Horse blood	40	50	Hly	100.00	100.00

Most transconjugants selected using tetracycline were resistant to all donor markers after both broth and filter matings (84.9% and 63.43% respectively). After filter matings many transconjugants were co-resistant to erythromycin (19.43%) and some were in addition β-haemolytic (14.86%) while after broth matings, these phenotypes were rare (2.83% and 0.94% respectively) and transconjugants solely resistant to tetracycline were more common (8.49%). None of the transconjugants selected on streptomycin, kanamycin or neomycin was resistant exclusively to these antibiotics. After broth matings, the majority (99.52%) were resistant to all the donor markers although a very small percentage (0.48%) were susceptible to tetracycline while after filter matings the ratio of the percentages of these phenotypes was less - 69.78% resistant to all donor markers and 29.61% susceptible to tetracycline. Furthermore, after filter matings, a small percentage (0.61%) was found to be susceptible to tetracycline but produced β-haemolysin. As was found with strain DS5, transconjugants of K87 isolated for β-haemolytic activity were all antibiotic susceptible whether obtained from broth or filter matings.

The total number of transconjugants obtained with erythromycin or tetracycline selection was small after matings in broth culture with K88 donors (Table 9e). On selection with erythromycin, resistance was always associated with tetracycline and chloramphenicol resistance after either broth or filter matings. The majority of tetracycline resistant transconjugants was resistant only to tetracycline after both broth and filter matings (75% and 83.85% respectively) and the remainder were co-resistant to erythromycin and chloramphenicol. All transconjugants selected on chloramphenicol after broth matings were also resistant to erythromycin and tetracycline as were 98.76% of those obtained after filter matings, the remaining 1.23% being co-resistant to tetracycline but susceptible to erythromycin and streptomycin. Streptomycin selected transconjugants were

Table 9e. Phenotypes of transconjugants obtained after matings in broth culture and on membrane filters with strain K88 donors

Selective antibiotic	Number of transconjugants tested		Phenotype observed	Percentage	
	Broth	Filter		Broth	Filter
Em or Lm	9	184	Em Tc Cm	100.00	100.00
Tc	4	130	Tc	75.00	83.85
			Em Tc Cm	25.00	16.15
Cm	73	81	Em Tc Cm	100.00	98.76
			Tc Cm	0	1.23
Sm	49	75	Sm	100.00	100.00

always susceptible to all the other donor markers.

In matings involving SB94 donors, almost half the erythromycin selected transconjugants were resistant to erythromycin alone and half resistant to all the donor markers. A small percentage from both broth and filter matings (0.44% and 1.90% respectively) however, were susceptible to tetracycline although displaying insensitivity to streptomycin, kanamycin and neomycin. All the tetracycline resistant transconjugants selected after broth matings had received donor markers en bloc while after filter matings 9.68% were solely resistant to tetracycline. Selection of streptomycin, kanamycin or neomycin resulted, for the majority of transconjugants (98.12% for broth matings and 96.59% for filter matings), in concomitant resistance to all the donor markers, the remainder being susceptible to tetracycline but co-resistant to erythromycin. (Table 9f).

The data for matings involving SB69 donors is not shown. Tetracycline resistant transconjugants were selected after two of four filter mating experiments and all were exclusively resistant to tetracycline.

Response of strains to clump inducing agent produced by JH2-1

CIA was prepared from a culture of JH2-1 as described and the clumping response of donor group D streptococci and selected transconjugants was tested. The response titres from duplicate experiments of the donor strains are shown in Table 10. The highest titre obtained was that of DS5 response at 32 while the other S. faecalis strains had titres of 16 (K87) and 4 (K55, K60, K88 and SB94). JH2-1 CIA had no visible effect on either S. faecium strains K46 or SB69, or on the producer strain JH2-1 itself.

In addition to the above donor strains representative transconjugants from matings with JH2-2 or JH2-17 recipients were also tested for response to JH2-1 CIA. Where possible transconjugants displaying only a single donor phenotypic marker were tested and assays were carried out in duplicate

Table 9f. Phenotypes of transconjugants obtained after matings in broth culture and on membrane filters with strain SB94 donors

Selective antibiotic	Number of transconjugants tested		Phenotype observed	Percentage	
	Broth	Filter		Broth	Filter
Em or Lm	453	211	Em	49.88	50.24
			Em Sm Km Nm	0.44	1.90
			Em Tc Sm Km Nm	49.67	47.87
Tc	43	124	Tc	0	9.68
			Em Tc Sm Km Nm	100.00	90.32
Sm Km or Nm	160	293	Em Sm Km Nm	1.88	3.41
			Em Tc Sm Km Nm	98.12	96.59

Table 10. Response of donor strains to CIA produced by JH2-1

Strain	Response titre
DS5	32
K55	4
K60	4
K87	16
K88	4
SB94	4
K46	<2
SB69	<2

with three transconjugants of each phenotype. No clumping was observed with any of the transconjugants regardless of donor with the following exceptions. One erythromycin resistance transconjugant from a filter mating of DS5 with JH2-17 had a titre of 4, one erythromycin resistant transconjugant of a broth mating of K60 with JH2-17 had a titre of 2 and one erythromycin resistant transconjugant from a filter mating of K87 with JH2-17 had a titre of 2.

Retransfer of markers from transconjugants

In order to determine whether the markers transferred from original donors were self-transmissable, transconjugants obtained from matings with JH2-17 recipients i.e. with chromosomal resistance to rifampicin and thymine requirement, and displaying a single donor marker were mated with JH2-1 (chromosomal fusidic acid resistance) in broth culture and on membrane filters. These results including the transfer frequencies are shown in Table 11. When the retransfer frequency was low, the phenotype of the transconjugants obtained was checked by replicating master plates on to the donor marker, rifampicin, fusidic acid and DST agar. Only those which grew well on the donor marker, fusidic acid and DST agar (without thymine supplement) were considered to be transconjugants.

As expected from published observations (Dunny and Clewell, 1975), the haemolysin marker of DS5 was again transferable in broth culture at high frequency but unlike the original broth matings, no decrease in recipient viable count was observed (data not shown). In the absence of transfer inhibition by the haemolysin plasmid, transfer of erythromycin resistance was observed in broth culture at low frequency but no transfer of tetracycline resistance was observed after either broth or filter matings. Likewise, there was no retransfer of either streptomycin or tetracycline resistance originating from strain K55. Retransfer of the erythromycin resistance determinant originating from strain K60 was observed in broth

Table 11. Retransfer to JH2-1 recipients of markers from transconjugants obtained after matings with JH2-17

Parental strain (initial mating method)	Transconjugant designation (donor)	Phenotype*	Retransfer frequency per donor colony forming unit (retransfer mating method)
DS5 (broth)	SSJ1	Hly	4.10×10^{-3} (broth)
DS5 (broth)	SSJ2	Hly	1.25×10^{-2} (broth)
DS5 (broth)	SSJ3	Hly	2.70×10^{-2} (broth)
DS5 (filter)	SSJ4	Em	3.30×10^{-7} (broth)
DS5 (filter)	SSJ5	Em	3.40×10^{-7} (broth)
DS5 (filter)	SSJ6	Em	7.70×10^{-7} (broth)
DS5 (broth)	SSJ7,SSJ8 or SSJ9	Tc	no transconjugants detected
K55 (broth)	SSK1,SSK2 or SSK3	Sm	no transconjugants detected
K55 (filter)	SSK4,SSK5 or SSK6	Tc	no transconjugants detected
K60 (broth)	SSL1	Em	6.70×10^{-6} (broth)
K60 (broth)	SSL2	Em	9.85×10^{-7} (broth)
K60 (broth)	SSL3	Em	9.69×10^{-8} (broth)
K60 (broth)	SSL4 or SS15	Tc	no transconjugants detected
K87 (broth)	SSM1	Em	1.45×10^{-2} (broth)
K87 (broth)	SSM5	Em	2.15×10^{-3} (broth)
K87 (broth)	SSM2,SSM3 or SSM4	Tc	no transconjugants detected
K87 (broth)	SSM6	Hly	3.10×10^{-6} (broth)
K87 (broth)	SSM7	Hly	no transconjugants detected
K87 (broth)	SSM8	Hly	2.30×10^{-6} (broth)
K87 (broth)	SSM9	Hly	2.85×10^{-6} (broth)
K88 (filter)	SSN1	Tc	3.95×10^{-8} (filter)
K88 (filter)	SSN2	Tc	3.00×10^{-7} (filter)
K88 (filter)	SSN3	Tc	1.34×10^{-7} (filter)
K88 (broth)	SSN4	Sm	1.22×10^{-5} (broth)
K88 (broth)	SSN5	Sm	1.83×10^{-5} (broth)
K88 (broth)	SSN6	Sm	2.33×10^{-5} (broth)
SB94 (filter)	SSO1,SSO2 or SSO3	Tc	no transconjugants detected

* All transconjugants obtained from original matings were in addition chromosomally resistant to rifampicin and thymine requiring.

matings at intermediate and low frequency, these transfer frequencies being 1000- to 100,000-fold less than those observed in the original broth matings, and no retransfer of the tetracycline determinant was observed. The ability of the streptomycin, kanamycin and neomycin markers to retransfer was not tested as no transconjugants were originally obtained which were not also erythromycin resistant.

The retransfer of the haemolysin marker originating from strain K87 was observed at intermediate frequency from three of four transconjugants representing a decrease in frequency of 10-to 10,000-fold compared with the original matings. This decrease in frequency could account for the apparent inability of SSP2 to transfer haemolysin as in the absence of a counter-selective agent, the background of non-haemolytic recipients can become so high that haemolysin detection is extremely difficult. In all four matings, as in the original broth matings between K87 and JH2-17, a decrease in the viable count of recipient was noted from an initial count of 2.0 to 3.6×10^{7} cfu ml^{-1} to 2.0 to 5.6×10^{4} cfu ml^{-1}, presumably due to the production of bacteriocin by the donor during mating. This effect was not seen during retransfer matings involving erythromycin or tetracycline resistant transconjugants. The erythromycin resistance marker from K87 was retransferable from both strains tested in broth culture at high frequency compared with the intermediate transfer frequency obtained in the original matings but no retransfer of the tetracycline resistance marker was observed in either broth or filter matings. Retransfer of K87 streptomycin, kanamycin and neomycin markers not tested for the same reason as that given for K60.

The tetracycline resistance marker originating from K88 did not transfer from transconjugants to JH2-1 during matings in broth culture but low frequency transfer was obtained after filter matings within the frequency range observed in the original filter matings. Strain K88

streptomycin resistance marker was retransferable during broth matings at intermediate frequencies 100-fold higher than those obtained in two of five original broth mating experiments and in all original filter mating experiments. No transconjugants resistant to erythromycin alone were available for testing.

Strain SB94 tetracycline resistance marker did not retransfer in either broth or filter matings. Unfortunately, no transconjugants resistant to erythromycin only were obtained from the original mating with JH2-17 and in the absence of a suitable recipient for the erythromycin marker in a JH2-2 background (rifampicin and fusidic acid chromosomal resistance), retransfer was not tested. Retransfer of the streptomycin, kanamycin and neomycin markers was not tested for the reason given above for K60.

The retransfer of tetracycline resistance originating from SB69 was not tested because transconjugants were not obtained after mating with JH2-17 and a suitable recipient was not available.

ELIMINATION OF ANTIBIOTIC RESISTANCE AND HAEMOLYTIC ACTIVITY

Loss of resistance or haemolysin production during storage

Cultures which had been stored in drug free sloppy Luria agar for up to one year were grown overnight in nutrient broth and the viable counts estimated. Master plates prepared as described previously were replicated on to agar containing appropriate antibiotics and the percentage of those clones which had lost antibiotic resistance or the ability to produce haemolysin was calculated and is shown in Table 12. Of the MLS antibiotics, only erythromycin was used in experiments for elimination of resistance, it being assumed that there would be concomitant loss of the other MLS resistances.

Loss of haemolytic activity was very high, 96% in total, from strain DS5 while only 0.5% of colonies tested had lost erythromycin resistance and none was tetracycline susceptible. Of the colonies grown from the storage culture of K55, 1.5% were resistant to tetracycline alone while 0.5% had lost streptomycin resistance only. No tetracycline susceptible colonies were obtained and the majority of colonies tested had the parental phenotype. Almost half (52.3%) of K60 colonies tested were no longer streptomycin, kanamycin and neomycin resistant but none was observed which was erythromycin or tetracycline susceptible. Likewise, no erythromycin or tetracycline susceptible colonies were obtained from the K87 culture although streptomycin, kanamycin and neomycin resistances were lost in 0.9% of K87 derived colonies. A total of 12.1% were non-haemolytic, a value which was much lower than the percentage of haemolysin negative derivatives of DS5. During incubating in BHI broth of DS5 and K87 cultures from sloppy Luria, K87 was seen to form cell aggregates while DS5 did not, aggregation possibly reflecting transfer of markers from haemolytic to non-haemolytic derivatives of K87, thus effectively reducing the percentage of colonies

Table 12. Percentage of different phenotypes obtained after storage of cultures at room temperature for 10 to 12 months.

Parental Strain (phenotype)	Number of cfu tested	Phenotype observed*	Percentage
DS5 (EmTcHly)	200	Tc	0.5
		EmTc	95.5
		EmTcHly	4.0
K55 (EmTcSm)	200	Tc	1.5
		EmTc	0.5
		EmTcSm	98.00
K60 (EmTcSmKmNm)	199	EmTc	52.3
		EmTcSmKmNm	47.7
K87 (EmTcSmKmNmHly)	215	EmTcHly	0.9
		EmTcSmKmNm	12.1
		EmTcSmKmNmHly	87.0
K88 (EmTcSmCm)	200	TcSm	0.5
		EmTcCm	1.0
		EmTcSmCm	98.5
SB94 (EmTcSmKmNm)	200	TcSmKmNm	29.0
		EmTc	5.5
		EmTcSmKmNm	65.5
K46 (EmTcSmApPn)	200	EmTcApPn	0.5
		EmTcSmApPn	99.5
SB69 (TcSmApPn)	200	TcSmApPn	100.0

* Antibiotic resistant or haemolysin producing.

showing mitotic segregation of markers.

Of the colonies tested from the culture of K88, 0.5% were simultaneously erythromycin and chloramphenicol susceptible, while 1% were no longer streptomycin resistant. The remaining 98.5% had the parental phenotype, no loss of tetracycline resistance being observed. Derivatives of SB94 susceptible to erythromycin were observed in 29% of colonies tested and a further 5.5% were resistant to erythromycin and tetracycline but susceptible to streptomycin, kanamycin and neomycin. Again no tetracycline susceptible derivatives were observed. Of the colonies of K46 none was susceptible to erythromycin, tetracycline, ampicillin or penicillin but 0.5% were found to have lost resistance to streptomycin. No segregation of antibiotic resistance was observed among colonies of SB69.

Novobiocin treatment

Table 13 shows the highest concentrations of novobiocin which permitted visible growth of strains i.e. half MIC and the viable counts at those concentrations from duplicate experiments. All the half MIC values were either the same or within one doubling concentration between experiments and for S. faecalis strains the difference was only one doubling concentration 3.2 to 6.4µg ml^{-1} within experiments, values which are in the expected range for this species (McHugh and Swartz, 1977). The S. faecium strains K46 and SB69 were more sensitive to the antibiotic with half MIC concentrations of 0.4µg ml^{-1}. From initial bacterial inocula of approximately 10^5 cfu ml^{-1}, most cultures incubated overnight with novobiocin at half MIC concentration showed only 10- to 1000-fold increase in viable count.

Single colonies obtained while estimating the above viable counts were used to prepare master plates which were replicated on to agar containing appropriate antibiotics or blood. The phenotypes of several hundred such colonies for each parental strain were determined and the percentages of each phenotype are shown in Table 14.

Table 13. Concentration of novobiocin and viable count of group D streptococci used for elimination of phenotype markers.

	Novobiocin ($\mu g\ ml^{-1}$)		Viable count ($cfu\ ml^{-1}$)	
Strain	Expt. 1	Expt. 2	Expt. 1	Expt. 2
DS5	3.2	6.4	$4.4x10^6$	$2.8x10^6$
K55	6.4	12.8	$3.1x10^6$	$3.0x10^5$
K60	6.4	6.4	$7.8x10^7$	$3.2x10^6$
K87	6.4	6.4	$9.2x10^7$	$1.4x10^7$
K88	3.2	6.4	$9.2x10^7$	$1.1x10^7$
SB97	6.4	6.4	$5.8x10^7$	$1.2x10^7$
K46	0.4	0.4	$7.2x10^7$	$1.0x10^8$
SB69	0.4	0.4	$9.4x10^6$	$2.0x10^7$

As had been observed after storage, the majority of DS5 colonies tested (86.1%) were resistant to erythromycin and tetracycline but did not produce haemolysin and a further 0.4% were haemolysin negative and erythromycin susceptible. A small percentage (0.2%) were haemolytic and tetracycline resistant giving a total of 0.6% susceptible to erythromycin, but none was found to be cured of tetracycline resistance. Only 13.3% showed the parental phenotype. Erythromycin and streptomycin resistances were eliminated by novobiocin treatment in 19.2% of colonies from the K55 cultures, the remainder showing the parental resistance pattern. Unlike the results obtained after storage, all the erythromycin susceptible colonies were streptomycin susceptible. The majority of colonies from K60 cultures (66%) were susceptible to streptomycin, kanamycin and neomycin, with a further 0.2% no longer resistant to erythromycin. The remainder showed the parental phenotype and none was tetracycline susceptible. The percentage curing of haemolytic activity from K87 was lower after novobiocin treatment (1.5%) compared with results from storage (13%) and no colonies were found antibiotic susceptible. A small percentage of treated K88 colonies (0.2%) were resistant to tetracycline alone and 1% were co-resistant to erythromycin and chloramphenicol giving a total of 1.2% elimination of streptomycin resistance. In 2.7% of colonies tested, erythromycin resistance had been eliminated with concomitant loss of chloramphenicol resistance. The remaining colonies had the parental phenotype, no elimination of tetracycline resistance being observed. Indeed, strain SB94 was the only S. faecalis strain which yielded a tetracycline susceptible derivative at a frequency of 0.2% after novobiocin treatment. This derivative was also susceptible to the other parental markers. A further 0.6% were tetracycline resistant but susceptible to erythromycin and aminoglycosides while the remainder had the parental phenotype. In contrast to the situation observed after storage of K46, novobiocin treatment yielded a variety of phenotypes, ampicillin and penicillin resistances being the only markers

Table 14. Percentage of different phenotypes obtained after novobiocin treatment

Parental Strain (phenotype)	Number of cfu tested	Phenotype observed*	Percentage
DS5 (EmTcHly)	460	Tc	0.4
		TcHly	0.2
		EmTc	86.1
		EmTcHly	13.3
K55 (Em Tc Sm)	464	Tc	19.2
		EmTcSm	80.8
K60 (EmTcSmKmNm)	420	Tc	0.2
		EmTc	66.0
		EmTcSmKmNm	33.8
K87 (EmTcSmKmNmHly)	538	EmTcSmKmNm	1.5
		EmTcSmKmNmHly	98.5
K88 (EmTcSmCm)	488	Tc	0.2
		TcSm	2.7
		EmTcCm	1.0
		EmTcSmCm	96.1
SB94 (EmTcSmKmNm)	523	-**	0.2
		Tc	0.6
		EmTcSmKmNm	99.2
K46 (EmTcSmApPn)	316	EmApPn	0.3
		TcApPn	0.3
		EmSmApPn	2.2
		EmTcAPPn	1.3
		EmTcSmApPn	95.9
SB69 (TcSmApPn)	417	TcSmApPn	100.0

* antibiotic resistant or haemolysin producing

** antibiotic susceptible

not eliminated. A small percentage was susceptible to tetracycline and streptomycin (0.3%) or erythromycin and streptomycin (0.3%) and a further 2.2% susceptible to tetracycline only. Streptomycin resistance was cured in 1.2% of colonies tested and the rest showed parental phenotype. Consistent with results obtained after storage, none of the colonies from treated cultures of SB69 had lost any of the parental antibiotic resistances.

Response of antibiotic susceptible and non-haemolytic derivatives to JH2-1 pheromone

Representative derivatives of parental strains obtained after storage and novobiocin treatment were tested in duplicate for their response to the CIA produced by JH2-1 and the titres are shown in Table 15. Of the DS5 derivatives, only those with the erythromycin and tetracycline co-resistance phenotype did not respond to CIA. Two tetracycline resistant derivatives showed CIA titres of 8, a quarter of the parental strain, and the other a titre of 4 while the erythromycin resistant derivative responded with a titre of 16. All K55 derivatives, which were susceptible to streptomycin, displayed clumping within one doubling concentration of the parental strain response titre of 4. K60 derivatives resistant to erythromycin and tetracycline aggregated within one doubling dilution or concentration of the parental strain and only the derivative susceptible to erythromycin showed no response. The response of non-haemolytic derivatives of K87 was low (titre 2) compared to the parental response titre of 16 while aminoglycoside susceptible, haemolytic derivatives had titres of 8 and 16. The only response of K88 derivatives was seen in two of three erythromycin, tetracycline and chloramphenicol co-resistant derivatives, erythromycin and chloramphenicol susceptible derivatives did not respond to CIA. Tetracycline and erythromycin co-resistant derivatives of SB94, as well as those resistant to tetracycline alone, responded within one doubling dilution or concentration of the parental response titre of 4.

Table 15. Response of antibiotic susceptible and non-haemolytic derivatives of parental strains to CIA produced by JH2-1

Parental strain	Derivative designation	Phenotype*	Response titre to CIA
DS5		EmTcHly	32
	DS5C1,DS5C2,DS5C3	EmTc	<2
	DS5C4	Tc	8
	DS5C8	Tc	8
	DS5C10	Tc	4
	DS5C9	TcHly	16
K55		EmTcSm	4
	K55C1,K55C4,K55C5, K55C6,K55C7	Tc	2
	K55C2	Tc	4
	K55C3	EmTc	2
K60		EmTcSmKmNm	4
	K60C1	EmTc	4
	K60C2	EmTc	8
	K60C3	EmTc	2
	K60C4	Tc	<2
K87		EmTcSmKmNmHly	16
	K87C1,K87C3,K87C6, K87C7,K87C8	EmTcSmKmNm	2
	K87C4	EmTcHly	8
	K87C5	EmTcHly	16
K88		EmTcSmCm	4
	K88C14	Tc	<2
	K88C1,K88C11,K88C12, K88C13	TcSm	<2
	K88C2,K88C3	EmTcCm	2
	K88C6	EmTcCm	<2
SB94		EmTcSmKmNm	4
	SB94C1	Tc	2
	SB94C2	Tc	8
	SB94C3	Tc	2
	SB94C3,SB94C5,SB94C6	EmTc	2
	SB94C7	-**	<2

* antibiotic resistant or haemolysin producing

** antibiotic susceptible

Only the antibiotic susceptible derivative SB94C7 showed no aggregation on incubation with JH2-1 CIA. Controls consisting of JH2-1 as responder cells to JH2-1 CIA likewise did not aggregate.

BACTERIOCIN PRODUCTION BY STRAINS DS5, K87 AND DERIVATIVES

Bacteriocin production was initially tested using JH2-1 as indicator organism. However, during the course of experiments to eliminate phenotypic markers, it was observed that non-haemolytic derivatives of K87 appeared to inhibit surrounding colonies particularly when replicated on to agar containing streptomycin. Consequently, eight "surrounding colonies", which otherwise appeared identical to the parental K87, were grown for use as indicator organisms, K87IO, in tests for bacteriocin production by strains DS5, SB94, K87 and its representative derivatives.

Bacteriocin production by DS5 and derivatives against JH2-1

Table 16 shows the activity of bacteriocin produced by DS5, strains derived from matings with JH2-17 and cured derivatives against the streptococcal recipient strain JH2-1. Strain DS5 produced a definite zone of clearing but the haemolytic transconjugants and two of the tetracycline resistant transconjugants were only weakly bacteriocinogenic while the remaining tetracycline resistant transconjugant tested, SSJ9, did not show any activity. Erythromycin resistant transconjugants exhibited conspicuous clear zones but of smaller diameter than DS5. Of the cured derivatives, DS5C1, DS5C2 and DS5C3 non-haemolytic strains produced zone diameters greater than those of the parental strain while strains with the same phenotype, DS5C5, DS5C6 and DS5C7 had the same zone diameters as DS5. The only difference between the two sets of derivatives was that the former were obtained after storage and the latter by novobiocin treatment. Two of three tetracycline resistant derivatives and a haemolytic, tetracycline resistant derivative obtained after novobiocin treatment appeared to have the same activity as DS5 while DS5C4, a tetracycline resistant derivative obtained after storage, had no detectable activity. All four derivatives obtained after storage were surrounded by opaque zones extending outwards

Table 16. Bacteriocin production by DS5 and derivatives against JH2-1

Strain*	Phenotype	Bacteriocin against JH2-1**
DS5	Em Tc Hly	++
SSJ1	Hly	±
SSJ2	Hly	±
SSJ3	Hly	±
SSJ4	Em	+
SSJ5	Em	+
SSJ6	Em	+
SSJ7	Tc	±
SSJ8	Tc	±
SSJ9	Tc	-
DS5C1	Em Tc	+++ O
DS5C2	Em Tc	+++ O
DS5C3	Em Tc	+++ O
DS5C4	Tc	- O
DS5C5	Em Tc	++
DS5C6	Em Tc	++
DS5C7	Em Tc	++
DS5C8	Tc	++
DS5C9	Tc Hly	++
Ds5C1O	Tc	++

* Prefix SSJ denotes transconjugant of DS5 x JH2-17 mating, chromosomally resistant to rifampicin and thymine requiring. Prefix DS5C denotes strains derived from curing experiments.

**-, no bacteriocin detected; ±, barely detectable clear zone diameter <0.5mm; +, zone clear diameter 0.5 to 2mm, ++ clear zone diameter 2 to 4mm; +++, clear zone diameter 4 to 6mm; O, opaque zone 7 to 9mm diameter.

from the clear zones produced by bacteriocin activity. A control stab of JH2-1 did not show any activity, neither clear nor opaque zones.

Bacteriocin production by DS5, SB94, K87 and its derivatives against JH2-1 and K87IO

The presence or absence of bacteriocin production by test strains against the indicator organisms JH2-1 and K87IO is shown in Table 17. Against JH2-1, K87 was moderately active while K87IO had barely detectable activity. Only the haemolytic transconjugants were bacteriocinogenic, with zone diameters larger than the parental strain and of the same size as DS5. All the cured derivatives and SB94 showed some bacteriocin activity against JH2-1 albeit very weak except for K87C3, K87C4 and K87C5. As seen with cured derivatives of DS5, some cured derivatives of K87 as well as the haemolytic transconjugants were surrounded, in addition to a clear zone, by an opaque zone the diameter of which was unrelated to the diameter of the clear zone. All the cured derivatives which were surrounded by opaque zones were obtained after storage.

Against K87IO, neither K87 nor K87IO showed any activity. Unlike the situation with JH2-1 indicator, none of the haemolytic transconjugants was bacteriocinogenic, tetracycline resistant transconjugants were bacteriocinogenic as were the erythromycin resistant transconjugants of K87 matings with JH2-2 but not of a K87 and JH2-17 mating. Also in contrast to results with JH2-1 indicator, only the cured derivatives K87C6, K87C7 and K87C8 showed any activity. DS5 was bacteriocinogenic with K87IO indicator as was SB94 which had a much more obvious clear zone on K87IO. As above, haemolytic transconjugants and those derivatives obtained after storage had opaque zones around the inocula.

As described previously, K87IO was taken from replica plates containing streptomycin on which the non-haemolytic derivatives of K87 appeared to be very bacteriocinogenic. Bacteriocin production was therefore tested by

Table 17. Bacteriocin production by DS5, SB94, K87 and its derivatives against JH2-1 and K87IO

Strain*	Phenotype	Bacteriocin against**	
		JH2-1	K87IO
K87	EmTcSmKmNmHly	+	-
K87IO	EmTcSmKmNmHly	±	-
SSM1	Em	-	-
SSM2	Tc	-	±
SSM3	Tc	-	±
SSM4	Tc	-	±
SSM6	Hly	++ O	- O
SSM7	Hly	++ O	- O
SSM8	Hly	++ O	- O
SSM9	Hly	++ O	- O
SSG12	Em	-	+
SSG13	Em	-	+
K87C1	EmTcSmKmNm	± O	- O
K87C2	EmTcSmKmNm	± O	- O
K87C3	EmTcSmKmNm	+ O	- O
K87C4	EmTcHly	+ O	- O
K87C5	EmTcHly	+ O	- O
K87C6	EmTcSmKmNm	±	±
K87C7	EmTcSmKmNm	±	±
K87C8	EmTcSmKmNm	±	±
DS5	EmTcHly	++	+
SB94	EmTcSmKmNm	±	++

* Prefix SSM denotes transconjugant of K87 x JH2-17 mating, chromosomally resistant to rifampicin and thymine requiring, and SSG of K87 x JH2-2 mating chromosomally resistant to rifampicin and fusidic acid. Prefix K87C denotes strains derived from curing experiments. K87IO described in text.

** -, no bacteriocin detected; ± barely detectable clear zone diameter <0.5 mm; +, clear zone diameter 0.5 to 2mm; ++ clear zone diameter 2 to 4mm; +++, clear zone diameter 4 to 6mm; O, opaque zone diameter 7 to 8.

stab inoculating strains into K87IO overlaid on BHI agar containing streptomycin, erythromycin or tetracycline at the concentrations used for selection of transconjugants and the results are shown in Table 18. K87 did not produce bacteriocin when there was no antibiotic present or on streptomycin but was weakly bacteriocinogenic on erythromycin and tetracycline. The indicator strain was not bacteriocinogenic with or without antibiotic. The erythromycin resistant transconjugant SSM1 was also non-bacteriocinogenic as were SSG12 and SSG13 and the tetracycline resistant transconjugants except when there was no antibiotic present. There was however a striking difference in activity between antibiotic free and streptomycin containing agar with haemolytic transconjugants and cured derivatives. In the presence of streptomycin, the previously non-bacteriocinogenic transconjugants and cured derivatives became highly bacteriocinogenic and K87C6, K87C7 and K87C8 which were weak producers without antibiotic, also appeared to increase activity. Derivatives K87C4 and K87C5 were cured of streptomycin resistance and so should not have grown but close examination of the inocula showed that there was slight growth in the overlay agar although none in the streptomycin agar. Possibly therefore a few cell divisions took place before the drug diffused completely into the overlay. On erythromycin, none of the transconjugants was active but all the cured derivatives were weakly bacteriocinogenic, while on tetracycline only K87C6, K87C7 and K87C8 showed weak activity. The opaque zones seen without antibiotic around haemolytic transconjugants and derivatives obtained after storage were not visible on streptomycin or erythromycin but were present on tetracycline. Strain DS5, bacteriocinogenic against K87IO without antibiotic and with tetracycline, did not produce bacteriocin or appear to grow at all (compare K87C4 and K87C5) on streptomycin. On erythromycin however, bacteriocin production was greatly enhanced. SB94 produced bacteriocin without antibiotic, showed slight enhancement in the presence of streptomycin, and was bacteriocinogenic also on erythromycin and tetracycline.

Table 18. Bacteriocin production by DS5, SB94, K87 and its derivatives against K87IO on agar containing streptomycin, erythromycin or tetracycline.

Strain*	Phenotype	Bacteriocin production ** with			
		no antibiotic	Sm	Em	Tc
K87	EmTcSmKmNmHly	-	-	±	±
K87IO	EmTcSmKmNmHly	-	-	-	-
SSM1	Em	-	-	-	-
SSM2	Tc	±	-	-	-
SSM3	Tc	±	-	-	-
SSM4	Tc	±	-	-	-
SSM6	Hly	- O	++++	-	- O
SSM7	Hly	- O	++++	-	- O
SSM8	Hly	- O	++++	-	- O
SSM9	Hly	- O	++++	-	- O
SSG12	Em	+	-	-	-
SSG13	Em	+	-	-	-
K87C1	EmTcSmKmNm	- O	++++	±	- O
K87C2	EmTcSmKmNm	- O	++++	±	- O
K87C3	EmTcSmKmNm	- O	++++	±	- O
K87C4	EmTcHly	- O	++++	±	- O
K87C5	EmTcHly	- O	++++	±	- O
K87C6	EmTcSmKmNm	±	+++	±	±
K87C7	EmTcSmKmNm	±	+++	±	±
K87C8	EmTcSmKmNm	±	+++	±	±
DS5	EmTcHly	+	-	++++	+
SB94	EmTcSmKmNm	++	+++	+	+

* See Table 17.

** See Table 17; ++++, clear zone diameter 6 to 9mm.

PREPARATION OF PLASMID DNA

To obtain a method of preparation of streptococcal plasmid DNA which was quick with as few steps in the procedure as possible, was reproducible and suitable for all the strains under study, preliminary experiments were carried out using several well documented methods.

On two separate occasions, attempts were made to prepare plasmid DNA from strains DS5, K87, JH2-2 and transconjugant derivatives of matings between DS5 and JH2-2 by the Vapnek method. The samples thus obtained were run on horizontal 0.8% (w/v) agarose gels and the DNA visualised in ultraviolet light after staining with ethidium bromide. All samples had an identical diffuse band of DNA which co-migrated with that obtained from the plasmid free strain JH2-2 and was, therefore, most likely chromosomal DNA.

The lysis of cells directly in the slots of a vertical gel as described by Eckhardt was tested as a rapid method of plasmid isolation which should have minimised nuclease digestion and the possibilities of mechanical shearing of large plasmids. The strains used in the first experiment were DS5, JH2-2 and two tetracycline resistant transconjugants from a mating of these strains, and the procedure was repeated using strains DS5, JH2-2, K55 and K60. On both gels, the position of chromosomal DNA was taken as those bands co-migrating with that from the plasmid free JH2-2. Such a band was seen in DS5 and transconjugants on the first gel but only in JH2-2 on the second. On the first gel, a faint band which had migrated faster than chromosomal DNA was seen in all samples except JH2-2 and it was assumed that this was the tetracycline resistance plasmid pAMα1 but other bands representing pAMγ1 or pAMβ1 were not seen in the DS5 sample. The second gel gave a more promising result for DS5 with pAMα1 clearly visible and a faint band migrating more slowly than chromosomal DNA, probably pAMγ1, although neither K55 nor K60 samples showed any DNA bands.

Assuming that under the conditions used lysis of cells was obtained it is possible that no plasmid DNA was seen in K55 and K60 because the concentration of cells was too high resulting in a reduced yield of plasmid (Eckhardt, 1978) although a band of chromosomal DNA would have been expected in these circumstances. Alternatively, if the cell concentration was too low and low copy number plasmids present, the concentration of DNA released would probably have been insufficient to visualise after staining.

The mutanolysin lysis method was used to isolate plasmid DNA from strains DS5, K88 and K87 in a series of experiments. As stated previously, density gradient centrifugation steps were omitted and replaced by phenol and chloroform extractions. This lysis method must have released a large amount of soluble protein as, on addition of phenol for the first extraction, the samples became completely white due to precipitated protein. A further phenol extraction followed by two chloroform extractions were necessary to produce a clear aqueous phase from which DNA was precipitated with ethanol. Figure 4 is a representation of the band patterns in 0.6% (w/v) agarose obtained from the above strains and the plasmid free JH2-1 on four separate occasions. As the running conditions were slightly different on each occasion, band sizes were calculated from the standard DNA samples on the respective gels and re-drawn in the appropriate position. The pattern of bands obtained from DS5 was similar in both experiments with bands at positions expected for ccc DNA of pAMα1 (9.1kb) pAMβ1 (26.5kb) and possibly one or more of the pAMγ1 plasmids (54kb). The other bands in the samples were either chromosomal DNA or other forms of these plasmids. The procedure was less successful with strain K88 - apparently only chromosomal DNA was isolated in the first experiment. However three bands, one probably chromosomal, were isolated when the procedure was repeated. The most striking feature of the K87 samples was the large amount of chromosomal DNA present. Band patterns were similar in both experiments but what the

Figure 4. Schematic representation of DNA samples prepared by the mutanolysin method after electrophoresis in 0.6% agarose gels.

Numbers in parenthesis indicate the experiment number. Arrow indicates chromosomal DNA and γ,β,α show the positions of pAMγ, pAMβ1 and pAMα1 of DS5 respectively.

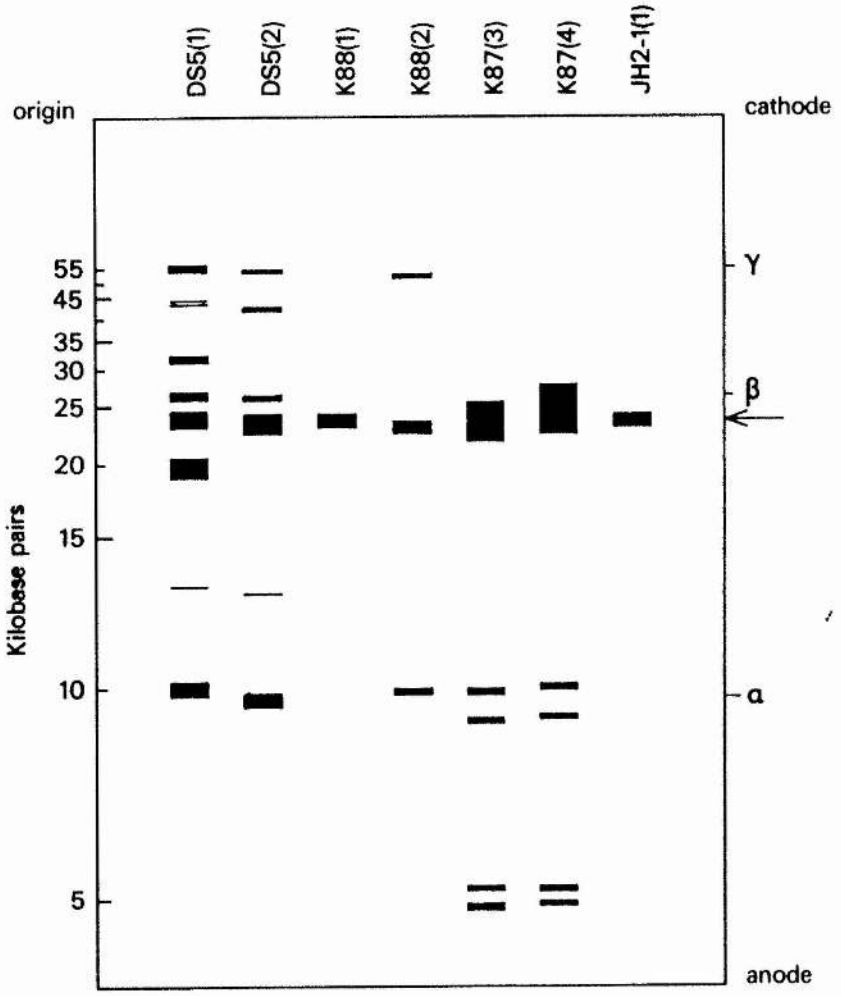


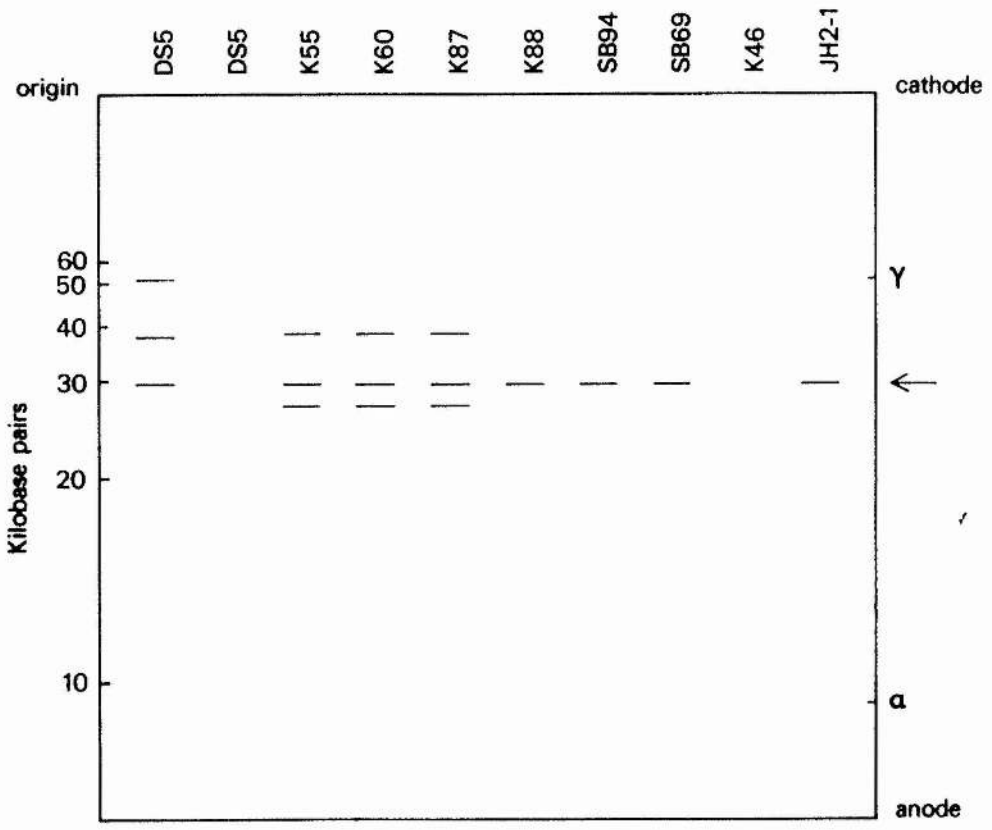
figure does not show was the distortion of the four small bands of K87 which was a characteristic of these samples and possibly caused by incomplete deproteinisation or overloading due to the high proportion of chromosomal DNA.

The method was successful in isolating several plasmid species from the strains tested but there was also a large amount of chromosomal DNA in the samples which could conceal the presence of co-migrating plasmid DNA. Another disadvantage of the method was the inefficient separation of cccDNA from open circular or linear forms of plasmids, exemplified in DS5 samples where there were several bands in addition to those expected.

The method recommended by Crosa and Falkow (1981) for isolation of large plasmids was tested twice using all the parental antibiotic resistant strains and the plasmid free strain JH2-1 and the appearance of the samples from the first experiment after electrophoresis in a 0.6% (w/v) agarose gel is shown in Figure 5. Two DS5 samples, one of which was prepared from double the cell density, were included in the preparation and only the sample from the higher DS5 cell density showed any bands, one of which was probably chromosomal DNA. The probable positions of pAMγ and pAMα1 are marked but there was no DNA visible at the position expected for pAMα1 or pAMβ1. Samples prepared from K55, K60 and K87 all had the same band pattern which differed markedly from that obtained from K87 using the mutanolysin procedure. Only chromosomal DNA was isolated from K88, SB94, SB69 and JH2-1 while no DNA was visible in the K46 sample. In general, the DNA bands obtained were very faint. At the second attempt to isolate plasmids using this method, no DNA was seen in any of the samples with the exception of K87 which had one band of approximately 26kb. The most probable reasons for the failure of this method were either not enough starting material i.e. insufficient cells to yield detectable DNA at the end of the procedure, or incomplete lysis of cells in the absence of lysozyme treatment leading to a

Figure 5. Schematic representation of DNA samples prepared by the Crosa and Falkow method after electrophoresis in a 0.6% agarose gel.

Arrow indicates the position of chromosomal DNA and γ, α the probable positions of DS5 plasmids pAMγ and pAMα1 respectively.

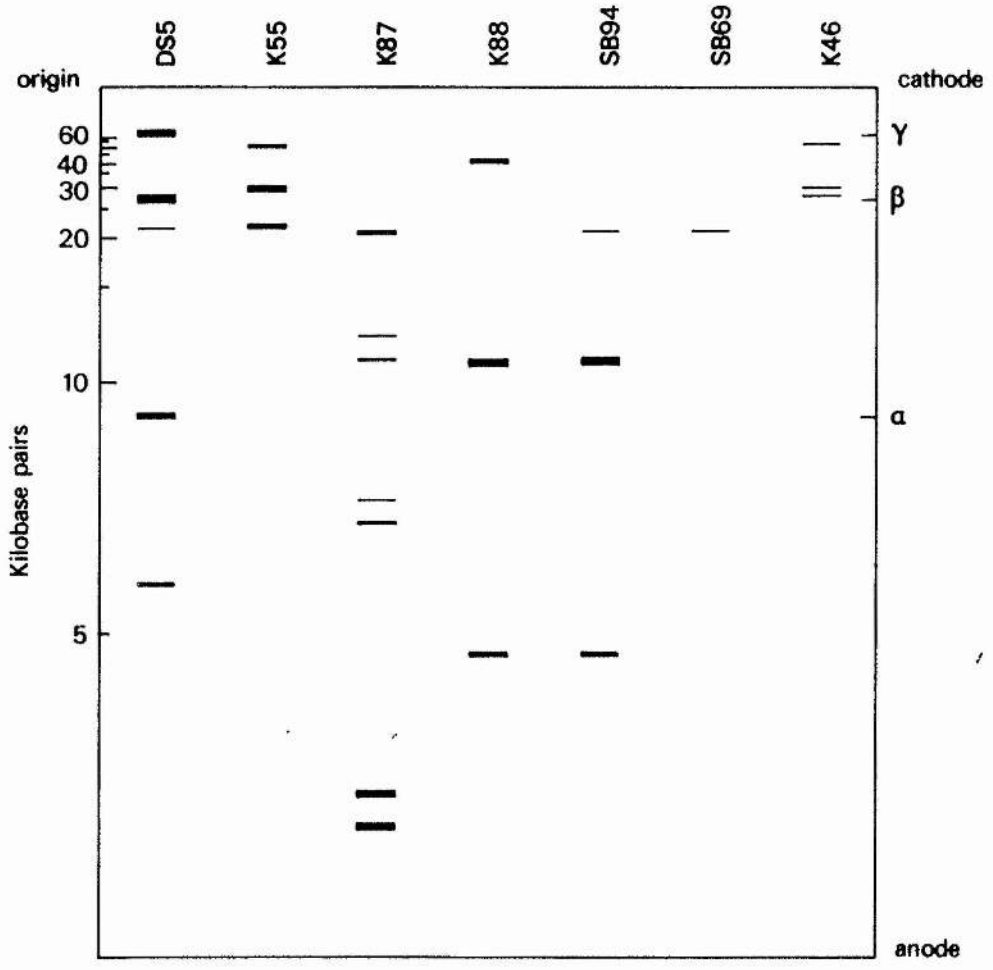


low DNA yield.

Large scale preparations from 1000ml culture which included a caesium chloride-ethidium bromide density gradient centrifugation were carried out once for strains DS5, K87, K88, SB94, twice for strains K55, SB69 and K46 and three times for K60. After centrifugation, all gradients except those of K60, SB69 and one of K46 had two and sometimes three bands visible with ultraviolet light. The uppermost band contained mainly chromosomal DNA and the lowest band the ccc plasmid DNA. Gradients from K60 preparations did not have any visible DNA bands while SB69 and one of the K46 preparations had one faintly fluorescent band at a density equivalent to that of the chromosomal DNA in the other preparations. Since samples were run on different agarose gels with slightly altered running conditions, the sizes of the bands obtained have been estimated from standard size DNA samples on the individual gels and re-drawn in Figure 6 at the appropriate positions. Samples DS5, K55, K87, SB94 and SB69 all had bands of approximately 21kb which may have been contaminating chromosomal DNA. As the method is designed to separate the linear chromosomal DNA from ccc plasmids, however, it is possible, especially in samples K55 and K87 where the bands were clear and sharp, that these bands represent plasmids. In addition to this band, the DS5 sample contained bands migrating to positions expected for pAMγ, pAMβ1 and pAMα1 with a further band of 5.5kb. This latter could have been the 5.1kb autonomously replicating dissociation product of pAMα1 described by Perkins and Youngman (1983). Both preparations of K55 yielded three bands. Strain K87 had, in addition to the 21kb band, six others arranged in three pairs none of which was the same size as those obtained by the mutanolysin method. The K88 sample had three bands, two of which co-migrated with bands obtained from strain SB94. SB69 had as expected only one faint diffuse band which was probably chromosomal DNA. Only one of the K46 density gradients contained ccc plasmid DNA which after electrophor-

Figure 6. Schematic representation of DNA samples obtained from 1000ml cultures after electrophoresis in 0.8% agarose gels.

Probable positions of DS5 plasmids pAMγ, pAMβ1 and pAMα1 are indicated by γ, β and α respectively.



esis was found to be composed of three bands.

Table 19 shows the average sizes of bands estimated on 0.6% agarose gels from several Dunny preparations. Between preparations, there were variations in band patterns particularly with respect to the larger bands. Presumably, such large plasmids are more susceptible to shear during preparation or alternatively, these bands could represent nicked forms of smaller plasmids, multimers or concatamers. Analysis of restriction endonuclease digestions of DS5 plasmids has revealed that the largest band obtained on gels is in fact composed of three plasmids ranging from 60 to 45.8kb (Clewell et al., 1982) and in three preparations, two bands were seen but resolution was poor. Additionally, most preparations had a band co-migrating with JH2-1 DNA and therefore presumably chromosomal DNA. pAMβ1 was usually resolved below this band at this gel concentration but the most distinct band in all DS5 preparations was always that corresponding to pAMα1.

Of the three or four bands visible in samples from strain K55, only band 1 was always present. Two preparations consisted of bands 1, 2 and 3, two of bands 1, 3 and 4 one one of bands 1, 2 and 4. Band 3 of K55 was possibly chromosomal DNA. Strain K87 samples resolved into five or six bands of which band 2 was the most prominent and was within the size range for chromosomal DNA. The sizes of the two pairs of bands, 3 and 4, and 5 and 6, were in agreement with those obtained previously by the multanolysin method. More bands were detected from K88 samples prepared by the Dunny method than by either the multanolysin or large scale methods and only bands 2, 5 and 6 were of similar sizes to those observed previously. Either band 3 or 4 could have been chromosomal DNA. SB94 samples had three or four DNA bands, two of which (3 and 4) corresponded to bands obtained by the large scale method and one of which (band 2) was probably chromosomal DNA.

Table 19. Mean sizes of bands observed in 0.6% agarose gels from samples prepared by the Dunny method.

Strain	Number of preparations	Band designation	Mean size of band (kb ± standard deviation)	Number of times band observed
DS5	10	pAMγ	53.3 ± 5.6	8
		pAMβ1	25.8 ± 3.1	10
		pAMα1	9.7 ± 0.8	10
K55	5	1	51.5 ± 5.9	5
		2	37.0 ± 4.4	3
		3	23.8 ± 1.6	4
		4	16.9 ± 0.9	3
K87	5	1	59.7 ± 3.3	3
		2	26.7 ± 1.8	5
		3	9.9 ± 0.7	5
		4	9.0 ± 0.8	5
		5	5.1 ± 0.5	5
		6	4.7 ± 0.4	5
K88	8	1	58.3 ± 6.4	6
		2	38.8 ± 2.8	7
		3	28.5 ± 2.6	6
		4	22.3 ± 2.4	5
		5	10.2 ± 0.5	8
		6	5.2 ± 0.4	8
SB94	4	1	53.8 ± 2.0	3
		2	23.2 ± 1.6	4
		3	10.9 ± 2.3	4
		4	5.2 ± 0.4	4
JH2-1	8		26.7 ± 3.4	8

Not included in Table 19 are the results for strains K60, K46 and SB69. In five of nine K60 preparations, no plasmid DNA was detected while in the remaining four, two or three bands were visible one of which always migrated with DNA obtained from plasmid free strains and was assumed to be chromosomal DNA. In addition to this band, two preparations had each one band of size 60.5kb and 65.5kb respectively and two preparations had two bands of sizes 120 and 97.5kb, and 94.5 and 53.7kb respectively. The relationship of these different estimated sizes was not clear.

Only two preparations from strain K46 produced bands on agarose gels, and in each, four bands were obtained. The first preparation run on 0.6% agarose had band sizes estimated at 120, 97.5, 41 and 28kb while the second preparation run on 0.35% agarose had band sizes estimated at 80, 61, 38 and 17.2kb. Again, the relationship of the bands observed in the different preparations was unclear. As with all the previous methods, no plasmid DNA could be isolated from four preparations of strain SB69.

To summarise therefore, it was found that the Vapnek, Eckhardt and Crosa and Falkow methods were unreliable and/or not reproducible. The mutanolysin method was efficient for isolating plasmid DNA from strains DS5 and K87 but a large, sometimes unacceptable, amount of chromosomal DNA was also isolated. The large scale method while producing "clean" preparations from strains DS5, K55, K87, K88 and SB94 was not successful in isolating large plasmids such as those found in strains K60 and K46. Additionally, the inclusion of density gradient centrifugation was prohibitive both in terms of time and cost. The Dunny method was therefore considered the most efficient, reproducible and useful method and was used throughout for the analysis of plasmid content from transconjugant and cured derivatives.

PLASMID CONTENT OF PARENTAL STRAINS AND DERIVATIVES

In order to relate the phenotypes of the parental strains to their respective plasmids, the plasmid content of a selection of transconjugants and cured derivatives of each strain was investigated using the DNA preparation method of Dunny (1981[b]).

Figure 7(a) shows the appearance of samples from K55 and transconjugants of matings between K55 and JH2-2 after electrophoresis in 0.5% agarose. Figure 7(b) shows samples from a second preparation of K55 along with transconjugants from matings with JH2-17 and cured derivatives of K55, after electrophoresis in 0.6% agarose. The band numbers given correspond to those assigned in Table 19. In the first preparation (Figure 7a), the band pattern obtained for K55 was 1, 2 and 4 while in the second preparation (Figure 7b) was 1, 3 and 4. The band at 27.5kb in Figure 7(a) which was present in transconjugant strains was most likely chromosomal DNA as was the band at 21.8kb in Figure 7(b). However, it is conceivable that these bands could consist of chromosomal DNA and a co-migrating plasmid, particularly in Figure 7(b) where the amount of DNA in this band from transconjugants is far greater than that of plasmid free recipient JH2-17. Tetracycline resistant transconjugants SSD3, SSD4 and SSD18 had similar band patterns to the parental strain although only SSD3 included band 4. The apparent variation in the migration of bands 1 and 2 was assumed to be due to differences in DNA concentration rather than actual size differences. The erythromycin resistant transconjugants SSD8 and SSD9 and the transconjugants co-resistant to erythromycin and streptomycin, SSD14 and SSD15, all contained band 1 only while the streptomycin resistant transconjugants SSD22 and SSD23 exhibited the same band pattern as SSD4 and SSD18. Of the samples in the second preparation, streptomycin resistant SSK1 and SSK2 had only band 3 (possibly chromosomal DNA) and SSK3 contained bands 2 and 3 (band 2 was not present in the corresponding K55 sample). Like those tetracycline resistant transcon-

Figure 7. Plasmid preparations of strain K55 and derivatives after electrophoresis in 0.5% agarose. (a) K55 and transconjugants from matings with JH2-2.

Values to the left of the figure are the sizes (kb) of standard plasmid molecules. These were, from the top, RP4, pDB248, pBR325/Bam and pBR322. Arrow indicates position of chromosomal DNA. Sample volumes were 20µl.

Strain	Derivation	Phenotype	Bands observed
K55	parental	Em Tc Sm	1, 2, 4
SSD3	K55 x JH2-2	Tc	1, 2, 4
SSD4, SSD18	"	Tc	1, 2
SSD8, SSD9	"	Em	1
SSD14, SSD15	"	Em Sm	1
SSD22, SSD23	"	Sm	1, 2

Band numbers refer to Table 19.

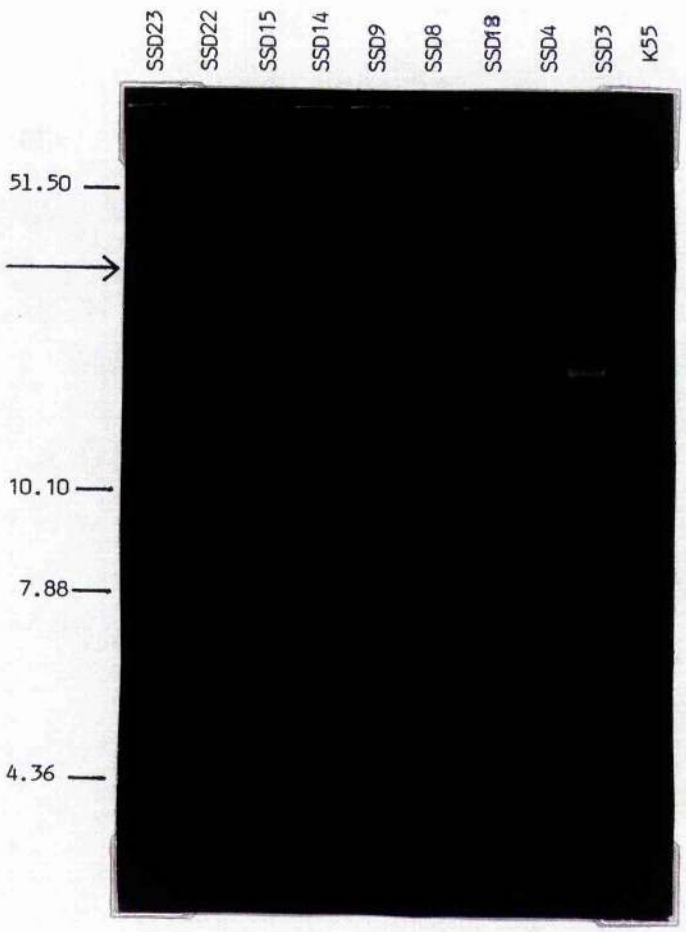
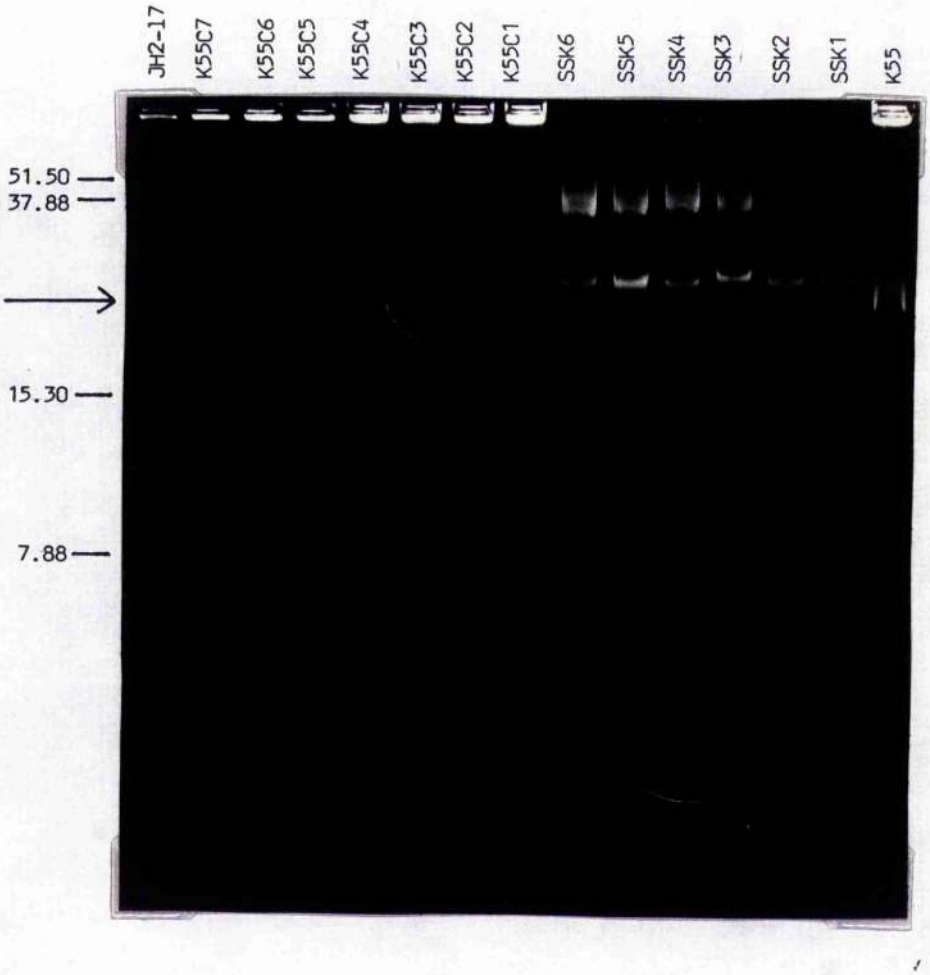

SSD23
SSD22
SSD15
SSD14
SSD9
SSD8
SSD18
SSD4
SSD3
K55
51.50
10.10
7.88
4.36

Figure 7. Plasmid preparations of strain K55 and derivatives after electrophoresis in 0.6% agarose. (b) K55, transconjugants from matings with JH2-17 and cured derivatives.

Values to the left of the figure are the sizes (kb) of standard plasmid molecules. These are, from the top, RP4, Sa, pAJ50 and pBR325/Bam. Arrow indicates position of chromosomal DNA. Sample volumes were 30µl.

Strain	Derivation	Phenotype	Bands observed
K55	parental	Em Tc Sm	1, 3, 4
SSK1, SSK2	K55 x JH2-17	Sm	3
SSK3	"	Sm	2, 3
SSK4, SSK5, SSK6	"	Tc	2, 3
K55C1, K55C2	Storage cured derivatives	Tc	1, 3
K55C4, K55C5, K55C6, K55C7	Novobiocin cured derivatives	Tc	1, 3
K55C3	Storage cured derivative	Em Tc	1, 3, 4

Band numbers refer to Table 19.


JH2-17
K55C7
K55C6
K55C5
K55C4
K55C3
K55C2
K55C1
SSK6
SSK5
SSK4
SSK3
SSK2
SSK1
K55
51.50
37.88
15.30
7.88

jugants of JH2-2 matings, band 2 was observed in SSK4, SSK5 and SSK6 but instead of band 1, band 3 was seen. The samples from derivatives of K55 cured of erythromycin and streptomycin resistance - K55C1, K55C2, K55C4, K55C5, K55C6 and K55C7 - all consisted of bands 1 and 3 and K55C3, the single derivative cured of streptomycin resistance alone, had the same band pattern as K55 namely bands 1, 3 and 4.

Two plasmid preparations of strain K60 and derivatives in 0.6% agarose gels are shown in Figure 8(a) and (b). Electrophoresis was carried out for 10 hours longer in Figure 8(b) than Figure 8(a). Neither preparation yielded any plasmid DNA from strain K60 and in both preparations, samples from transconjugant strains contained a prominent band co-migrating with DNA from JH2-17which was probably chromosomal DNA. Of the transconjugants of K60 and JH2-2, all had a band of approximately 55.0kb, the differences in migration probably being due to disparity of DNA concentrations rather than actual size variation. Strain SSF18 however, contained additional bands of sizes 9.4 and 5.6kb neither of which had ever been observed in K60 preparations. Transconjugants of K60 and JH2-17 displayed basically the same band patterns as those above although the size of the band was higher at approximately 65.0kb and there was a larger band visible at approximately 140.0kb which may have been a dimer or open circular form of the smaller band. Derivatives K60C1, K60C2 and K60C3 cured of streptomycin resistance during storage had a different band pattern to those derivatives K60C5, K60C6, K60C7 and K60C8 obtained by novobiocin treatment. Excluding the probable chromosomal band, the former samples contained bands of sizes 65.9 and 12.0kb while the latter contained bands of sizes 75.2, 39.8, 15.5 and 14.6kb. The sample from strain K60C4, a derivative susceptible to erythromycin and streptomycin obtained by novobiocin treatment, appeared to consist of only the putative chromosomal DNA band.

Figure 8. Plasmid preparations of strain K60 and derivatives after electrophoresis in 0.6% agarose.(a) K60 and transconjugants from matings with JH2-2.

Values to the left of the figure are the sizes (kb) of standard plasmid molecules. These are. from the top, RP4, pDB248, pBR325/Bam and pBR322. Arrow indicates position of chromosomal DNA. Sample volumes were 20µl.

Strain	Derivation	Phenotype	Band sizes (kb)
K60	parental	Em Tc Sm Km Nm	None visible
SSF2, SSF3, SSF17	K60 x JH2-2	Em Tc Sm Km Nm	55.0, 23.2
SSF18	"	Em Tc Sm Km Nm	55.0, 26.0, 23.2, 9.4, 5.6
SSF4, SSF5, SSF11, SSF12	"	Em Sm Km Nm	55.0, 23.2
SSF9, SSF10	"	Em Tc	55.0, 23.2
SSF13, SSF14, SSF15, SSF16	"	Em	55.0, 23.2

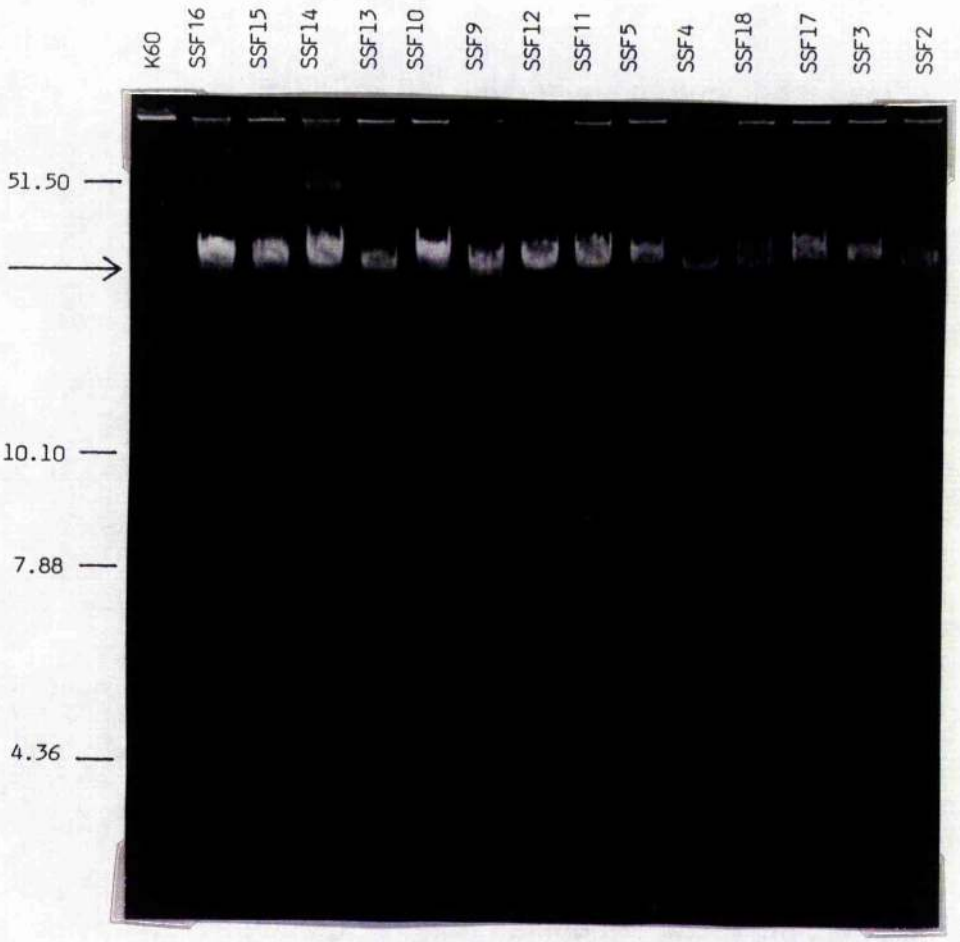
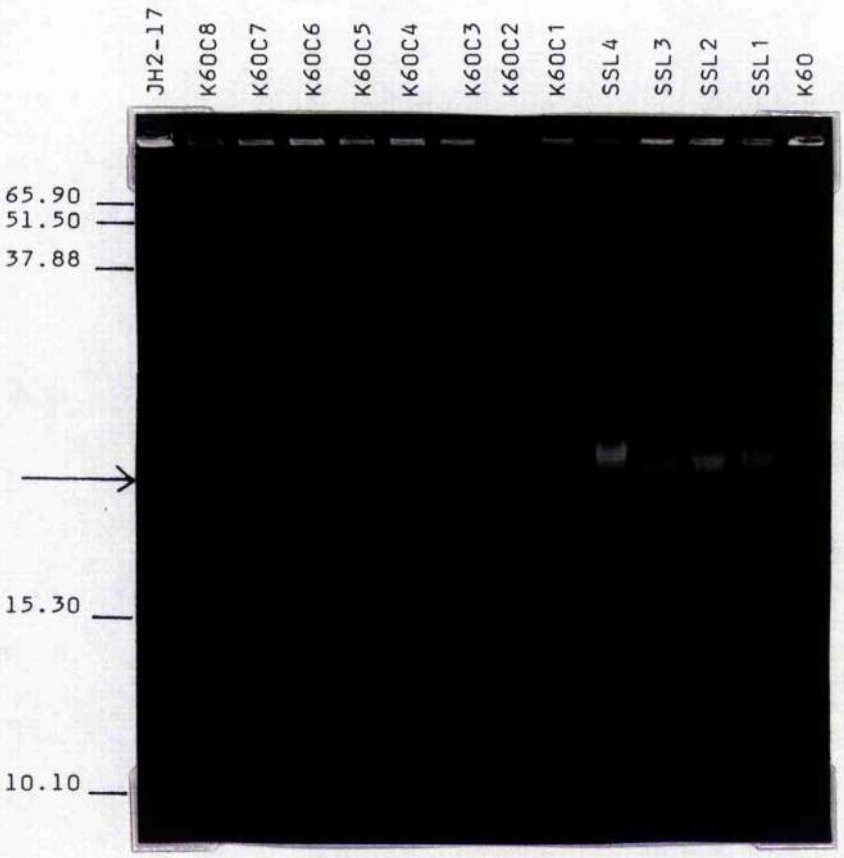

K60
SSF16
SSF15
SSF14
SSF13
SSF10
SSF9
SSF12
SSF11
SSF5
SSF4
SSF18
SSF17
SSF3
SSF2
51.50
10.10
7.88
4.36

Figure 8. Plasmid preparations of strain K60 and derivatives after electrophoresis in 0.6% agarose. (b) K60, transconjugants of matings with JH2-17 and cured derivatives.

Values to the left of the figure are the sizes (kb) of standard plasmid molecules. These are, from the top, pGC9114, RP4, Sa, pAJ50 and pDB248. Arrow indicates position of chromosomal DNA. Sample volumes were 30µl.

Strain	Derivation	Phenotype	Band sizes (kb)
K60	parental	Em Tc Sm Km Nm	None visible
SSL1, SSL2, SSL3	K60 x JH2-17	Em	140.0, 65.0, 17.2
SSL4	"	Tc	65.0, 17.2
K60C1, K60C2, K60C3	Storage cured derivative	Em Tc	65.9, 17.2, 12.0
K60C4	Novobiocin cured derivative	Tc	17.2
K60C5, K60C6, K60C7, K60C8	"	Em Tc	75.2, 39.8, 17.2, 15.5, 14.6


JH2-17
K60C8
K60C7
K60C6
K60C5
K60C4
K60C3
K60C2
K60C1
SSL4
SSL3
SSL2
SSL1
K60
65.90
51.50
37.88
15.30
10.10

Two plasmid preparations, one of K87 and transconjugants of matings with JH2-2 the other of K87 with transconjugants of matings with JH2-17 and cured derivatives of K87 are shown in Figure 9. The derivation of the strains and the bands present in the preparations are also shown. In the first preparation (Figure 9a) band 1 was not visible in the K87 sample even with 20µl sample but was present in several of the transconjugant strains although it was not consistently associated with any particular phenotype. Band 2 was present in all the strains and as this band had been found previously to co-migrate with chromosomal DNA from plasmid free strains, was thought perhaps to be chromosomal DNA. The staining of this band, however, was much more intense in most of the transconjugant strains than in the K87 sample and in JH2-17 samples (not shown) of equivalent volume. None of the transconjugant samples contained bands 3, 4, 5 or 6. In the second preparation (Figure 9b and c), band 1 was just visible in the K87 sample of Figure 9(c) when 40µl was applied to the gel. This band was also seen in several transconjugant strains, including SSG12 which had contained this band in the first preparation also, but as before it was not associated with any particular phenotype. Band 2 was again present in all the samples and although it appeared to co-migrate with the DNA isolated from plasmid free JH2-17, the amount of DNA in band 2 was far in excess of that in the chromosomal DNA from JH2-17. As before, the staining intensity of the band was variable, again conflicting with the assumption that this band represented linear chromosomal DNA as the samples were treated alike, in as much as was possible, and should therefore have contained roughly the same amount of linear DNA. None of the transconjugant strains contained bands 3, 4, 5 or 6 but the tetracycline resistant transconjugants SSM3 and SSM4 had bands of 32.6kb and 13.0kb respectively which were not present in the parent K87. In contrast, bands 3, 4, 5 and 6 were present in all the cured derivatives with the exception of K87C8 in which only bands 4 and 6 were visible. All the cured derivatives contained band 2 but only those

Figure 9. Plasmid preparations of strain K87 and derivatives after electrophoresis in 0.6% agarose. (a) K87 and transconjugants from matings with JH2-2.

Values to the left of the figure are the sizes (kb) of standard plasmid molecules. These are, from the top, RP4, pBR325/Hind, pBR325/Bam and pBR322. Arrow indicates position of chromosomal DNA. With the exception of the K87 samples indicated, sample volumes were 10µl.

Strain	Derivation	Phenotype	Bands observed
K87	parental	Em Tc Sm Km Nm Hly	2, 3, 4, 5, 6
SSG15, SSG29	K87 x JH2-2	Em Sm Km Nm	1, 2
SSG30	"	Em Sm Km Nm	2
SSG8, SSG9, SSG14	"	Em Tc Sm Km Nm	2
SSG16, SSG28	"	Em Tc Sm Km Nm	1, 2
SSG1, SSG2	"	Hly	1, 2
SSG12	"	Em	1, 2
SSG17	"	Tc	2
SSG23	"	Em Hly	2
SSG26	"	Em Tc	2

Band numbers refer to Table 19.

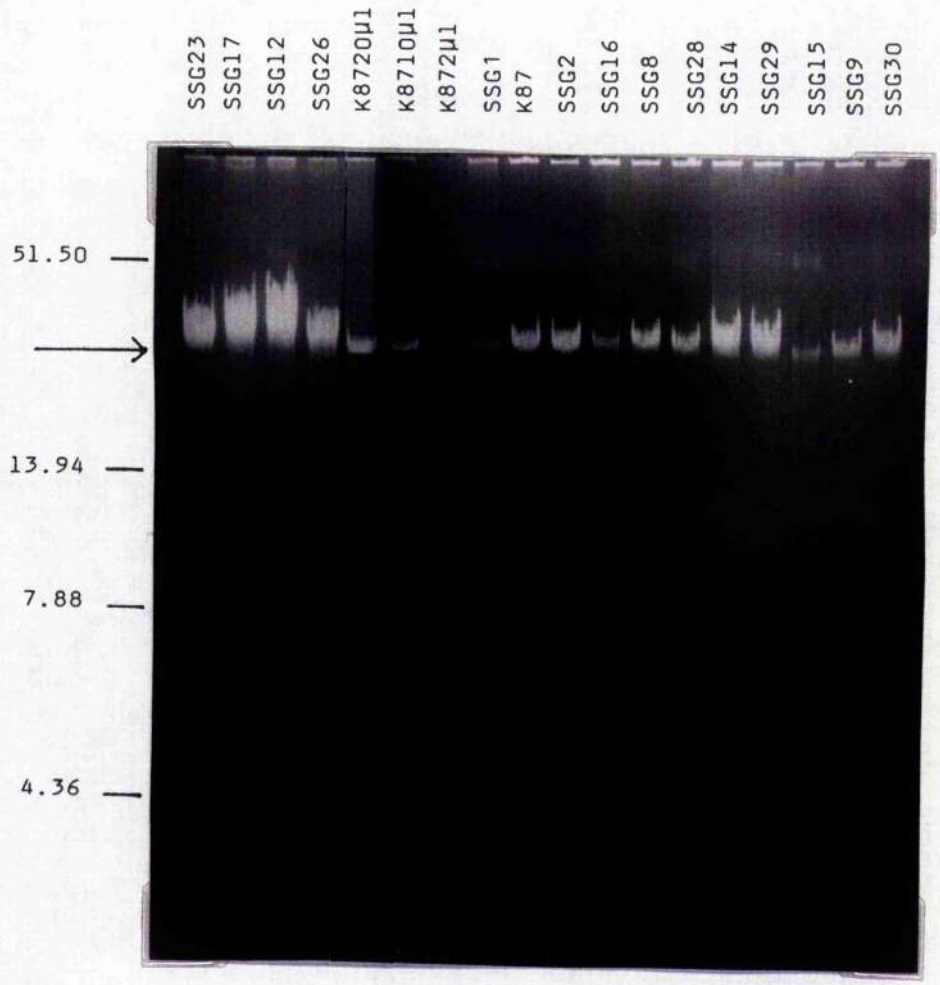

SSG23
SSG17
SSG12
SSG26
K8720µl
K8710µl
K872µl
SSG1
K87
SSG2
SSG16
SSG8
SSG28
SSG14
SSG29
SSG15
SSG9
SSG30
51.50
13.94
7.88
4.36

Figure 9. Plasmid preparations of strain K87 and derivatives after electrophoresis in 0.6% agarose. (b) K87 and transconjugants from matings with JH2-17 and JH2-2.

Values to the left of the figure are the sizes (kb) of standard plasmid molecules. These are, from the top, pGC9114, RP4, Sa, pAJ50, pDB248, and pHC79. Arrow indicates the position of chromosomal DNA. Sample volumes were 30µl.

Strain	Derivation	Phenotype	Bands observed
K87	parental	Em Tc Sm Km Nm Hly	2, 3, 4, 5, 6
SSM1	K87 x JH2-17	Em	1, 2
SSG12, SSG13	K87 x JH2-2	Em	1, 2
SSM2	K87 x JH2-17	Tc	2
SSM3	"	Tc	1, 2, (32.6)
SSM4	"	Tc	2, (13.0)
SSM6, SSM7	"	Hly	1, 2
SSM8, SSM9	"	Hly	2

Band numbers refer to Table 19 while values in parenthesis are sizes (kb) of novel bands.

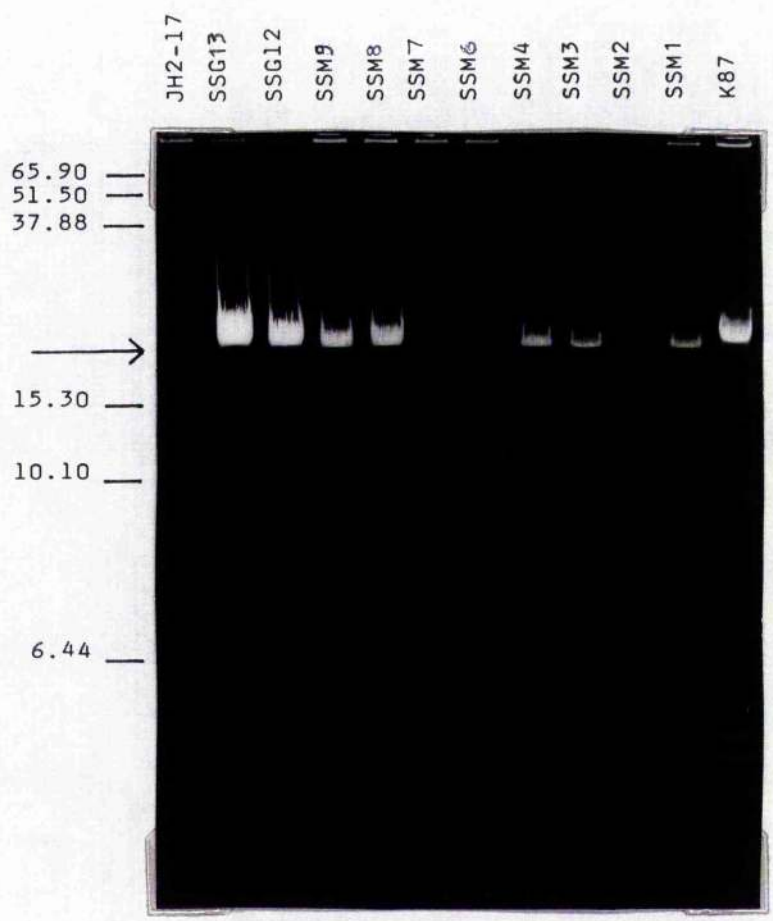
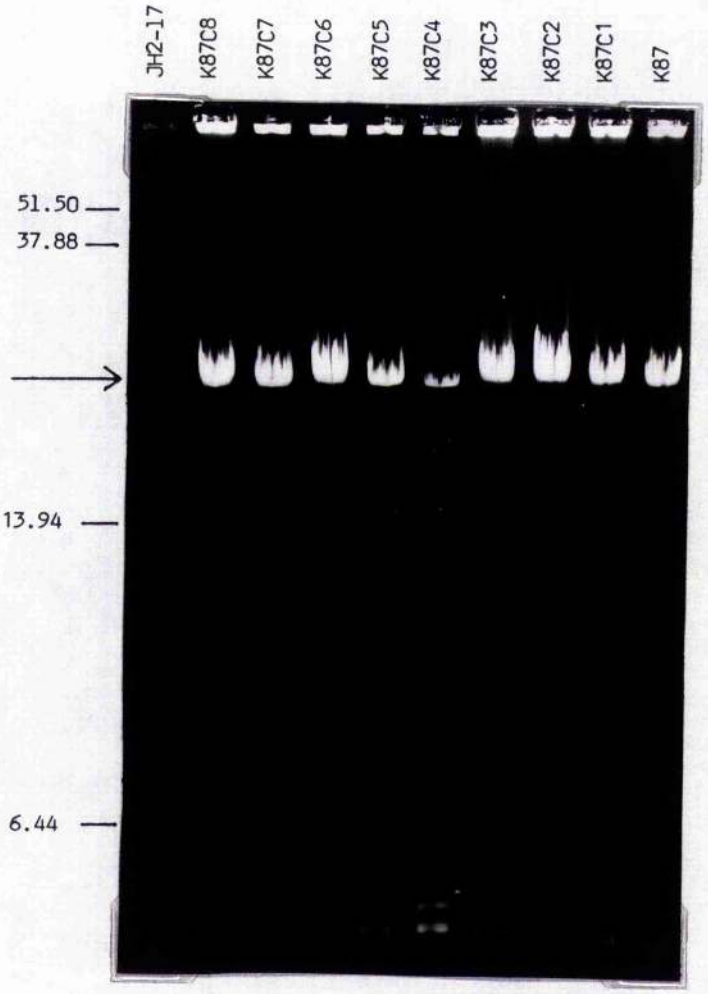

JH2-17
SSG13
SSG12
SSM9
SSM8
SSM7
SSM6
SSM4
SSM3
SSM2
SSM1
K87
65.90
51.50
37.88
15.30
10.10
6.44

Figure 9. Plasmid preparations of strain K87 and derivatives after electrophoresis in 0.6% agarose. (c) K87 and cured derivatives.

Values to the left of the figure are the sizes (kb) of standard plasmid molecules. These are, from the top, RP4, Sa, pBR325/Hind and pHC79. Arrow indicates position of chromosomal DNA. Sample volumes were 40µl.

Strain	Derivation	Phenotype	Bands observed
K87	Parental	Em Tc Sm Km Nm Hly	1, 2, 3, 4, 5, 6
K87C1, K87C2, K87C3	Storage cured derivative	Em Tc Sm Km Nm	2, 3, 4, 5, 6
K87C6, K87C7	Novobiocin cured derivative	Em Tc Sm Km Nm	2, 3, 4, 5, 6
K87C8	Novobiocin cured derivative	Em Tc Sm Km Nm	2, 4, 6
K87C4, K87C5	Storage cured derivative	Em Tc Hly	1, 2, 3, 4, 5, 6

Band numbers refer to Table 19.


JH2-17
K87C8
K87C7
K87C6
K87C5
K87C4
K87C3
K87C2
K87C1
K87
51.50
37.88
13.94
6.44

haemolytic strains had band 1.

Since the mutanolysin method of plasmid preparation had been relatively successful for strain K87, it was used to prepare DNA from K87, SSM1, SSM2, SSM3, SSM4, SSM7, SSM8 and K87C1. Examination of the 0,6% agarose gel (not shown) revealed that the results obtained with the Dunny preparation were reproducible by this method with the exceptions that SSM4 in addition to band 2 and the 13.0kb band also contained a faint band of 28.0kb and that K87C1 did not appear to have bands 4 and 6.

Samples from two plasmid preparations are shown, the first of strain K88 with transconjugants from JH2-2 matings in Figure 10(a) and the second of K88 with transconjugants from JH2-17 matings in Figure 10(b) and with cured derivatives in Figure 10(c). Derivations of the strains and the sizes of bands observed are also given. As there were several strains which contained apparently novel bands, the band numbers allocated in the previous section (see Table 19) were not used but are shown at the side of the gel photographs. In the first preparation, band 1 of strain K88 appeared to be missing and there was an extra band at 15.4kb. The band of 20.8kb which was present in all the samples was possibly linear chromosomal DNA. Transconjugants SSE1, SSE5, SSE6, SSE7 and SSE17 resistant to erythromycin, tetracycline and chloramphenicol contained only a band of 52.5kb (K88 band 1) and the 20.8kb band. Of the streptomycin resistant transconjugants in this preparation, SSE2 and SSE3 contained only the 20.8kb band, SSE4 contained in addition a 35.0kb band and SSE16 two bands of 38.0 (K88 band 2) and 25.2kb. Repeated preparations of SSE2 also failed to reveal any band other than a possible chromosomal band. The largest bands in the tetracycline resistant transconjugants SSE8 and SSE15 were 42.8kb and 35.0kb respectively and both strains possessed bands of 20.8kb and 19.2kb (K88 band 4). The only transconjugant obtained which was co-resistant to tetracycline and chloramphenicol, SSE18, exhibited the same band pattern as SSE1, SSE5 etc., namely

Figure 10. Plasmid preparations of strain K88 and derivatives after electrophoresis in 0.6% agarose. (a) K88 and transconjugants of matings with JH2-2.

Values to the left of the figure are the sizes (kb) of standard plasmid molecules. These are, from the top, RP4, pDB248, pBR325/Bam and pBR322. Numbers to the right of the figure correspond to the band numbers of Table 19. Arrow indicates position of chromosomal DNA. Sample volumes were 20µl.

Strain	Derivation	Phenotype	Band sizes (kb)
K88	parental	Em Tc Sm Cm	38.8, 20.8, 19.2, 15.4, 8.5, 4.5
SSE1, SSE5, SSE6, SSE7, SSE17	K88 x JH2-2	Em Tc Cm	52.5, 20.8
SSE2, SSE3	"	Sm	20.8
SSE4	"	Sm	35.0, 20.8
SSE16	"	Sm	38.0, 25.2, 20.8
SSE8	"	Tc	42.8, 20.8, 19.2
SSE15	"	Tc	35.0, 20.8, 19.2
SSE18	"	Tc Cm	52.5, 20.8
SSE19	"	Tc	45.0, 20.8, 13.5

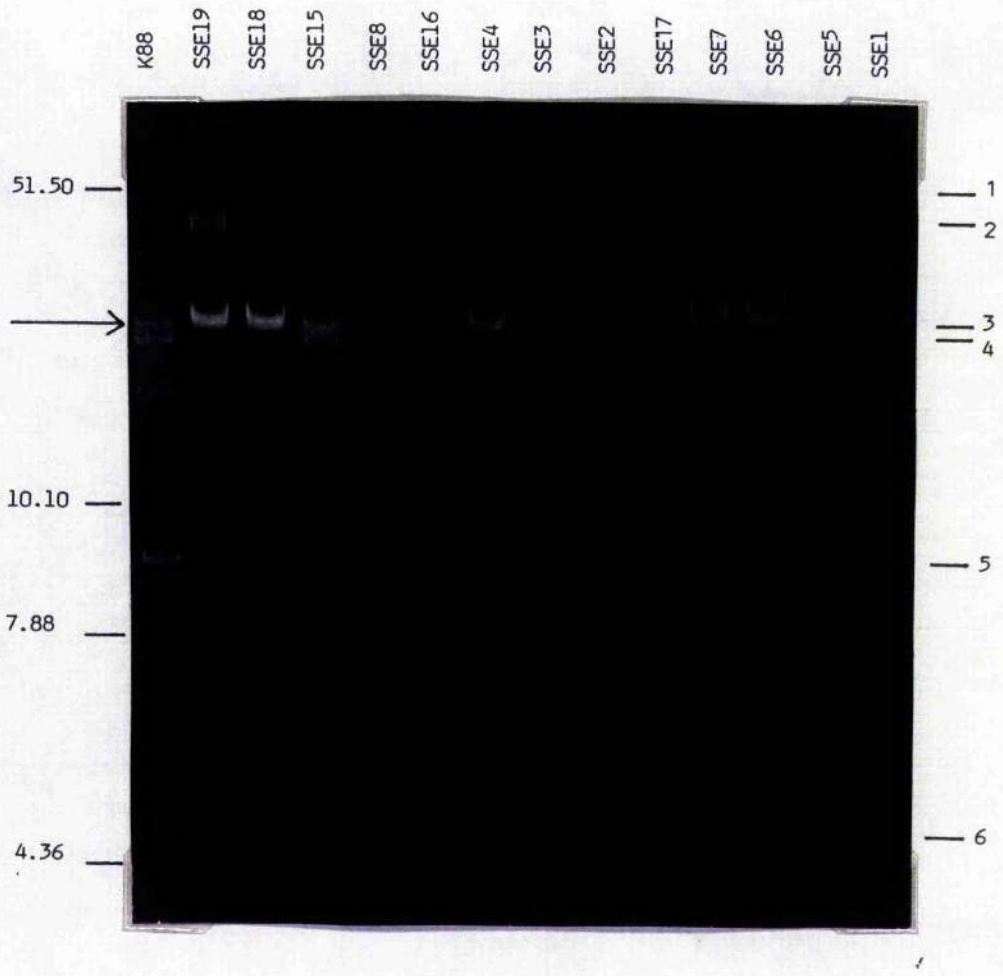

K88
SSE19
SSE18
SSE15
SSE8
SSE16
SSE4
SSE3
SSE2
SSE17
SSE7
SSE6
SSE5
SSE1
51.50
10.10
7.88
4.36
1
2
3
4
5
6

Figure 10. Plasmid preparations of strain K88 and derivatives after electrophoresis in 0.6% agarose. (b) K88 and transconjugants of matings with JH2-17.

Values to the left of the figure are the sizes (kb) of standard plasmid molecules. These are, from the top, pGC9114, RP4, Sa, pDB248 and pBR322. Numbers to the right of the figure correspond to the band numbers of Table 19. Arrow indicates position of chromosomal DNA. Sample volumes were 30µl.

Strain	Derivation	Phenotype	Band sizes (kb)
K88	parental	Em Tc Sm Cm	63.0,33.2,21.5,19.5,10.0,5.4
SSN1	K88 x JH2-17	Tc	63.0,54.0,21.5,19.5
SSN2	"	Tc	63.0,35.0,19.5
SSN3	"	Tc	63.0,21.5,19.5
SSN4, SSN5, SSN6	"	Sm	44.0,19.5

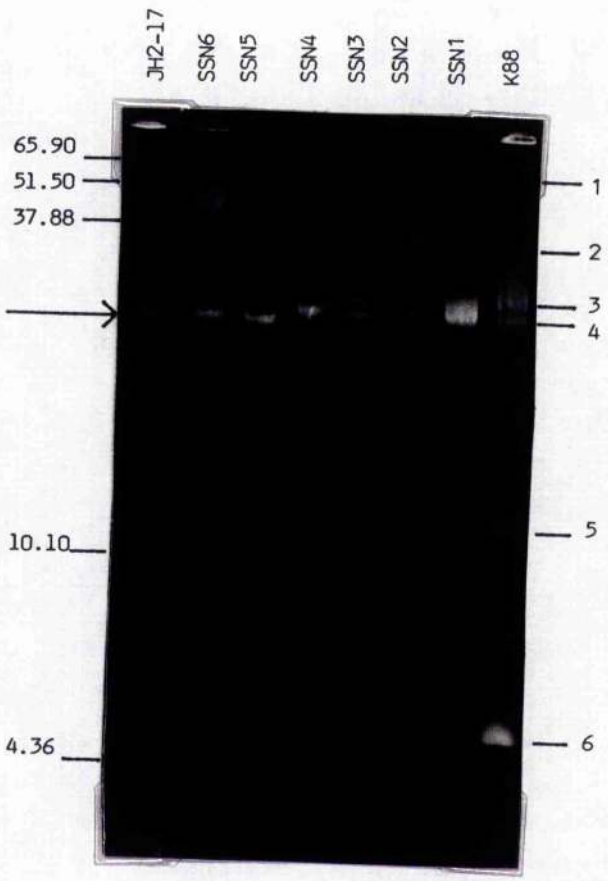
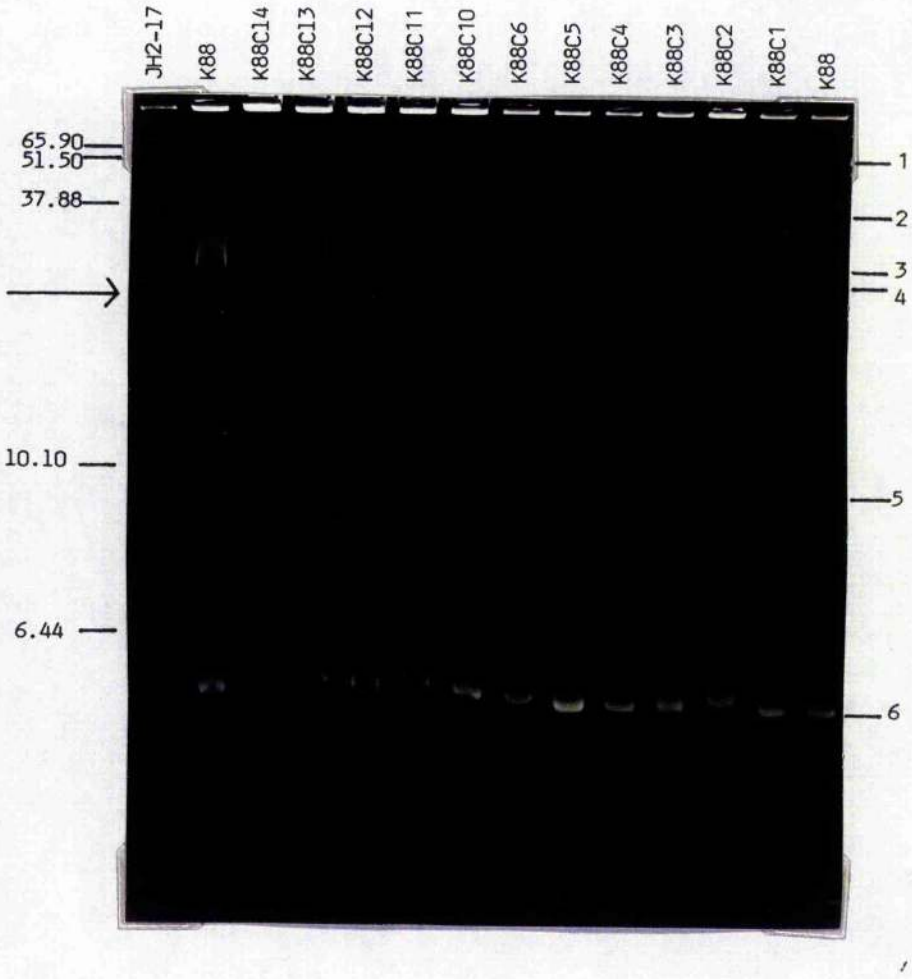

JH2-17
SSN6
SSN5
SSN4
SSN3
SSN2
SSN1
K88
65.90
51.50
37.88
10.10
4.36
1
2
3
4
5
6

Figure 10. Plasmid preparations of strain K88 and derivatives after electrophoresis in 0.6% agarose. (c) K88 and cured derivatives.

Values to the left of the figure are the sizes (kb) of standard plasmid molecules. These are, from the top, pGC9114, RP4, Sa, pDB248 and pHC79. Numbers to the right of the figure correspond to the band numbers of Table 19. Arrow indicates position of chromosomal DNA. Sample volumes were 30µl.

Strain	Derivation	Phenotype	Band sizes (kb)
K88	parental	Em Tc Sm Cm	63.0,35.7,23.0,20.7, 10.0,5.4
K88C1	Storage cured	Tc Sm	63.0,35.7,23.0,20.7, 10.0,5.4
K88C10,K88C11, K88C12	Novobiocin cured derivative	Tc Sm	23.0,20.7,10.0,5.4
K88C13	Novobiocin cured derivative	Tc Sm	35.7,23.0,20.7,10.0, 5.4
K88C14	Novobiocin cured derivative	Tc	23.0
K88C6	Novobiocin cured derivative	Em Tc Cm	63.0,23.0,20.7,10.0,5.4


JH2-17
K88
K88C14
K88C13
K88C12
K88C11
K88C10
K88C6
K88C5
K88C4
K88C3
K88C2
K88C1
K88
65.90
51.50
37.88
10.10
6.44
1
2
3
4
5
6

K88 band 1 and 20.8kb band. In two repeat preparations of this strain, this pattern was also obtained. None of the transconjugants contained K88 bands 5 or 6.

In the second preparation, the band obtained in all samples at 19.5kb in Figure 10(b) and 20.7kb in Figure 10(c) (K88 band 4) co-migrated with DNA obtained from the plasmid free JH2-17 and so was likely to be chromosomal DNA. Band 1 was visible in the K88 sample of this preparation. The tetracycline resistant transconjugants unlike those above, contained K88 band 1 but as above, SSN1 and SSN3 contained K88 band 3 in addition to the 19.5kb band although SSN1 had an extra band of 54.0kb. SSN2 in addition to band 1 and the 19.5kb band had a band of 35.0kb which was probably K88 band 2. The streptomycin resistant transconjugants were also different from those examined in the first preparation, having in addition to the 19.5kb band, one band of 44.0kb. As before bands 5 and 6 were not observed in transconjugants.

The derivative obtained after storage which was susceptible to erythromycin and chloramphenicol, did not appear to be different from the parental strain. In contrast, of those with the same phenotype obtained after novobiocin treatment, K88C10, K88C11 and K88C12 did not contain bands 1 or 2 and K88C13 was missing band 1. One derivative, K88C6 was obtained which had lost streptomycin resistance only and this strain did not contain band 2. The sole derivative which did not contain bands 5 and 6 was K88C14 which had been cured of all but tetracycline resistance. It also lacked bands 1 and 2 and only band 3, which had also been observed in four of five tetracycline resistant transconjugants, was present.

Plasmid preparations of SB94, transconjugants of JH2-2 and JH2-17 matings, and cured derivatives are shown in Figure 11 along with the derivations of the strains and the bands observed (numbered according to

Table 19). As noted previously, there is the possibility that the band 2, present in all the samples, is chromosomal DNA and it did indeed co-migrate with DNA from the plasmid free JH2-17. Both the erythromycin resistant and the tetracycline resistant transconjugant strains had the same bands present i.e. bands 1 and 2, but none had bands 3 or 4. Erythromycin resistant transconjugants contained in addition a novel band of 61.5kb which may have been an open circular form of band 1 although if this were so, it should have been visible in at least some of the other samples. Tetracycline resistant SSO2 appeared to have two bands with similar migration rates, one of which was probably band 1.

Among the cured derivatives, no difference in the bands present could be seen between the various phenotypes and SB94, even in the antibiotic susceptible strain although the amount of band 1, as judged by the intensity of staining, was variable being greatest in the derivatives which had lost erythromycin and streptomycin resistance during storage. An exception was the derivative cured of erythromycin and aminoglycosides resistances by novobiocin treatment, SB94C8, which in addition had a novel band of 18.2kb.

As no transfer of antibiotic resistance was obtained in matings with K46 donors, Figure 12 shows plasmid preparations of K46 and cured derivatives only, after electrophoresis in 0.35% (w/v) agarose. K46C1 was isolated after storage, the other derivatives were obtained by novobiocin treatment. The sizes of the bands observed are also given. It was not clear whether the faint bands in the K46 samples at 34.7 and 32.9kb were in fact the same as the major band at 38.5kb and an artefact caused perhaps by the low gel concentration, or were separate plasmid species. In support of the former, all four K46 samples, which had been processed individually, showed the same band pattern and a similar effect was seen with the cccDNA band of the standard plasmid pSa. Against this, however, was the fact that all the derivatives with the exception of K46C11 had similar but not identical

Figure 11. Plasmid preparations of strain SB94, transconjugants from matings with JH2-2 and JH2-17 and cured derivatives after electrophoresis in 0.6% agarose.

Values to the left of the figure are the sizes (kb) of standard plasmid molecules. These are, from the top, RP4, Sa, pBR325/Hind, pBR325/Bam and pHC79. Arrow indicates the position of chromosomal DNA. Sample volumes were 30µl.

Strain	Derivation	Phenotype	Bands observed
SB94	parental	Em Tc Sm Km Nm	1, 2, 3, 4
SSH5, SSH6, SSH7	SB94 x JH2-2	Em	(61.5), 1, 2
SSO1, SSO3	SB94 x JH2-17	Tc	1, 2
SSO2	SB94 x JH2-17	Tc	(54.0), 1, 2
SB94C1, SB94C2, SB94C3	Storage cured derivative	Tc	1, 2, 3, 4
SB94C8	Novobiocin cured derivative	Tc	1, 2, (18.2), 3, 4
SB94C4, SB94C5 SB94C6	Novobiocin cured derivative	Em Tc	1, 2, 3, 4
SB94C7	Novobiocin cured derivative	Antibiotic susceptible	1, 2, 3, 4

Band numbers refer to Table 19. Values in parenthesis are sizes (kb) of novel bands.

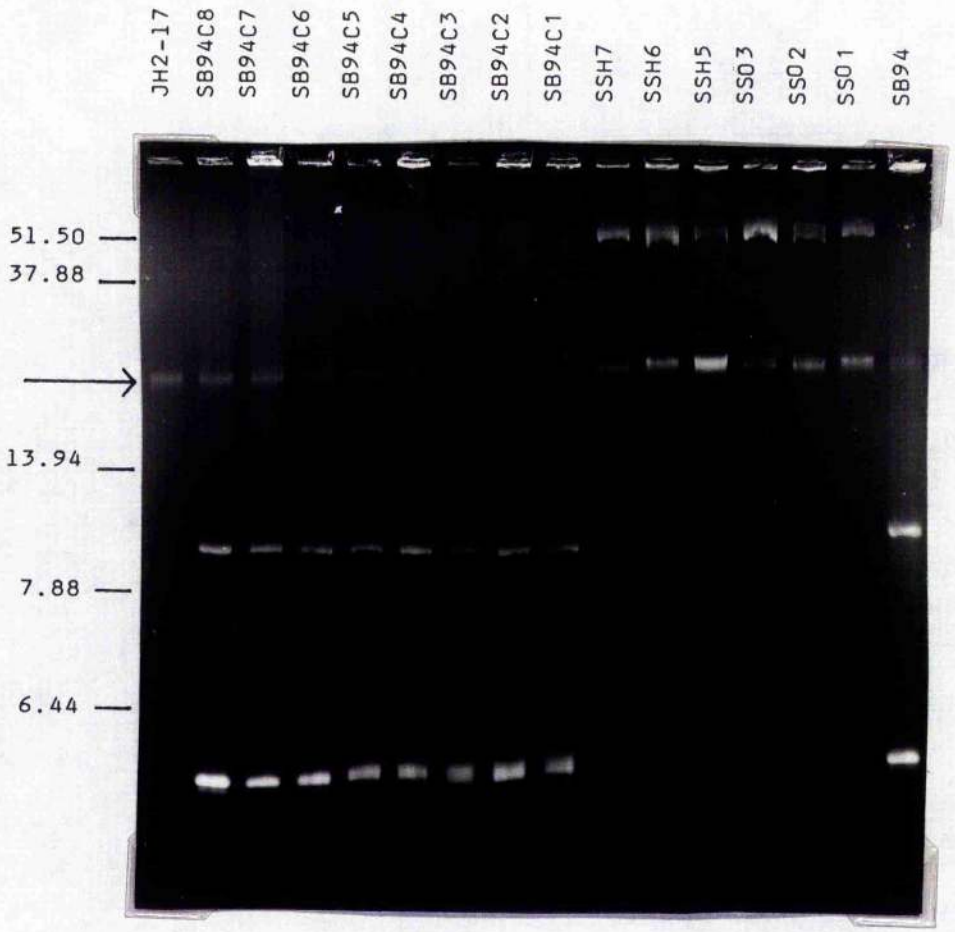
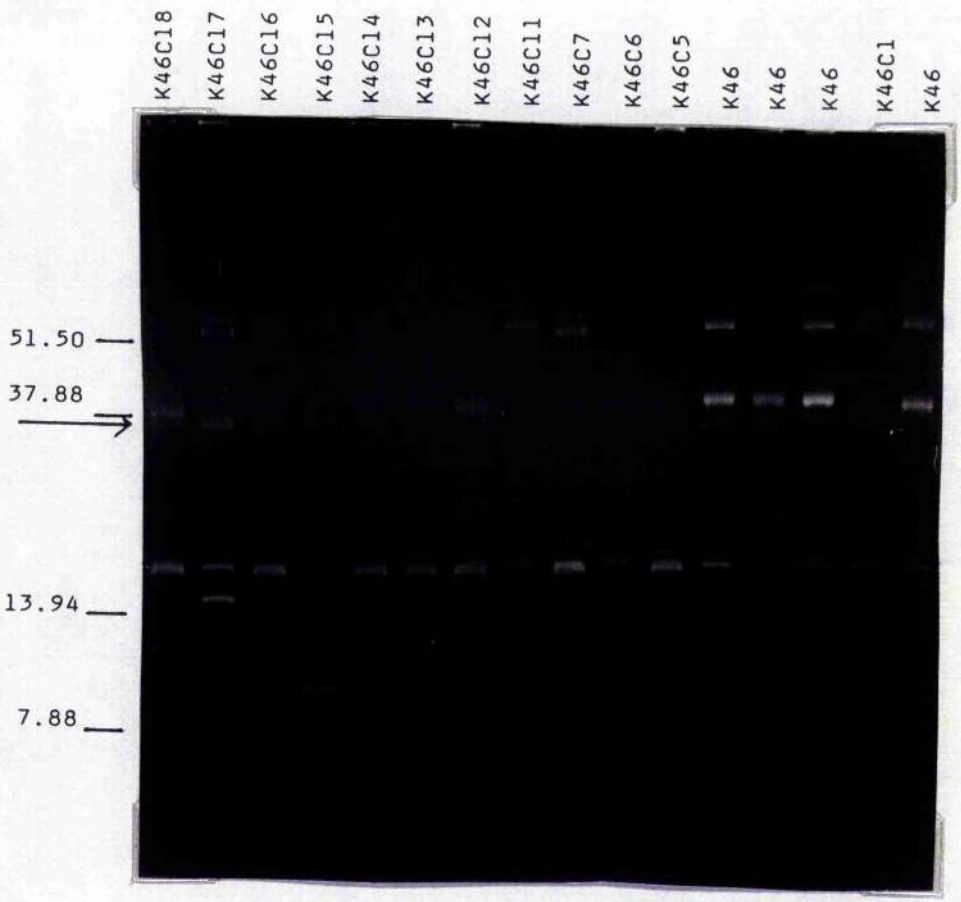

JH2-17
SB94C8
SB94C7
SB94C6
SB94C5
SB94C4
SB94C3
SB94C2
SB94C1
SSH7
SSH6
SSH5
SSO3
SSO2
SSO1
SB94
51.50
37.88
13.94
7.88
6.44

Figure 12. Plasmid preparations of strain K46 and cured derivatives after electrophoresis in 0.35% agarose.

Values to the left of the figure are the sizes (kb) of standard plasmid molecules. These are, from the top, RP4, Sa, pBR325/Hind and pBR325/Bam. Arrow indicates position of chromosomal DNA. Sample volumes were 50µl.

Strain	Phenotype	Band sizes (kb)
K46	Em Lm Tc Sm Km Nm Ap Pn	80.0,61.2,38.5,34.7,32.9,17.2
K46C1, K46C5, K46C7, K46C16, K46C18	Em Lm Tc Ap Pn	64.2,58.0,38.5,24.7,17.2
K46C6	Em Lm Tc Ap Pn	61.2,38.5,34.7,25.8,17.2
K46C17	Em Lm Tc Ap Pn	64.2,58.0,38.5,34.7,17.2,14.3
C46C11	Em Lm Ap Pn	61.2,34.7,17.2
K46C12,K46C13,K46C14	Em Lm Sm Km Nm Ap Pn	64.2,58.0,38.5,34.7,17.2
K46C15	Tc Ap Pn	64.2,59.4,38.5,34.7,23.2,17.2, 9.0


K46C18
K46C17
K46C16
K46C15
K46C14
K46C13
K46C12
K46C11
K46C7
K46C6
K46C5
K46
K46
K46
K46C1
K46
51.50
37.88
13.94
7.88

patterns in that the 38.5 and 34.7kb bands were visible but never the 32.9kb band. Also the relative intensity of these two bands was variable between derivatives which was not the case in the K46 samples. All the K46 samples contained bands at 80.0 and 61.2kb but none of the derivatives contained the 80.0kb band. As to the 61.2kb band, only K46C6 and K46C11 contained one band of the same size, all the other derivatives having two bands of 64.2 and 58.0kb. The band of 17.2kb which migrated fastest in K46 was also observed in all the derivatives although in some, noteably K46C5, K46C7, K46C12, K46C13, K46C14, K46C16 and K46C18 and to a lesser extent K46C1, this band was more prominent and possibly composed of two co-migrating bands, This did not however relate to any particular resistance phenotype. The band patterns of all the derivatives were therefore very similar with only four exceptions. K46C6 unlike the other strains of the same phenotype, had an additional band of 25.8kb while another of the same phenotype, K46C17, had a novel band of 14.3kb. The derivative cured of tetracycline and aminoglycosides resistance, K46C11, had only bands at 61.2, 34.7 and 17.2kb, missing the 38.5kb band. K46C15 which was susceptible to erythromycin, lincomycin and aminoglycosides, had a band of 64.2kb but, instead of an accompanying band of 58.0kb, had a band of 59.4kb. In addition to bands of 38.5, 34.7 and 17.2kb this strain also had two bands of 23.2 and 9.0kb. It seems probable that these extra bands, particularly the smaller ones, were fragments of the larger plasmids.

RESTRICTION ENDONUCLEASE DIGESTION OF PLASMID PREPARATIONS

From the preceding plasmid preparations, it was not possible to relate the resistance phenotypes of the parental strains to the plasmids which they contained in particular because, in many instances, there was no apparent difference between the bands observed in parental and derivative strains or between transconjugants of different phenotypes. It was thought therefore that a study of the fragments obtained by restriction endonuclease digestion of preparations from parental strains and their derivatives could serve to clarify the situation, perhaps by indicating insertions into or deletions from plasmids found in derivatives or by demonstrating the presence of different plasmids of the same apparent size. After digestion and electrophoresis in 0.8% agarose gels, the sizes of fragments in the samples were estimated using a standard HindIII digest of λ to construct standard curves.

Restriction digests of K55 and derivatives using the enzymes EcoR1 and Pst1 were carried out and are shown in Figure 13 with undigested samples containing the same amount of DNA included for comparison. Figure 13(a), (c), (d) and (e) shows K55 samples which were prepared and digested individually and the fragment sizes given in Table 20 are the mean of those calculated from (a), (c), (d) and (e) for EcoR1 digests but only (c), (d) and (e) for Pst1 digests as the K55 Pst1 digestion in (a) was incomplete, probably due to the presence of inhibitor such as a trace of ethanol. Under the conditions used, Pst1 would therefore appear to be more sensitive to inhibition and so digestion in other samples may likewise have been fallible. There was no reason to suppose however that the activity of EcoR1 was affected.

Both EcoR1 and Pst1 digests of K55 produced nine visible fragments designated A to I (Table 20), the former enzyme giving in total estimated

Figure 13. Restriction endonuclease digestion patterns of plasmid preparations from K55 and derivatives after electrophoresis in 0.8% agarose.

Lane at the far right of figures contains HindIII digest of λ. Sample volumes were 50µl. Letters to the left correspond to K55 PstI digestion fragments and letters to the right, to K55 EcoRI digestion fragments.

(a) Lanes 1, 4, 7 and 10 contain undigested preparations from strains K60 (Em Tc Sm), SSK1 (Sm), SSK2 (Sm) and SSK3 (Sm) respectively. Lanes 2, 5, 8 and 11 contain the respective preparations digested with EcoRI and lanes 3, 6, 9 and 12 contain the preparations digested with PstI.

(b) Lanes 2, 4, 7 and 10 contain undigested preparations from strains K55C3 (Em Tc), K55C5 (Tc), K55C6 (Tc) and K55C7 (Tc) respectively. Lanes 2, 5, 8 and 11 contain the respective preparations digested with EcoRI and lanes 3, 6, 9 and 12 contain the preparations digested with PstI.

(c) Lanes 1, 4, 7 and 10 contain undigested preparations from strains K55 (Em Tc Sm), SSK4 (Tc), SSK5 (Tc) and SSK6 (Tc) respectively, Lanes 2, 5, 8 and 11 contain the respective preparations digested with EcoRI and lanes 3, 6, 9 and 12 contain the preparations digested with PstI.

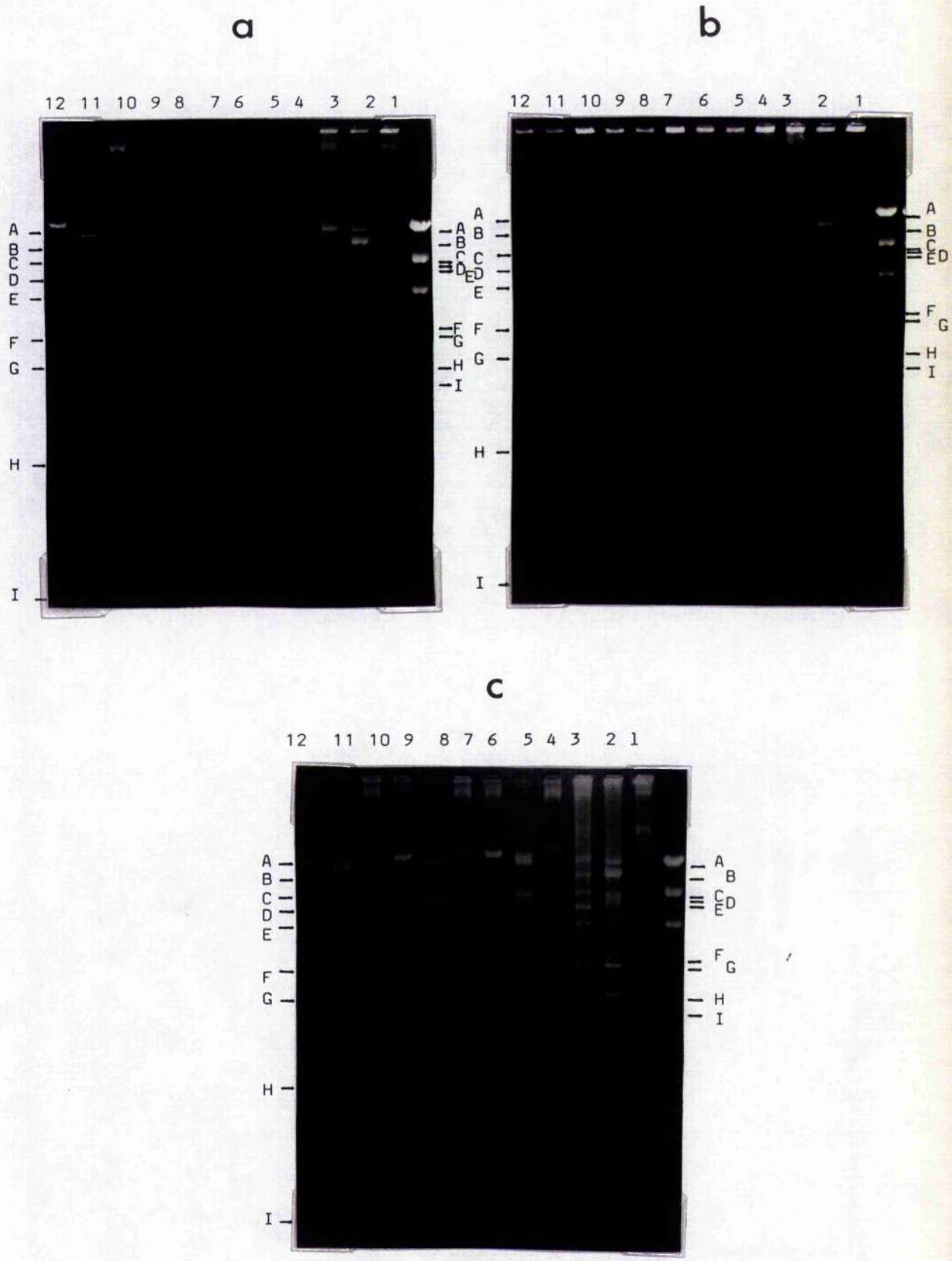
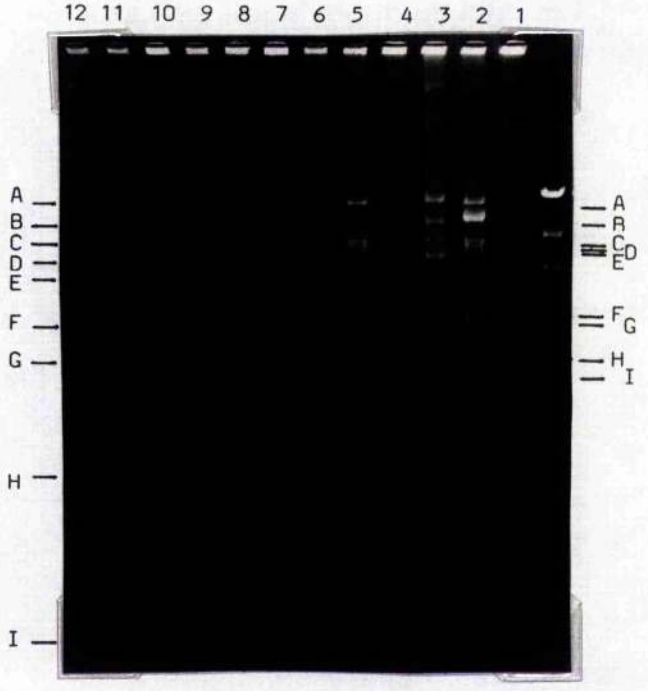

a
b
c
12 11 10 9 8 7 6 5 4 3 2 1
A
B
C
D
E
F
G
H
I

Figure 13 (continued)

(d) Lanes 1, 4, 7 and 10 contain undigested preparations from strains K55 (Em Tc Sm), K55C1 (Tc), K55C2 (Tc) and K55C4 (Tc) respectively. Lanes 2, 5, 8 and 11 contain the respective preparations digested with EcoR1 and lanes 3, 6, 9 and 12 contain the preparations digested with Pst1.

(e) Lanes 1, 3, 6 and 9 contain undigested preparations from strains K55 (Em Tc Sm), SSD6 (Em), SSD10 (Em) and K55 respectively. Lanes 2, 4, 7 and 10 contain the respective preparations digested with EcoR1 and lanes 5, 8 and 11 contain SSD6, SSD10 and K55 respectively digested with Pst1.

d


12 11 10 9 8 7 6 5 4 3 2 1
A
B
C
D
E
F
G
H
I
A
B
C
D
E
F G
H I

e

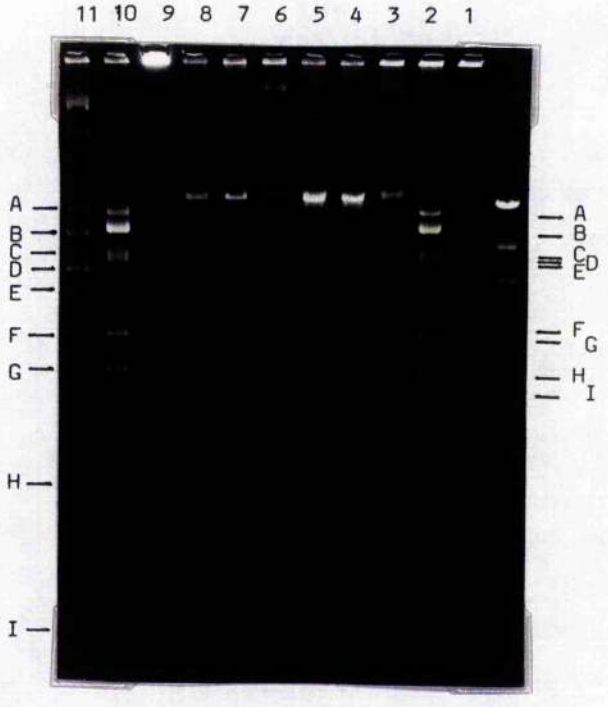

11 10 9 8 7 6 5 4 3 2 1
A
B
C
D
E
F
G
H
I
A
B
C
D
E
F G
H I

Table 20 Restriction endonuclease fragments of strain K55.

Fragment	Fragment size (kb) after digestion with	
	EcoRl	Pstl
A	23.21 ± 0.91	25.60 ± 2.72
B	14.29 ± 0.95	14.05 ± 1.18
C	10.00 ± 0.20	9.93 ± 0.30
D	9.45 ± 0.26	8.00 ± 0.20
E	8.62 ± 0.28	6.60 ± 0.06
F	4.84 ± 0.06	4.50 ± 0.00
G	4.58 ± 0.06	3.80 ± 0.06
H	3.73 ± 0.06	2.38 ± 0.12
I	3.42 ± 0.15	1.50 ± 0.10
TOTAL	82.14 ± 2.93	76.36 ± 4.74

size of 82.14kb while the latter gave a size of 76.36kb. Although somewhat lower than the EcoR1 estimate, it was possible that one of the Pst1 bands could have represented two fragments of the same size, a good candidate being the 8.0kb fragment D which consistently appeared more fluorescent after staining. Nevertheless, both estimates fell short of the expected total had all four undigested bands in Table 19 actually been different plasmid species and supported the view that one or more of these bands represented open circular on linear DNA.

Digestion of DNA from streptomycin resistant transconjugants SSK1 and SSK2 with EcoR1, shown in Figure 13(a), resulted in elimination of the band suggesting that this was not plasmid but chromosomal DNA although Pst1 digestion had no effect, probably because of inhibition of activity. The remaining streptomycin resistant transconjugant, SSK3, which in Figure 7(b) had two bands, yielded EcoR1 fragments A, C, D, E, F and I with a total size of 59.54 $\pm$ 1.86kb. The same EcoR1 pattern was seen in all those cured derivatives susceptible to streptomycin and erythromycin, resistant to tetracycline, Figure 13(b) and (d), and in tetracycline resistant transconjugants SSK4, SSK5 and SSK6, Figure 13(c), although SSK4 and SSK6 had additional fragments at 33.60 and 4.20kb. The Pst1 digest of SSK3 contained one band of large size near the origin which was probably undigested DNA and another band of 38.0kb at approximately the same position as the putative chromosomal DNA. However, the staining of this band was much more intense than that of the undigested sample indicating that at least partial digestion had taken place. A similar effect was seen with Pst1 digestion of all the other transconjugant strains, tetracycline as well as erythromycin resistant, and EcoR1 digestion of erythromycin resistant transconjugants, but was not observed when cured derivatives were digested with either enzyme. As all the samples were prepared at the same time using the same materials, method etc., the reason for this discrepancy between parental strain and cured

derivatives on the one hand and transconjugants on the other was not clear.

Pstl digestion of derivatives cured of streptomycin and erythromycin resistance, K55C1, K55C2, K55C4, K55C5, K55C6 and K55C7, yielded fragments B to I of total size 50.76 ± 2.02kb. Without taking into account the possibility of two fragments at D, this value was comparable with that estimate (59.5 ± 1.86kb) for the EcoR1 fragments observed in these strains and this total was in turn similar to the estimated size of band 1 (51.5 + 5.9kb) in undigested K55 preparations. Only one derivative, K55C3 was obtained which was susceptible to streptomycin but remained resistant to erythromycin and tetracycline and, on digestion, samples of this strain had exactly the same restriction profiles for both enzymes as the parental strain. It therefore appeared that EcoR1 fragments B, G and H total size 22.6 ± 2.72 were equivalent to Pstl fragment A, size 25.60 ± 2.72kb, and probably associated with erythromycin resistance. A band of size 23.75 ± 1.60kb (band 3) was observed in undigested K55 preparations although in previous preparations of K55C3 itself, Figure 7(b), bands 1, 3 and 4 were observed.

Samples of strain K60 and derivatives before and after endonuclease digestion are shown in Figure 14. EcoR1 and Pstl digestion of K60 produced sixteen and eight fragments respectively with total fragment sizes of 127.45kb and 99.45kb. As above with the K55 samples, there was some doubt as to the efficiency of digestion by Pstl especially in the transconjugant samples. The erythromycin resistant transconjugants of matings with JH2-17 shown in Figure 14(a), SSL1, SSL2 and SSL3 had similar EcoR1 fragment patterns to K60 but fragments A, C, G, D and L were missing. The total size of the remaining fragments was 68.10kb which corresponds well with the band size 65.0kb observed previously in these strains (Figure 8b). The Pstl digests were probably incomplete under the conditions used but nevertheless, fragments A, D, E, F, G and H were clearly visible in SSL1 and SSL2 with a total size of 60.50kb. In contrast, the tetracycline resistant transconjugant SSL4

Figure 14. Restriction endonuclease digestion patterns of plasmid preparations from K60 and derivatives after electrophoresis in 0.8% agarose.

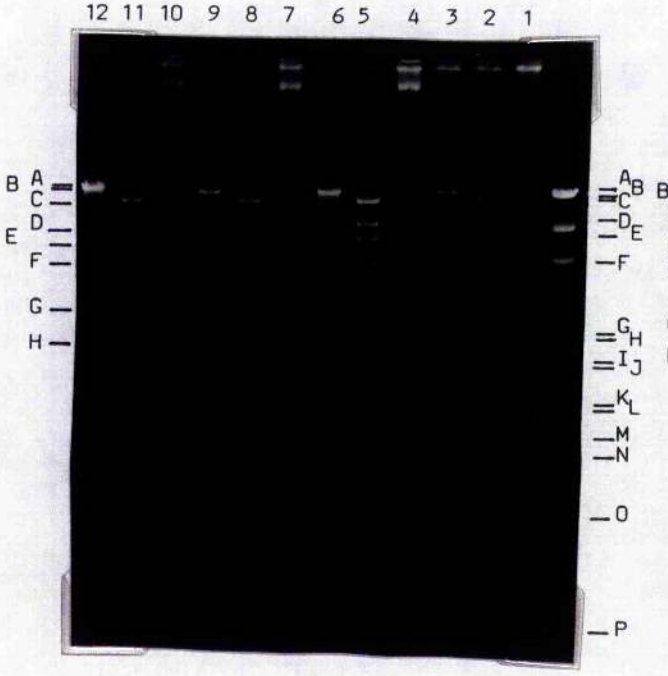
Lane at the far right of figures contains HindIII digest of λ. Sample volumes were 50µl. Letters to the left correspond to K60 PstI digestion fragments and letters to the right, to K60 EcoRI digestion fragments.

(a) Lanes 1, 4, 7 and 10 contain undigested preparations from strains K60 (Em Tc Sm Km Nm), SSL1 (Em), SSL2 (Em) and SSL3 (Em) respectively. Lanes 2, 5, 8 and 11 contain the respective preparations digested with EcoRI and lanes 3, 6, 9 and 12 contain the preparations digested with PstI.

(b) Lanes 1, 4, 7 and 10 contain undigested preparations from strains SSL4 (Tc), K60C1 (Em Tc), K60C2 (Em Tc) and K60C3 (Em Tc) respectively. Lanes 2, 5, 8 and 11 contain the respective preparations digested with EcoRI and lanes 3, 6, 9 and 12 contain the preparations digested with PstI.

(c) Lanes 1, 4, 7 and 10 contain undigested preparations from strains K60C4 (Tc), K60C5 (Em Tc), K60C6 (Em Tc) and K60C7 (Em Tc) respectively. Lanes 2, 5, 8 and 11 contain the respective preparations digested with EcoRI and lanes 3, 6, 9 and 12 contain the preparations digested with PstI.

a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B A
C
D
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F
G
H
A B
C
D E
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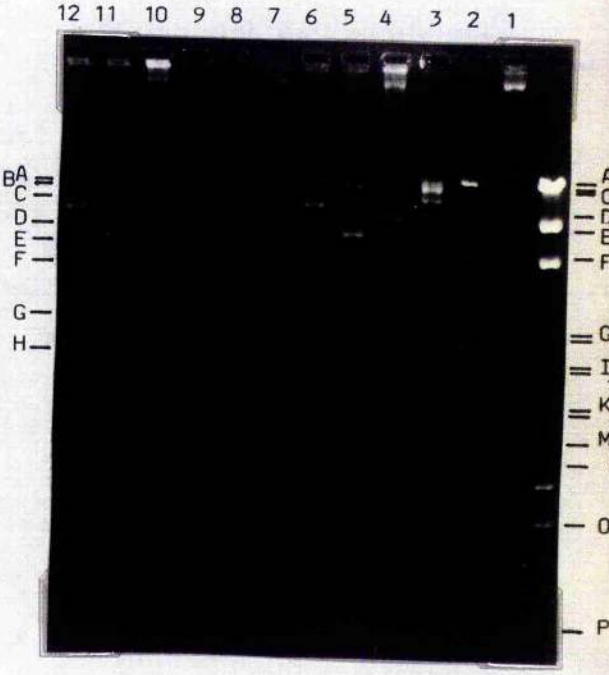
I J
K L
M
N
O
P

b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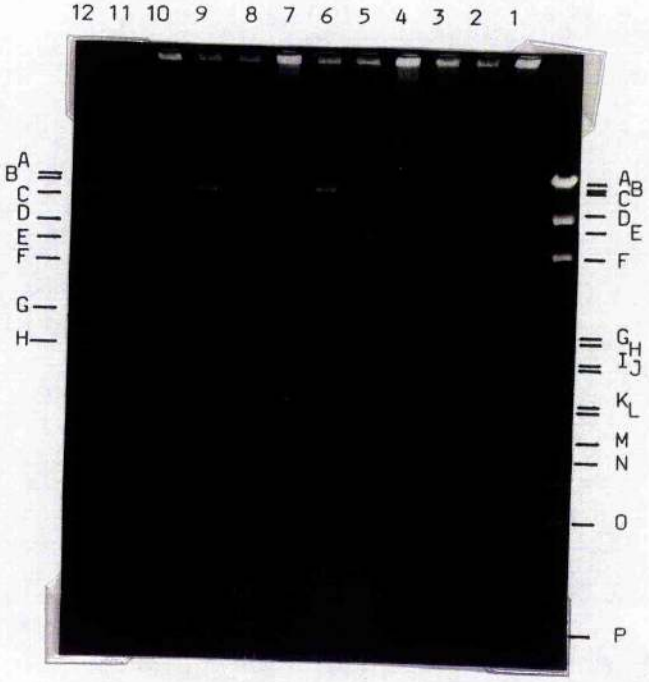
B A
C
D
E
F
G
H
P

c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B A
C
D
E
F
G
H
A B
C
D E
F
G H
I J
K L
M
N
O
P

Table 21. Restriction endonuclease fragments of strain K60.

Fragment	Fragment size (kb) after digestion with	
	EcoR1	Pst1
A	29.25	29.25
B	21.75	23.50
C	19.20	15.45
D	11.50	9.80
E	9.10	7.90
F	6.85	6.35
G	4.15	4.40
H	3.85	2.80
I	3.60	
J	3.50	
K	3.00	
L	2.90	
M	2.65	
N	2.45	
O	2.00	
P	1.70	
TOTAL	127.45	99.45

yielded manifestly different restriction profiles. EcoR1 fragments A, F, G, H and L, total size 47.00kb, were present but there were in addition seven fragments of sizes 5.45, 4.70, 4.30, 3.95, 1.90, 1.75 and 1.60, total 23.65kb, giving an overall size of 70.65 which was comparable to that obtained for SSL1, SSL2 and SSL3. Pst1 digestion of SSL4 appeared to give fragments A, B, C, D and E and novel fragments of 11.20, 6.20 and 4.95kb, total 22.35kb, but the combined size at 108.25kb was greater than that calculated for EcoR1 fragments. This could possibly reflect incomplete digestion of the larger fragments.

Derivatives of K60 which had lost streptomycin resistance on storage (Figure 14b), K60C1, K60C2 and K60C3, had EcoR1 restriction profiles resembling those of the erythromycin resistant transconjugants but additional novel fragments of 8.50 and 1.75kb as well as fragment L were present. The sum of the novel fragments was 10.25kb giving a total size of 81.25kb which compared favourably with the sum (77.9kb) of the sizes of suspected plasmid bands in these strains (Figure 8b). The Pst1 digests contained fragments A, D, E, F, G and H with a novel fragment of 14.3kb giving a total size of 74.80kb. Derivatives with the same phenotype in Figure 14(c) but which had been obtained by novobiocin treatment, K60C5, K60C6 and K60C7 had slightly different restriction profiles. As with the derivatives obtained on storage, the EcoR1 digests contained the same fragments as those observed in transconjugants SSL1, SSL2 and SSL3 but had in addition, fragment L and novel fragments of 8.30, 3.95, 1.75 and 1.65kb. The sum of the novel fragments was 15.65kb, the combined size of the EcoR1 fragments being 86.50kb. The Pst1 digests were also similar but the novel fragment was larger at 17.50kb giving a total fragment size of 78.00kb. Although the sums of the fragments obtained from derivatives of both sources were similar the sizes of the respective novel fragments were obviously different from those obtained on storage. The combined size of the bands in undigested

K60C5, K60C6 and K60C7, excluding the probable chromosomal DNA, was 145.10kb, Figure 8b, indicating that not all the bands were cccDNA. In contrast to the tetracycline resistant SSL4, K60C4 which was resistant to tetracycline alone did not appear to contain any plasmid as neither undigested nor digested samples had visible bands or fragments.

Plasmid preparations of strain K87 with transconjugants and cured derivatives before and after digestion with EcoR1 and Pst1 are shown in Figure 15. The samples of K87 shown in Figure 15(a), (c), (d) and (e) were processed individually, the fragment sizes estimated and the mean and standard deviations calculated (Table 22), with the exception of EcoR1 fragment M which was very faint and visible only in (a). EcoR1 digestion of K87 samples yielded thirteen fragments designated A to M with a total size of 89.42kb. Comparison with undigested sample, showed that band 5 was not digested - the faint band seen under this and band 6 was assumed to be an artefact due to the high plasmid concentration at this agarose concentration. Addition of band 5 to the total (94.52kb) resulted in a value 20.58kb less than the total sizes of bands 1 to 6 (Table 19) suggesting either that one or more of the fragments was not clearly separated or that not all the bands listed in Table 19 were cccDNA. In this respect, bands 3 and 4 could have been open circular or linear forms of bands 5 and 6 or band 2 could have been chromosomal DNA. Nevertheless, the total of EcoR1 fragment sizes was such as to suggest that, although band 1 was not visible in undigested K87 samples of this preparation, there was sufficient present to produce visible fragments after digestion, as more ethidium bromide could intercalate into the linearised DNA. Digestion of K87 with Pst1 produced only three fragments of sizes 6.10kb, 4.92kb and 4.66kb. Bands 2 and 6, possibly band 4 and, presumably, band 1 were not digested by this enzyme. The fact that band 2 was not digested was contrary to the suggestion that it may have been chromosomal DNA. It was likely that fragment B

Figure 15. Restriction endonuclease digestion patterns of plasmid preparations from K87 and derivatives after electrophoresis in 0.8% agarose.

Lane at the far right of figures contains HindIII digest of λ. Sample volumes were 50µl. Letters to the left correspond to K87 PstI digestion fragments and letters to the right, to K87 EcoRI digestion fragments.

(a) Lanes 1, 4, 7 and 10 contain undigested preparations from strains K87 (Em Tc Sm Km Nm Hly), SSM1 (Em), SSG12 (Em) and SSG13 (Em), respectively. Lanes 2, 5, 8 and 11 contain the respective preparations digested with EcoRI and lanes 3, 6, 9 and 12 contain the preparations digested with PstI.

(b) Lanes 1, 4, 7 and 10 contain undigested preparations from strains SSM6 (Hly), SSM7 (Hly), SSM8 (Hly) and SSM9 (Hly) respectively. Lanes 2, 5, 8 and 11 contain the respective preparations digested with EcoRI and lanes 3, 6, 9 and 12 contain the preparations digested with PstI.

(c) Lanes 2, 4, 7 and 10 contain undigested preparations from strains K87 (Em Tc Sm Km Nm Hly), SSM2 (Tc), SSM3 (Tc) and SSM4 (Tc) respectively. Lanes 2, 5, 8 and 11 contain the respective preparations digested with EcoRI and lanes 3, 6, 9 and 12 contain the preparations digested with PstI.

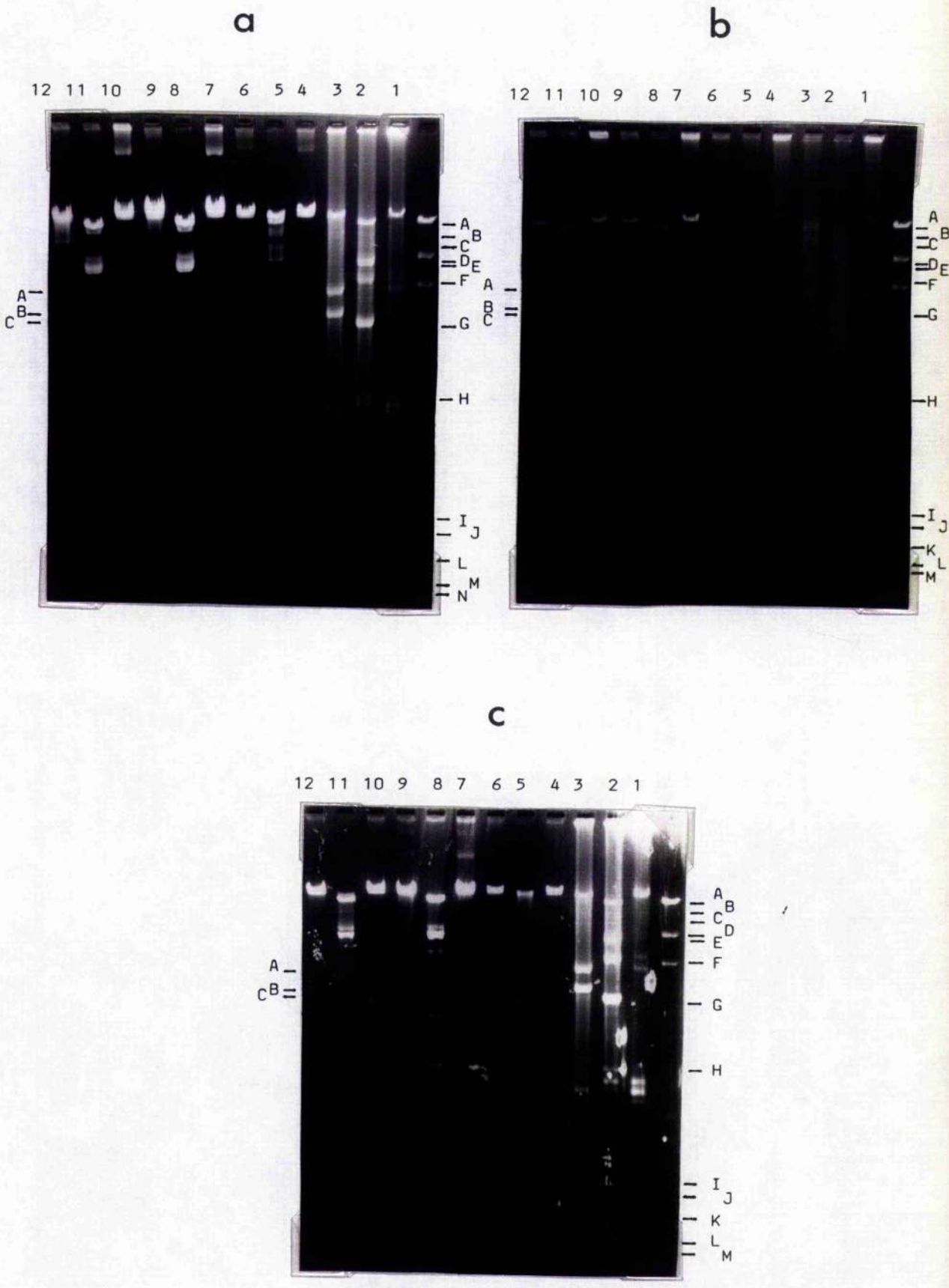
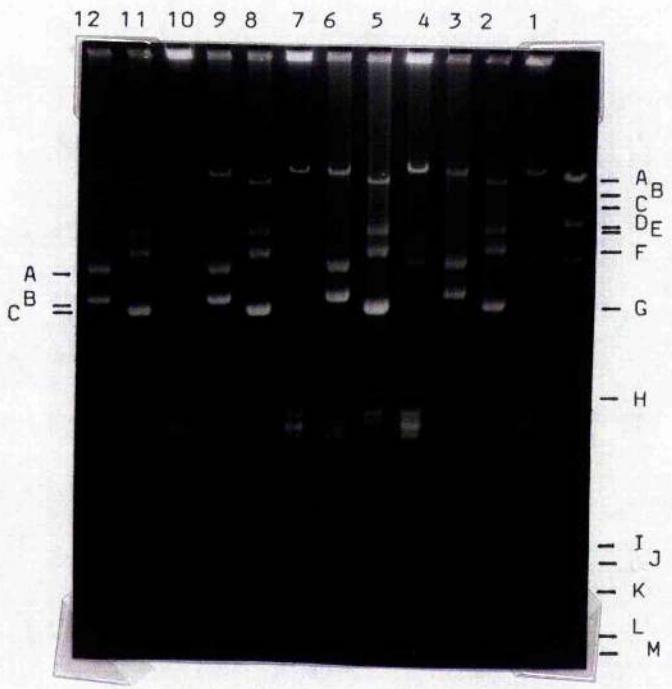

a
12 11 10 9 8 7 6 5 4 3 2 1
A
B
C
A
B
C
D
E
F
G
H
I
J
L
M
N
b
12 11 10 9 8 7 6 5 4 3 2 1
A
B
C
A
B
C
D
E
F
G
H
I
J
K
L
M
c
12 11 10 9 8 7 6 5 4 3 2 1
A
B
C
A
B
C
D
E
F
G
H
I
J
K
L
M

Figure 15 (continued)

(d) Lanes 1, 4, 7 and 10 contain undigested preparations from strains K87 (Em Tc Sm Km Nm Hly), K87C1 (Em Tc Sm Km Nm), K87C2 (Em Tc Sm Km Nm) and K87C3 (Em Tc Hly) respectively. Lanes 2, 5, 8 and 11 contain the respective preparations digested with EcoR1 and lanes 3, 6, 9 and 12 contain the preparations digested with PstI.

(e) Lanes 1, 4, 7 and 10 contain undigested preparations from strains K87 (Em Tc Sm Km Nm Hly), K87C5 (Em Tc Hly), K87C6 (Em Tc Sm Km Nm) and K87C8 (Em Tc Sm Km Nm) respectively. Lanes 2, 5, 8 and 11 contain the respective preparations digested with EcoR1 and lanes 3, 6, 9 and 12 contain the preparations digested with PstI.

d


12 11 10 9 8 7 6 5 4 3 2 1
A
B
C
D
E
F
G
H
I
J
K
L
M

e

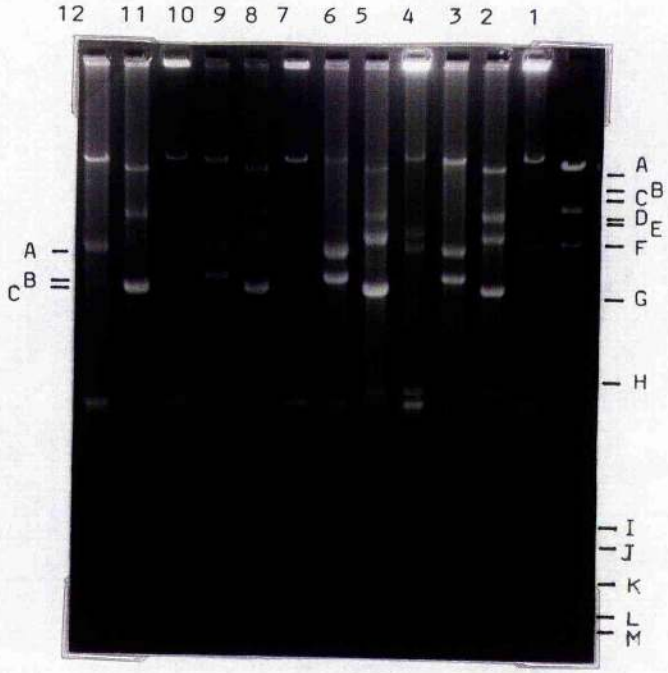

12 11 10 9 8 7 6 5 4 3 2 1
A
B
C
D
E
F
G
H
I
J
K
L
M

Table 22. Restriction endonuclease fragments of strain K87.

Fragment	Fragment size (kb) after digestion with	
	EcoR1	Pst1
A	21.60 ± 0.33	6.10 ± 0.20
B	15.84 ± 0.43	4.92 ± 0.10
C	12.50 ± 0.25	4.66 ± 0.08
D	9.56 ± 0.32	
E	8.95 ± 0.25	
F	6.86 ± 0.30	
G	4.40 ± 0.26	
H	2.80 ± 0.03	
I	1.70 ± 0.03	
J	1.55 ± 0.13	
K	1.28 ± 0.10	
L	1.23 ± 0.15	
M	1.15	
TOTAL	89.42	15.68

represented band 5 with one PstI site but the origin of fragments A and C was not clear.

Digestion with EcoRI of the sample prepared from strain SSM1, an erythromycin resistant transconjugant from a mating with JH2-17, produced poorly resolved fragments indicative of incomplete digestion while with PstI, no digestion was observed. This was probably due to inhibitory substances present in the sample. Strains SSG12 and SSG13, erythromycin resistant transconjugants from matings with JH2-2, each showed the same EcoRI digestion fragments. Only fragments A, E, J, K, L and M visible in K87 samples were seen in SSG12 and SSG13. Between fragments A and E were two novel fragments of 17.90kb and 10.50kb and between E and J were fragments of 3.30, 2.90, 2.75, 2.55, 2.45 and 2.10kb giving a total of 80.21kb. PstI digestion of SSG12 and SSG13 appeared to be incomplete although five fragments of sizes 18.20, 10.90, 7.00, 5.80 and 3.50kb, none of which corresponded to fragments in K87 digests, were visible. Including the undigested band 2, the sum was 72.10kb.

Of the haemolytic transconjugants, SSM6 and SSM7 were clearly different from SSM8 and SSM9. Digestion of the former with EcoRI produced fragments B and C with novel fragments of 10.70, 9.20 and 7.50kb giving a total size of 55.74kb. PstI digestion yielded three fragments of 23.50, 17.40 and 15.10kb with a total size of 56.00kb corresponding to that of EcoRI fragments. This value was less than expected if these samples contained both K87 band 1 and band 2 as observed in Figure 9(b). However, preparations from SSM6 and SSM7 had reproducibly much less DNA in the band 2 position than other K87 derivatives and so it is possible that in these strains band 2 was composed of chromosomal DNA only. In contrast, SSM8 and SSM9 digested with EcoRI had fragments A, B, C, D and E with one novel fragment of 2.65kb. In addition, barely visible were fragments J, K, L and M giving a total size of 76.31kb. No digestion of these samples by PstI was observed.

The sample from the tetracycline resistant transconjugant SSM2 was inefficiently digested with EcoR1 and not digested at all by Pst1. The EcoR1 digests of SSM3 and SSM4 however were similar having the same major fragments, but were not identical with many minor fragments, some of which were probably incomplete digestion products. In addition to EcoR1 fragments A and E, the sizes of the prominent fragments of SSM3 were 11.0, 7.70, 5.65, 4.50, 3.90, 3.15 and 2.80kb. Fragments I, J and K were also visible giving a total size of 73.78kb. The EcoR1 digest of SSM4 also had fragments A, E, I, J and K and fragments of sizes 11.0, 3.90, 3.15 and 2.80kb in common with SSM3 but fragments of 16.20, 4.25 and 3.30kb were present. The sum of these fragments was slightly higher than SSM3 at 79.68kb. In neither case did the sum of the fragment sizes add up to what would have been expected if the bands seen in undigested samples (Figure 9b) were different plasmid species i.e. 119.00kb for SSM3 and 39.80kb for SSM4. Neither SSM3 nor SSM4 was digested with Pst1.

Non-haemolytic derivatives K87C1 and K87C2, and streptomycin susceptible derivatives K87C4 and K87C5 all had identical Pst1 digestion profiles to the parental K87 and the EcoR1 profiles differed only in that fragments B and C were not visible in the derivatives. The EcoR1 profile of the non-haemolytic derivative K87C6 was the same as K87C1 and K87C2 but the Pst1 digestion appeared different due to the incomplete digestion of bands 3 and 5. K87C8 which was also non-haemolytic and in undigested samples lacked bands 3 and 5, had a similar EcoR1 profile but in addition to B, C and undigested band 5, was missing only fragment F. The size of this fragment, 6.86kb, was larger than the estimated size of band 5 and smaller than theestimated size of band 3 but the fact that only one fragment appeared to be missing lent support to the suggestion that band 3 may represent open circular band 5. In the Pst1 digest of K87C8, only fragment B was missing. Since bands 2 and 6 were not digested by Pst1

and K87C8 did not contain band 1, Pst1 fragments A and C may have originated from band 4. Bands 4 and 6 could not in that case represent different forms of the same plasmid.

Restriction digests of K88, transconjugants of matings with JH2-1 and cured derivatives are shown in Figure 16. Each of the gels contains K88 samples which were prepared and digested individually and the mean estimated sizes of the fragments are shown in Table 23.

EcoR1 digestion of K88 yielded fifteen fragments designated A to O and undigested band 6 with a combined size of 109.16kb which is considerably lower than the combined size of 163.3kb of bands 1 to 6 in Table 19. Fragment O and fragments M, N and O are outwith the area of Figure 16(a) and (c) respectively and not visible in Figure 16(b). There was no visual evidence that any of the larger bands were not digested and so it would appear that not all K88 bands 1 to 6 in Table 19 represent ccc plasmid DNA. The total of the estimated fragment sizes of K88 digested with Pst1 was 83.51kb but certain of the large bands were not digested. The undigested bands, band 1 in Figure 16 (a), bands 1 and 2 in Figure 16 (b) and (c), varied between preparations, due perhaps to incomplete digestion of band 2. If this were so, the combined size of fragments and band 1 was 141.93kb.

On digestion with EcoR1, the tetracycline resistant transconjugants SSN1, SSN2 and SSN3 exhibited only fragment A, the size of which corresponded to that of band 4 in Table 19, and no undigested bands (Figure 16a). This was in spite of the fact that they had two or more bands in the undigested samples and would imply that these bands were linear, open circular or multimeric forms of the same plasmid species. Pst1 digestion of these samples resulted in alteration of the mobilities of the undigested bands, suggesting incomplete digestion, and the production of a fragment of similar size as EcoR1 fragment A. Digestion of the streptomycin resistant

Figure 16 Restriction endonuclease digestion patterns of plasmid preparations from K88 and derivatives after electrophoresis in 0.8% agarose.

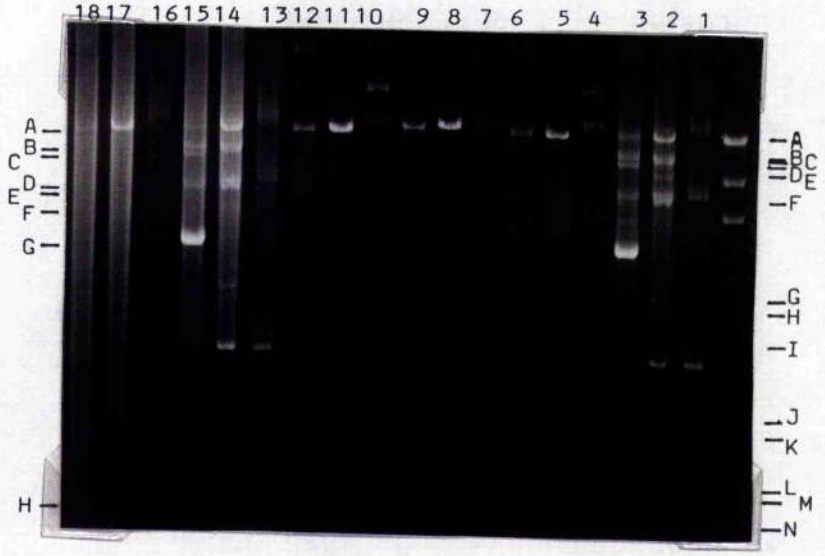
Lane at the far right of figures contains HindIII digest of λ. Sample volumes were 50µl. Letters to the left correspond to K88 PstI digestion fragments and letters to the right, to K88 EcoRI digestion fragments.

(a) Lanes 1, 4, 7, 10, 13 and 16 contain undigested preparations from strains K88 (Em Tc Sm Cm), SSN1 (Tc), SSN2 (Tc), SSN3 (Tc), K88C13 (Tc Sm) and K88C14 (Tc) respectively. Lanes 2, 5, 8, 11, 14 and 17 contain the respective preparations digested with EcoRI and lanes 3, 6, 9, 12, 15 and 18 contain the preparations digested with PstI.

(b) Lanes 1, 4, 7, 10, 13 and 16 contain undigested preparations from strains K88 (Em Tc Sm Cm), SSN4 (Sm), SSN5 (Sm), SSN6 (Sm), K88C2 (Em Tc Cm) and K88C3 (Em Tc Cm) respectively. Lanes 2, 5, 8, 11, 14 and 17 contain the respective preparations digested with EcoRI and lanes 3, 6, 9, 12, 15 and 18 contain the preparations digested with PstI.

(c) Lanes 1, 4, 7, 10, 13 and 16 contain undigested preparations from strains K88 (Em Tc Sm Cm), K88C6 (Em Tc Cm), K88C1 (Tc Sm), K88C10 (Tc Sm), K88C11 (Tc Sm) and K88C12 (Tc Sm) respectively. Lanes 2, 5, 8, 11, 14 and 17 contain the respective preparations digested with EcoRI and lanes 3, 6, 9, 12, 15 and 18 contain the preparations digested with PstI.

a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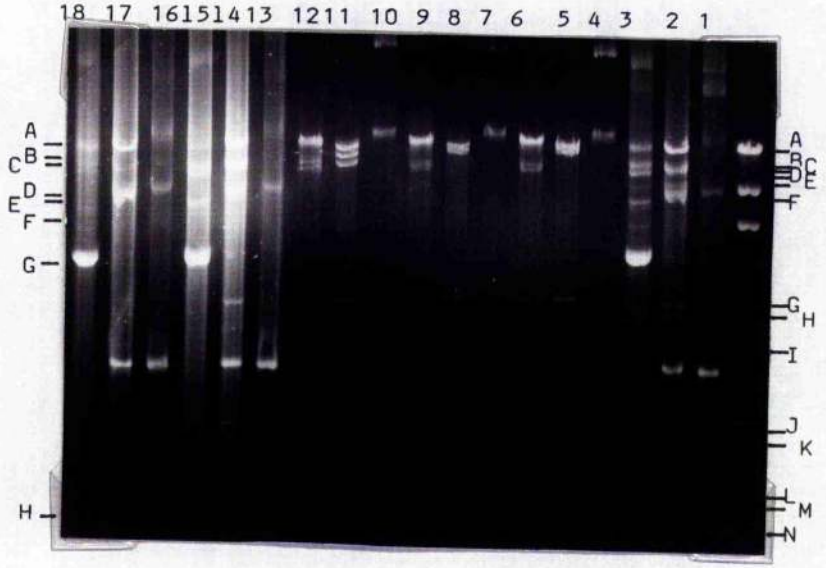
A
B
C
D
E
F
G
H
I
J
K
L
M
N

b


18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
A
B
C
D
E
F
G
H
I
J
K
L
M
N

c

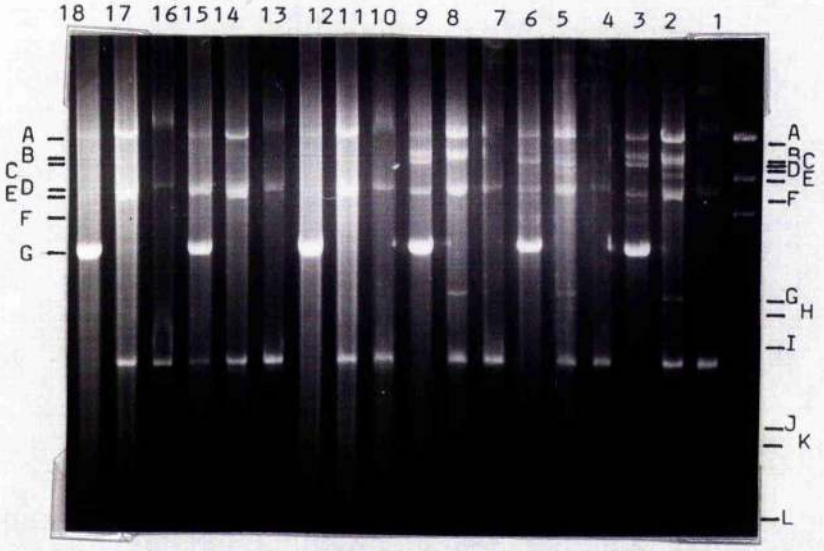

18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
A
B
C
D
E
F
G
H
I
J
K
L

Table 23. Restriction endonuclease fragments of strain K88

Fragment	Fragment size (kb) after digestion with	
	EcoR1	Pst1
A	23.50 ± 0.00	25.82 ± 0.82
B	14.77 ± 0.50	15.63 ± 0.77
C	13.83 ± 0.60	13.27 ± 0.73
D	12.63 ± 0.39	8.32 ± 0.40
E	10.88 ± 0.63	7.80 ± 0.36
F	8.20 ± 0.91	6.42 ± 0.35
G	3.67 ± 0.12	4.75 ± 0.18
H	3.42 ± 0.12	1.50
I	2.95 ± 0.05	
J	2.13 ± 0.06	
K	2.05 ± 0.08	
L	1.63 ± 0.10	
M	1.55	
N	1.45	
O	1.30	
TOTAL	103.96	83.51

transconjugants SSN4 and SSN5 with EcoR1 resulted in fragments A, G, J and L with two novel fragments of 21.7kb and 3.65kb giving a total of 56.28kb (Figure 16b). The EcoR1 digestion of SSN6 also produced fragments A, G, J and L but novel fragments of 19.80, 15.65 and 3.65kb were also present, giving a total of 70.03kb. Pst1 digestion of all three transconjugants resulted in three fragments and no undigested bands. The largest fragment (28.15kb) was slightly larger than Pst1 fragment A while the two smaller fragments of sizes 15.65kb and 14.00kb could have corresponded to fragments B and C. The total size of Pst1 fragments was 57.80kb which was comparable to the total of EcoR1 fragments from SSN4 and SSN5 but less than the EcoR1 total of SSN6.

One derivative, K88C14, was obtained which was resistant to tetracycline only. As with tetracycline resistant transconjugants, EcoR1 and Pst1 digestion (Figure 16a) produced a fragment of approximately 23.50kb although in the Pst1 digestion a large band appeared which was not present in the undigested sample. Presumably this was partially digested DNA of very large size which, in the undigested sample, would remain in the sample well. In the previous preparation (Figure 10c), this derivative contained only one band of 23.00kb.

By their restriction profiles, the tetracycline and streptomycin resistant derivatives cured of resistance to erythromycin and chloramphenicol could be divided into two groups. Derivatives K88C1 (Figure 16c) which was obtained after storage, and K88C13 (Figure 16a) obtained after novobiocin treatment, were found to have EcoR1 fragments A, B, C, F, G, J, L N with a total size of 69.18kb, and undigested band 6 (5.2kb) and Pst1 fragments A, B, C, D, G and H with a total size of 69.29kb. These strains had been shown previously to contain bands 2, 4, 5 and 6 although K88C1 also contained band 1. Derivatives K88C10, K88C11 and K88C12 (Figure 16c), obtained after novobiocin treatment showed only EcoR1 fragments A and F

giving a total of 31.70kb, and undigested band 6. On digestion with PstI, K88C10 and K88C12 had fragments A and G with a total of 30.57kb and while K88C11 had the same fragments, it also contained what appeared to be partially digested band 6 and band 5. That band 5 was present when PstI digestion of band 6 was incomplete but absent on complete digestion of band 6 as in K88C10 and K88C12 samples, indicated that band 5 could have been the open circular form of band 6. Almost certainly, PstI fragment G was linearised band 6. In the previous undigested preparation, K88C10, K88C11 and K88C12 were found to contain only bands 4, 5 and 6 which would result in a total size of 27.50kb if bands 5 and 6 were the same plasmid species.

The restriction profiles of the derivatives susceptible to streptomycin but resistant to erythromycin, tetracycline and chloramphenicol were variable (Figure 16b,c). EcoR1 digestion of K88C2, obtained after storage, yielded fragments A, B or C, F, G, J, L and undigested band 6 giving a total of approximately 59.10kb and PstI digestion yielded fragments A, B, C, D and G giving 67.79kb. K88C3 obtained after novobiocin treatment had EcoR1 fragments A, B, D, E, F, G, H, I, J, K, L, M and undigested band 6 with a combined total of 92.58kb. PstI digestion of both K88C3 and K88C6 yielded fragments A, B, C, E, F and G with a total of 73.69kb. It appeared however that these digests also contained undigested band 1. Previous preparation of K88C6 had shown the presence of bands 1, 4, 5 and 6 and assuming 5 and 6 to be the same plasmid, the combined sizes of these bands would be 85.80kb which is close to the values estimated from EcoR1 digestion fragments.

Figure 17 shows three individual SB94 samples with transconjugants and cured derivatives before and after digestion with EcoR1 and PstI. Although a large proportion of high molecular weight DNA was isolated in the SB94 sample of Figure 17(c) which caused the smearing seen in the digested

Figure 17. Restriction endonuclease digestion patterns of plasmid preparations from SB94 and derivatives after electrophoresis in 0.8% agarose.

Lane at the far right of figures contains HindIII digest of λ. Sample volumes were 50µl. Letters to the left correspond to SB94 PstI digestion fragments and letters to the right, to SB94 EcoRI digestion fragments.

(a) Lanes 1, 4, 7, 10, 13 and 16 contain undigested preparations from strains SB94 (Em Tc Sm Km Nm), SSO1 (Tc), SSO2 (Tc), SSO3 (Tc), SB94C1 (Tc) and SB94C2 (Tc) respectively. Lanes 2, 5, 8, 11, 14 and 17 contain the respective preparations digested with EcoRI and lanes 3, 6, 9, 12, 15 and 18 contain the preparations digested with PstI.

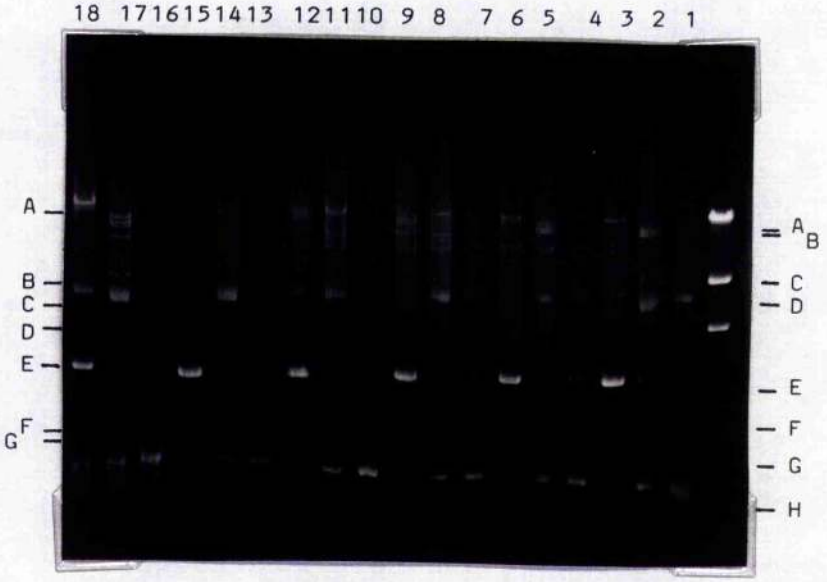
(b) Lanes 2, 4, 7, 10, 13 and 16 contain undigested preparations from strains SB94 (Em Tc Sm Km Nm), SB94C3 (Tc), SB94C4 (Em Tc), SB94C5 (Em Tc), SB94C6 (Em Tc) and SB94C7 (antibiotic susceptible), respectively. Lanes 2, 5, 8, 11, 14 and 17 contain the respective preparations digested with EcoRI and lanes 3, 6, 9, 12, 15 and 18 contain the preparations digested with PstI.

(c) Lanes 1, 4, 7, 10 and 13 contain undigested preparations from strains SB94 (Em Tc Sm Km Nm), SSH5 (Em), SSH6 (Em), SSH7 (Em) and SB94C11 (Tc) respectively. Lanes 2, 5, 8, 11 and 14 contain the respective preparations digested with EcoRI and lanes 3, 6, 9, 12 and 15 contain the preparations digested with PstI.

a


18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
A
B
C
D
E
F
G
A B
C
D
E
F
G
H

b


18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
A
B
C
D
E
F
G
A B
C
D
E
F
G
H

c

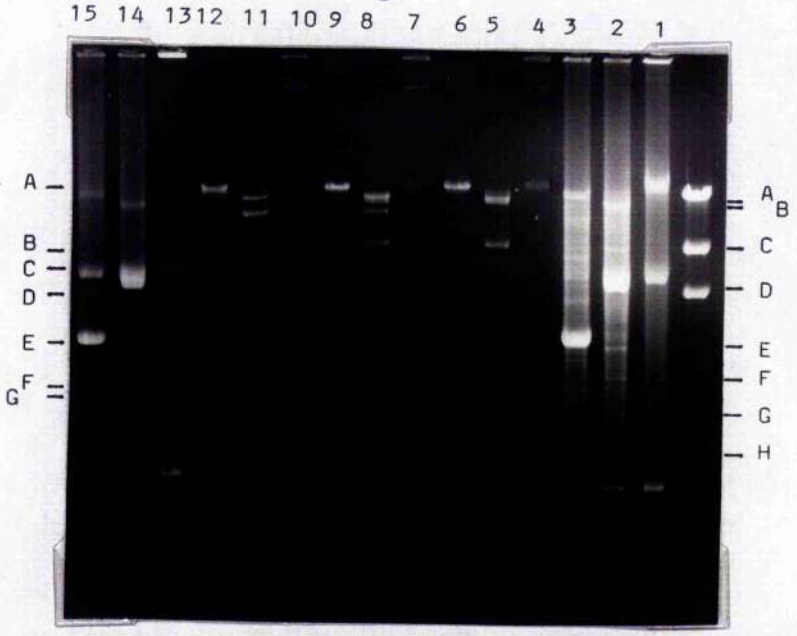

15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
A
B
C
D
E
F
G
A B
C
D
E
F
G
H

Table 24. Restriction endonuclease fragments of strain SB94

Fragment	Fragment size (kb) after digestion with	
	EcoRl	Pstl
A	19.93 ± 1.15	24.08 ± 1.01
B	18.35 ± 0.95	8.97 ± 0.55
C	9.90 ± 0.41	7.57 ± 0.33
D	7.33 ± 0.46	6.25 ± 0.20
E	4.72 ± 0.19	4.77 ± 0.15
F	4.00 ± 0.10	3.70 ± 0.23
G	3.37 ± 0.12	3.50 ± 0.20
H	2.87 ± 0.12	
TOTAL	70.47 ± 3.50	58.84 ± 2.67

aliquots, the relevant fragments could still be distinguished. Digestion of SB94 with EcoR1 produced eight fragments A to H and with Pst1, seven fragments A to G. Band 4 of the undigested sample was not digested by EcoR1 but did appear to have one Pst1 site producing fragment E. The sizes of the fragments were estimated relative to the HindIII digest of λ and the mean sizes from the three samples are shown in Table 24. The combined total of EcoR1 fragments (70.47kb) was higher than that of Pst1 fragments (58.84kb) possibly because band 3 (10.9kb in Table 19) was undigested by Pst1 and its presence concealed by fragment B. Alternatively if band 3 were the open circular form of band 4 and EcoR1 fragment D was also open circular band 4, the values would be more comparable. In support of the latter, in almost all the samples containing band 4, there appeared to be a decrease in the amount of the band in EcoR1 aliquots and a concomitant increase in the amount of fragment D although why there should have been such a conversion from cccDNA to open circular form was not clear. Also, between Figure 17(a) and (c) and Figure 17(b) there was a small but discernable decrease in migration rate of band 3 in the undigested sample and a corresponding decrease in migration rate of EcoR1 fragment D.

EcoR1 digestion profiles of tetracycline resistant transconjugants (Figure 17a) were similar although not exactly the same and quite dissimilar to the parental strain. SSO2 contained fragments A, B, E and G but the majority of fragments obtained were not seen in SB94. The sizes of these fragments were 23.50, 14.70, 10.50, 4.30, 3.80, 3.30, 2.80, 2.35, 1.90, 1.75, 1.50, 1.30 and 1.25kb and, together with fragments A, B, E and G, the total size was 121.47kb which was close to the total expected (131.0kb) if each of the bands in the previous undigested preparation (Figure 11) represented a distinct plasmid species. Strains SSO1 and SSO3 fragment patterns were almost identical to each other. In common with SSO2, they

had only fragment E and fragments of sizes 14.70, 10.50, 4.30, 3.80, 2.15, 1.75 and 1.50kb but also had fragments of sizes 26.65, 23.50, 21.25, 16.35, 9.50, 8.60, 8.00, 7.10, 6.35, 5.40, 3.00 and 2.70kb giving a total size of 181.82kb. This value was far in excess of that expected from Figure 11 where undigested samples possessed only bands 1 and 2 with a total size of 77.0kb indicating, therefore, the presence in these strains of more than one plasmid of similar size. Nevertheless, as none of the samples was efficiently digested by PstI, it was conceivable that EcoR1 digestion was likewise inhibited and that some of these fragments were the results of only partial digestion.

Digestion of the erythromycin resistant transconjugants with EcoR1 (Figure 17c) produced profiles resembling the tetracycline resistant transconjugants but again unlike the parental strain. SSH5 had fragments of sizes 23.50, 22.00 (compare SS01, SS03, 23.50 and 21.25kb) 10.60 (SS01, SS02, SS03, 10.50kb) and 4.95kb; SSH6 had fragments of 23.50, 22.0, 17.40, 10.60 and 4.05kb; SSH7 had fragments of 23.50, 17.40, 10.60 and 4.95kb. The total sizes of SSH5, SSH6 and SSH7 were 61.05, 77.55 and 56.45kb respectively. The SSH6 profile was a 'composite' of the other two strains suggesting, if it was assumed that all the transconjugants contained the same plasmid, that some of the fragments were partial digestion products. The previous preparation showed that the strains contained a 61.5kb band, bands 1 and 2 (Figure 11) giving a total size therefore of 148.5kb. Possibly if band 2 were, as suspected, chromosomal DNA and the 61.5kb band was open circular band 1, the profile of SSH7 with a size of 56.45kb may represent the complete digestion of band 1.

All the cured derivatives had been shown previously to contain all the undigested parental bands (Figure 11). EcoR1 digestion of tetracycline resistant strains susceptible to erythromycin and streptomycin, SB94C1,

SB94C2, SB94C3 and SB94C11 produced profiles similar to each other and to the parent. SB94C1 and SB94C2 had EcoR1 fragments C, D and E as well as undigested band 4 but the larger fragments were different at 23.50 and 17.70kb. Pst1 digests resulted in fragments C, D, E and F as well as fragments of 21.25, 16.35 and 14.50kb. The combined sizes of fragments were 63.15kb and 74.39kb for EcoR1 (including fragment D) and Pst1 digests respectively. EcoR1 digestion of SB94C3 produced fragments A, B, C, D, E, G, H and undigested band 4 and additional fragments of 21.00, 13.30 and 3.60kb giving a total of 104.37kb, while Pst1 digestion gave all the fragments found in the parent strain and additional fragments of 19.95, 13.80, 12.50, 10.75, 7.40 and 6.25kb giving a total of 129.49kb. Digestion of SB94C11 with EcoR1 produced fragments A, B, D, E, G with a novel fragment of 5.25kb totalling 58.95kb and undigested band 4. Digestion with Pst1 was incomplete as seen by the presence of a faint band 4 and the band which appeared at the position of fragment C was probably open circular band 4. The only cured derivative obtained which was susceptible to tetracycline, SB94C7, was also susceptible to erythromycin and streptomycin. EcoR1 digestion produced fragments C, D, E F and G novel fragments of 22.85, 19.95 and 13.2kb with a total size of 85.32kb and undigested band 4. Unfortunately, the Pst1 digestion was incomplete and produced only partially digested band 4 and a fragment of 26.50kb.

The remaining derivatives SB94C4, SB94C5 and SB94C6 were all resistant to erythromycin and tetracycline and all had the same restriction profiles. Only EcoR1 fragments C and D were in common with the parental strain , there being novel EcoR1 fragments of 24.80, 21.00, 19.10, 16.65, 14.80, 13.30 and 3.65kb totalling 130.53kb as well as undigested band 4. Similarly, only Pst1 fragments C and E were seen in the derivatives but novel fragments of 21.00, 16.70, 12.95 and 10.50kb were produced giving a total of 73.49kb.

Two separate plasmid preparations were attempted in order to obtain samples of K46 and derivatives for endonuclease digestion. Unfortunately, neither preparation yielded sufficient material to be visible either before or after digestion.

DISCUSSION

This study was undertaken to investigate antibiotic resistant and haemolytic group D streptococci isolated locally and to examine the location, chromosomal or otherwise, of the relevant determinants. The five strains of S. faecalis and two of S. faecium were isolated and identified in a previous study in which the susceptibility of the strains to erythromycin, tetracycline, streptomycin, gentamicin, penicillin G and ampicillin was also tested (Blankson, 1981; Bayne et al., 1983). The strains were chosen for further study because they displayed resistance to two or more antibiotics in the original screen.

To expand the information available on the antibiotic resistance phenotype, the MICs for six more antibiotics were estimated. As expected, the five S. faecalis strains, originally found resistant to erythromycin, are co-resistant to lincomycin and the streptogramin B-type antibiotic, pristinamycin IA, and hence display the typical MLS resistance phenotype, the result of N^6, N^6-dimethylation of adenine in 23S rRNA (Graham and Weisblum, 1979) which prevents binding of the drugs to the ribosome (Shivakumar and Dubnau, 1981). The S. faecium strain K46 however, although resistant to erythromycin and lincomycin, is susceptible to pristinamycin possibly indicative of a different basis for resistance. This would be unlikely to be mutation causing altered ribosomal proteins since the binding sites for all the MLS antibiotics are the same or at least overlapping (Gale et al., 1981) but could possibly be due to differential permeability of the cytoplasmic membrane. Alternatively, this strain may be particularly susceptible to pristinamycin IIB (synonym for streptogramin A) which was present as a trace amount in the antibiotic preparation.

MLS resistance in S. faecalis can be expressed constitutively or can be induced by sub-inhibitory concentrations of erythromycin. An example of constitutive expression of resistance is that of strain DS5 and the response of this strain to challenge after growth in an inducing concen-

tration of erythromycin was compared with the responses of the six erythromycin resistant strains under the same conditions. The growth curves of the latter strains were the same as that for DS5 indicating that erythromycin resistance is constitutively expressed. This result does not exclude, however, the possibility that a strain may harbour more than one MLS determinant, one of which could be inducible since in such a circumstance, with the experimental conditions used, the inducible nature of a determinant would be obscured by the presence of constitutively expressed MLS resistance. In order to test such a possibility, it would be necessary to screen a number of transconjugant and cured derivatives for segregation of inducible and constitutive MLS resistance. Although several MLS resistance genes have been described in streptococci, both inducible, such as those located on Tn917 of S. faecalis (Tomich et al, 1980) and S. pyogenes plasmid pAC1 (Weisblum et al, 1979), and constitutive, for example those of S. faecalis plasmid pAMβ1 (Weisblum et al, 1979) and the chromosomally located transposon of S. pneumoniae, Tn1545 (Carlier and Courvalin, 1982) - there have nevertheless been no descriptions of strains which exhibit both modes of expression.

Tetracycline resistance is commonly encountered among enterococci (van Embden et al, 1977; Finland, 1979) and indeed, all the strains in this study were resistant to the drug. Investigation of the expression of resistance showed that all the strains display constitutive resistance although this again does not rule out the possibility of there being more than one type of determinant present. Indeed, unlike the situation for MLS resistance, there are examples of streptococcal strains which harbour different loci determining both constitutive and inducible tetracycline resistance, such as S. pneumoniae B109 (Inamine and Burdett, 1982) and S. faecalis JH1 (Banai and LeBlanc, 1983). Moreover, of 31 S. faecalis

strains examined by Burdett et al (1982b) for DNA homology with tetL (constitutive) and tetM (inducible) probes, 30 hybridised with both probes and a single isolate contained tetL alone. It would appear therefore that strains of S. faecalis which harbour only one type of determinant are exceptional.

The strains were tested for resistance to five aminoglycoside-aminocyclitol antibiotics. All were resistant to streptomycin, four were also resistant to kanamycin and neomycin but none was gentamicin or spectinomycin resistant. While recognising that, due to the resistance mechanism which involves a balance between drug uptake and inactivation, susceptibility to a particular drug need not necessarily correlate with absence of a modifying enzyme, it is nevertheless possible to speculate as to the classes of enzyme produced in these strains. Streptococcal aminoglycoside modifying enzymes constitute a sub-set of those produced by staphylococci and are different from those found in Gram-negative bacteria (Carlier and Courvalin, 1982). Using the compilation of modifying enzymes published by Foster (1983) and the information given by Carlier and Courvalin (1982), resistance to streptomycin is probably mediated by the enzyme AAD(6) which adenylates the 6-hydroxyl of the streptidine ring of streptomycin, and resistance to kanamycin and neomycin is mediated by an APH(3')(5") type III which phosphorylates 3'- and 5"-hydroxyl groups and which is commonly found in streptococci. It is of course possible that resistance to streptomycin may not be due to the production of a modifying enzyme but instead to ribosomal resistance caused by mutation resulting in an alteration in the ribosomal target site. Indeed, in the study by Eliopoulos et al. (1984), half the streptomycin resistant S. faecalis were ribosomally resistant while the others produced AAD(6). Only the latter resistance mechanism would however be transferable by conjugation.

None of the S. faecalis strains was found to be resistant to β-lactam antibiotics but the S. faecium strains K46 and SB69 were resistant to 40μg ml^{-1} penicillin and 10μg ml^{-1} ampicillin. No evidence of β-lactamase activity could be obtained using standard methods and so it is assumed that resistance in these strains is due to alteration in PBPs. A similar lack of β-lactamase activity has been shown in studies of penicillin resistant S. faecium (Le Bouguenec and Horodniceanu, 1982) and S. pneumoniae (Collatz et al. 1984).

One S. faecalis strain, K88, was resistant to chloramphenicol but the resistance was only fully expressed after growth in an inducing, sub-inhibitory concentration of drug. Although inducible resistance to chloramphenicol due to decreased cytoplasmic membrane permeability has been reported in Gram-negative organisms (Gaffney et al. 1981), resistance to the drug in streptococci has been found, without exception, to be associated with the induction of the inactivating enzyme CAT. In S. faecalis, chloramphenicol resistance has been shown to be transferable (van Embden, et al. 1977).

S. faecium strain SB69 in addition to being antibiotic resistant, was α-haemolytic on horse blood while S. faecalis strain K87 was β-haemolytic and hence assigned to the subspecies zymogenes. The latter strain appeared to be typical of β-haemolytic S. faecalis in that it was also bacteriocinogenic with activity against the plasmid free strain JH2-1. Previous studies of the haemolysin-bacteriocin traits of S. faecalis strains have indicated that they are two activities of the same molecule (Tomura et al. 1973; Dunny and Clewell, 1975) and in all strains examined, haemolysin-bacteriocin activity has been associated with a conjugative plasmid (Clewell, 1981; Borderon et al. 1982). One other S. faecalis strain SB94 showed weak bacteriocin produced against strain JH2-1 but no haemolytic activity. Several strains of S. faecalis have been described which produce

bacteriocin not associated with haemolysin, for example strain DS5 which in addition to pAMγ1 encoding haemolysin-bacteriocin, harbours pAMγ2 encoding a second bacteriocin (Clewell et al., 1982b), but in these strains a haemolysin-bacteriocin plasmid is also present (Clewell, 1981). Against a derivative of strain K87, strain SB94 was clearly bacteriocinogenic indicating the production of a bacteriocin at least different from that produced by K87 since K87 itself normally had no activity against this indicator.

To gain information on the relationship between and location of the different determinants in each strain the three approaches used were firstly to define their transfer characteristics in broth culture and on membrane filters, secondly, to determine the genetic stability of the phenotypic traits and thirdly, to correlate the phenotype of parental strains and their derivatives with plasmid content. Strain DS5 was included in the former sets of experiments as a well characterised control organism which has been shown to be an efficient donor of haemolysin and tetracycline resistance in broth culture (Dunny and Clewell, 1975) and capable of acting as a donor of erythromycin resistance in filter matings (Hershfield, 1979).

After mating experiments, transconjugant colonies were selected on nutrient agar containing the antibiotic of interest as well as either fusidic acid or rifampicin. Initially, donor and recipient strains were tested individually at the end of mating on selective antibiotic agar for spontaneously occurring mutations but this was found not to be a problem in most cases because the transfer frequency was far higher than the mutation rate. However, particularly in those matings which produced low frequency transfer of aminoglycoside resistance, for example in broth matings of K55 donors with JH2-2 and JH2-17, it is possible that colonies obtained on selective agar were in fact recipient mutants.

They were not donor mutants as all such colonies were resistant to the non-selected agent, rifampicin, in the case of JH2-2 matings or were thymine auxotrophs in the case of JH2-17 matings.

The mutation frequencies observed were comparable to published frequencies for S. faecalis spontaneous mutation to streptomycin resistance (10^{-9}; Horodniceanu et al., 1979a), fusidic acid resistance (4×10^{-9}; Jacob and Hobbs, 1974), erythromycin resistance ($< 10^{-10}$; Engel et al., 1980) and tetracycline resistance ($< 10^{-10}$; Franke and Clewell, 1981) which in this study was found to be extremely low (10^{-12}).

Evidence for the mechanism of transfer i.e. transformation, transduction or conjugation, was not obtained in this investigation. Rather the assumption was made that transfer occurred by a conjugal mechanism based on the extensive efforts of others which show that S. faecalis is not naturally transformable and that no transducing phages have been associated with antibiotic resistance transfer in this species (Jacob and Hobbs, 1974; Jacob et al., 1975; Dunny and Clewell, 1975). Clewell (1981) has reported that in a screen of 200 S. faecalis, none was transformed to antibiotic resistance under conditions in which transformation of S. sanguis was readily obtained although it has recently been reported that S. faecalis protoplasts may be transformed (Smith and Clewell, 1984).

The transfer frequencies obtained with DS5 donors in broth culture were comparable to those reported by Dunny and Clewell (1975). In this system, the MLS resistance plasmid pAMβ1, although conjugative, does not transfer due to inhibition by pAMγ1, while the non-conjugative tetracycline resistance plasmid pAMα1 is mobilised by pAMγ1, pAMγ2 or pAMγ3 at frequencies 100- to 1000-fold lower than haemolysin transfer. The phenotypes of the transconjugants were as expected, with haemolytic transconjugants susceptible to tetracycline and erythromycin although the percentage

of transconjugants selected on tetracycline which were haemolytic was higher than previously reported (Dunny and Clewell, 1975). One broth mating however, gave atypical results in that the transfer frequencies for haemolysin and tetracycline were increased and intermediate frequency transfer of erythromycin was detected. In addition, instead of the usual drop in recipient viable count due to sensitivity to the donor bacteriocin, the viable count actually increased. Analysis of the transconjugant phenotypes from this experiment showed no difference in the percentage of types selected on tetracycline or with fusidic acid, blood agar and of the six single colonies obtained on erythromycin, five were co-resistant to tetracycline and one resistant to tetracycline and haemolytic. The reason for this anomalous result is not clear but recipient growth implies that in the donor starter culture the bacteriocin encoded by pAMγ1 was in some way inactivated (the bacteriocin of pAMγ2 has no activity against JH2-2; Dunny and Clewell, 1975). For this to have happened, it would have to be assumed that either there was a substance inhibitory to bacteriocin in the culture medium on this one occasion or that all the colonies picked from stock culture plates were lacking the bacteriocin but not the haemolysin component of pAMγ1. Either a mutation resulting in inactivity is also associated with the relaxation of inhibition of pAMβ1 transfer or more likely, the mere fact that the recipient cells are no longer killed, results in increased transfer of all markers to such an extent that transfer of erythromycin is observed.

The transfer frequencies of DS5 markers after filter matings have not generally been reported although it is known that the recipient under these conditions is not noticeably susceptible to bacteriocin (LeBlanc and Lee, 1982) and that transfer of pAMβ1 can occur at low frequency (2×10^{-7} per donor; Hershfield, 1979). The transfer frequency for haemolysin remained unchanged but the frequency for tetracycline resistance transfer increased along with an increase in the percentage of non-haemolytic, tetracycline

resistant transconjugants.

Selected transconjugants were tested for bacteriocin production against JH2-1 and as anticipated, haemolytic transconjugants were also bacteriocinogenic. However, all the erythromycin resistant transconjugants and two of the tetracycline resistant transconjugants which were non-haemolytic nevertheless produced bacteriocin against JH2-1. Since pAMγ2 encoded bacteriocin is inactive against JH2 (Dunny and Clewell, 1975) and pAMγ3 does not encode bacteriocin (Clewell et al., 1982b), activity in these strains must arise from pAMγ1. However, as haemolysin and bacteriocin activities are two functions of the same protein encoded by pAMγ1 (Dunny and Clewell, 1975), this would require that there be a mutation in the plasmid solely affecting expression of haemolysin although no previous studies of haemolysin-bacteriocin plasmids have suggested that this may occur.

The response of DS5 and transconjugants to CIA produced by recipient strain JH2-1 was tested. The titre obtained for DS5 response was higher than that reported by Dunny et al. (1979) for a derivative of DS5 cured of pAMβ1 but it was not clear in the latter case if the strain was also cured of pAMγ2 or pAMγ3 which presumably contributed to the overall response of DS5. Response of transconjugants to JH2-1 CIA was not observed regardless of the phenotype. The same was true for almost all transconjugants tested in this study and it has been shown that JH2-2, harbouring a plasmid which encodes a pheromone response, does not aggregate due to an inability to recognise and take up exogenous CIA even although isogenic strains may exhibit normal responses (Ike and Clewell, 1984). It would therefore appear that JH2-17 is likewise unable to process efficiently exogenous CIA. Alternatively, it is possible that the aggregates are simply not visible without magnification and indeed, this is supported by the fact that the retransfer frequencies of the haemolysin marker in broth matings were comparable to those obtained originally. In the absence of a pheromone system

transfer in broth culture would be drastically reduced. From observations of bacteriocin activity the implication that erythromycin transconjugants contain a plasmid other than pAMγ1, is reinforced by the retransfer results which show low frequency transfer of erythromycin resistance in broth culture. Since pAMβ1 is inhibited by pAMγ1 and since detectable transfer of pAMβ1 normally requires cell-to-cell contact obtained on filters, it is probable that in these broth matings, the plasmid is utilising the gratuitous pheromone system of a plasmid such as pAMγ2 or pAMγ3. The reason why no retransfer of tetracycline resistance was observed is unclear as it seems likely that at least two of those transconjugant strains tested contained one of the pAMγ plasmids, all of which are capable of mobilising pAMα1 (Clewell et al., 1982b).

Growing cultures of DS5 overnight in the presence of acridine orange or ethidium bromide, Clewell et al. (1974) were unable to demonstrate loss of pAMγ1 or pAMα1 but pAMβ1 was cured in 1 to 2% of colonies tested while spontaneous loss of pAMβ1 occurred at a frequency of 0.1% or less. Although it is not possible to compare directly the curing frequencies obtained with different methods these results are nevertheless in marked contrast to those obtained in this study after storage of DS5 at room temperature for 12 months. Here, 96% of colonies tested were non-haemolytic. Presumably on storage at room temperature the viable count after 12 months would be extremely low and hence the culture which grew on transfer to fresh nutrient medium was derived from only a few cells. If most of these cells had lost pAMγ1 during storage, this method would select for a very high proportion of non-haemolytic cells in the culture. An alternative view could be that after 12 months storage, those surviving cells would be in a metabolically "poor" state and on transfer to fresh medium, use their remaining energy for chromosomal replication and possibly replication of smaller plasmids but, in the absence of environmental pressure

large plasmids would be lost. A similarly increased curing frequency of a 40kb conjugative antibiotic resistance plasmid from a strain of S. faecalis after storage has been reported (Marder and Kayser, 1977). Neither of the above explanations would apply to pAMβ1 and pAMα1 as they were maintained in 99.5% and 100% of colonies tested, comparable to the results of Clewell and coworkers (1974). Treatment with novobiocin resulted in a frequency of 86.5% non-haemolytic derivatives which is very much higher than the curing frequencies (1.6 to 3.4%) of the plasmids described by Borderon and coworkers (1982) after similar treatment. As with loss on storage, none of the colonies from the novobiocin treated culture had lost tetracycline resistance and only 0.6% were erythromycin sensitive.

Cured derivatives of DS5 were tested for response to JH2-1 CIA and, unlike transconjugants, were capable of responding. Those obtained after storage which were resistant to erythromycin and tetracycline did not aggregate indicating that, in addition to loss of pAMγ1, plasmids pAMγ2 and pAMγ3, which also specify pheromone response, were not present. One tetracycline resistant derivative obtained after storage and two such derivatives obtained after novobiocin treatment however, showed response titres of 4 or 8 which is less than the parent but implies the presence of pAMγ2 and/or pAMγ3. The haemolytic, tetracycline resistant derivative obtained after novobiocin treatment had response titre of 16 due, at least in part, to pAMγ1.

Surprisingly, when the cured derivatives were tested for bacteriocin activity against JH2-1, the three obtained after storage which had not responded to CIA were bacteriocinogenic and by the semi-quantitative criterion of zone diameter, appeared to be more bacteriocinogenic than the parent DS5, while the tetracycline resistant derivative obtained at the same time did not produce bacteriocin. Possible explanations are

that there is in the former strains a mutant pAMγ1 encoding defective haemolysin and pheromone response, or less likely, another gene for bacteriocin exists in DS5 unrelated to a plasmid encoding pheromone response. Derivatives obtained after novobiocin treatment were also bacteriocinogenic regardless of whether they produced haemolysin. In addition to observing clear zones where the growth of the indicator organism was inhibited, around the stabs of derivatives from storage, and extending further than the clear zone, were opaque zones. The nature of the opaque zones is unclear but they were not simply due to test organism spreading outward from the stab under the overlay and the opacity was in fact in the agar of the overlay. These zones were thought initially to resemble the "halos" described by Ike and coworkers (1983), but they related halo formation with response to CIA and three of the four derivatives in this study showed no pheromone response. Instead, it is possible that the opaque zones represent precipitation of a substance in the agar possibly due to liberation of excess acid in a process which is repressed in the parent.

Although strain DS5 was originally included in the study as a known plasmid containing control, a number of anomalous results have been obtained, particularly in relation to the three pAMγ1 plasmids which have only recently been described. In order to resolve the questions raised by this study, it would be necessary to investigate more deeply the plasmid content of the derivatives obtained in relation to their phenotypes. The scarcity of publications dealing with DS5 plasmids in their original host probably reflects the difficulty in interpretation of results due to interaction of the five plasmids.

The transfer of resistance markers from S. faecalis strain K55 was found to occur in broth culture at intermediate or low frequency. Erythromycin resistance consistently transferred at a frequency 10- to 100-fold higher than did tetracycline and streptomycin resistance suggesting that the

latter genes were at a different location from the erythromycin resistance determinant. With the streptomycin and, to a lesser extent, the tetracycline resistance genes transfer was not always detected, probably because of small but deleterious changes in experimental procedure. As the transfer frequencies of these markers were close to the level of detection in these experiments, a slight decrease in mating efficiency could easily result in no transconjugants being obtained.

Strain K55 responded to JH2-1 CIA with a titre of 4 which is a common value for antibiotic resistant S. faecalis isolated from clinical sources (Dunny et al., 1981b). However, transfer in broth culture of plasmids which specify response to pheromone normally occurs at frequencies at least 1000-fold higher than those observed for K55 markers, indicating that the K55 resistance loci do not reside on the plasmid which encodes pheromone response in this strain. Enhanced transfer of an erythromycin resistance plasmid by a plasmid encoding pheromone response has been reported (Dunny et al., 1981b) and it is probable that these conjugative elements in K55 make use of the cell-to-cell contact afforded by the pheromone system. The transfer frequencies on membrane filters which would, in the absence of pheromone assistance for broth transfer, be expected to be higher due to the enforced cell-to-cell contact, were in fact within the same frequency range with tetracycline and streptomycin markers again showing a 10- to 100-fold lower frequency compared with that for erythromycin resistance. Mating on membrane filters, however, did produce more reproducible results particularly for the tetracycline resistance determinant. This effect could simply be due to the increased cell numbers and incubation time used for membrane filter matings, or it could reflect a greater stability of mating pairs or aggregates on a solid support.

Analysis of the transconjugant phenotypes confirmed that erythromycin resistance could be transferred independent of the other resistance markers

although a high proportion of transconjugants selected on erythromycin were co-resistant to streptomycin. This was not foreseen especially in those transconjugants selected after broth culture matings in which the transfer frequencies of the two markers were so different, and suggests some temporary association between the determinants whereby the erythromycin gene is located on a plasmid which facilitates transfer of streptomycin resistance. This association could be mobilisation such as described for pAMα1 in DS5 (Dunny and Clewell, 1975) or formation of a cointegrate structure such as the intermediates described during transposition of Tn917 from pAD2 to pAD1 (Clewell et al., 1982a). The former is more likely in cases where transconjugants selected on streptomycin were resistant to that drug only. Again, this has been demonstrated in S. faecalis strain DS16 which harbours pAD1 and pAD2, where transfer of pAD2 usually occurs as a result of cointegrate formation but can occur at low frequency due to mobilisation without concomitant pAD1 transfer (Tomich et al., 1979). Study of the retransfer frequencies also suggests that the streptomycin gene must be mobilised by erythromycin resistance plasmid since the streptomycin resistance locus alone was incapable of retransfer. The frequency of retransfer erythromycin was not tested because of the difficulty in obtaining transconjugants resistant to the drug alone after matings between K55 and JH2-17. Regardless of the means of resistance transfer, however, the fact that such a high proportion of erythromycin selected transconjugants were streptomycin resistant implies that the transfer frequency of the latter should have been higher than that actually observed and hence that there is an initial delay in expression of streptomycin resistance on transfer to JH2-2.

None of the transconjugants selected on tetracycline was resistant to either erythromycin or streptomycin although the transfer frequencies for streptomycin had been similar. The tetracycline determinant, however, was not capable of self-transfer at least in a JH2-17 background.

Loss of antibiotic resistance was observed both after storage at room temperature and after novobiocin treatment. On storage, 2% of colonies tested were susceptible to streptomycin and 1.5% susceptible to streptomycin and erythromycin. With novobiocin treatment there was a high percentage curing (19.2%) of erythromycin and streptomycin resistance, again suggesting some, at least temporary, linkage of the two determinants. The lack of success in curing tetracycline resistance was not surprising in view of previous studies which have been unable to obtain curing of small, multicopy tetracycline resistance plasmids (Clewell et al., 1974; Burdett, 1980) or of conjugative elements located on the chromosome (Tomich et al., 1979; Smith et al., 1981) although in the latter case apparent curing due to point mutation could be obtained at a frequency of 10^{-3} (Smith et al., 1981). Curing of large conjugative tetracycline resistance plasmids such as pJH1 has been demonstrated (Jacob and Hobbs, 1974) although in its original host, curing would not have been observed under the conditions of this study as strain JH1 contains in addition a chromosomal tetracycline resistance locus (LeBlanc and Lee, 1982).

To relate the presence or absence of certain markers with pheromone response, cured derivatives were tested for response to JH2-1 CIA. Since erythromycin was probably the only determinant capable of self-transfer, it was expected to be the only resistance associated with pheromone response but this was clearly not the case. All the tetracycline resistant derivatives responded to the CIA which suggests, in agreement with transfer frequency values, that the strains contain another cryptic plasmid which encodes pheromone response. A less likely alternative is that the tetracycline resistant locus resides on a conjugative element which could be either an autonomous replicon or a chromosomal insertion but which specifies pheromone response. Presumably, in the former case, transfer to a JH2-17 host would involve loss of conjugative ability by deletion of transfer genes

or by integration into the chromosome of the new host as has been observed for tetracycline resistance plasmid pIP685 in S. faecalis (Horodniceanu et al., 1982b).

To locate the resistance genes, a study of the plasmid content of the parental strain and its transconjugants and cured derivatives was made. On agarose gels, four bands were observed in strain K55 although all four bands were not present in each preparation, which indicates that one or more of these bands could be open circular or linear forms of a plasmid. It was hoped that examination of the plasmid content of transconjugant strains and cured derivatives, particularly those which displayed resistance to a single antibiotic, would clarify this problem. Conceivably, band 1 could have represented a cointegrate structure between bands 2 and 4 such as was suggested by transfer experiments involving erythromycin and streptomycin resistance loci. However, since all tetracycline resistant transconjugants of matings with JH2-2 and all derivatives cured of erythromycin and streptomycin resistances also contained band 1, this therefore seemed unlikely. A further complication in the plasmid analysis was that some tetracycline resistant transconjugants appeared to contain exactly the same plasmids as streptomycin resistant transconjugants and one tetracycline resistant strain had a band pattern identical to K55, implying co-transfer of all the parental plasmids. Only one derivative cured of streptomycin resistance but resistant to erythromycin and tetracycline was obtained and this also had a band pattern identical to the parental strain. In this case, it is possible that loss of streptomycin resistance was due to mutation.

To resolve the potential problem of there being open circular forms of plasmid which were complicating the band patterns, samples were digested with the restriction endonucleases EcoR1 and Pst1. Digestion of the K55 sample with either enzyme resulted in nine fragments the sums of which were

comparable. The tetracycline resistant transconjugants and derivatives cured of erythromycin and streptomycin resistance all contained the same EcoR1 fragments and Pst1 fragments which gave a total size of 59.40 and 50.76kb respectively. Although the value for Pst1 is somewhat lower than for EcoR1, it is possible that one band may represent more than one fragment, for example Pst1 fragment D. Nevertheless, both estimations are close to the size calculated for band 1 of 51.5kb and the results suggest that this is a conjugative plasmid designated pSK551 which may encode tetracycline resistance. This would be supported by results of transfer experiments in as much as this determinant was always transferred alone but would not explain why the resistance was not retransferred. If pSK551 does specify tetracycline resistance, on transfer, a deletion affecting conjugative ability but which was too small to detect in these digests must have occurred. A conjugative tetracycline resistance plasmid, pCF-10, of similar size (53kb) has been described in S. faecalis (Dunny et al., 1979) but transfer in broth culture occurred at high frequency due to pCF-10 encoded pheromone response (Dunny et al., 1981b). In contrast, the low frequency transfer of K55 tetracycline resistance suggests that a pheromone system is not directly involved. It therefore seems more likely that pSK551 is in fact a conjugative cryptic plasmid which encodes pheromone response and that the tetracycline resistance determinant is located on the chromosome but capable of transfer by conjugation.

Of the streptomycin resistance transconjugants, endonuclease digestion showed that two contained only chromosomal DNA, as had been suggested in undigested preparations, and one contained EcoR1 fragments which appeared identical to those of pSK551, lending support to the proposal that this plasmid may not be associated with antibiotic resistance. Although it is possible that streptomycin resistance is chromosomally determined but

capable of transfer either by transposition to a conjugative plasmid or by conjugative transposition (Gawron-Burke and Clewell, 1982), this conclusion would be in conflict with curing data in which a high percentage of streptomycin susceptible derivatives were obtained. The streptomycin resistance gene could instead be located on a non-conjugative plasmid which is capable of existing as an autonomous replicon or as a cointegrate with an erythromycin resistance plasmid, but which on mobilisation, without concomitant transfer of erythromycin resistance, could integrate into the host chromosome. Integration of autonomous replicons is not an unknown phenomenon in streptococci (Horodniceanu et al., 1982b,c).

EcoR1 fragments B, G and H were present in all K55 samples and in the erythromycin resistant derivative cured of streptomycin resistance and probably represent another plasmid encoding erythromycin resistance, pSK552, which is approximately 22.60kb in size. It has one Pst1 site producing 25.60kb fragment A which is equivalent to band 3 in undigested K55 samples. A conjugative erythromycin resistance plasmid of this size would be within the size range of similar plasmids isolated from many different streptococcal groups (Clewell, 1981). The discrepancies in this proposal are the digested samples from erythromycin resistant transconjugants in which both enzymes produced only one novel fragment of size 38.5kb. The most likely explanation is that digestion in these samples was incomplete since otherwise the generation of such fragments would involve insertion of DNA with concomitant inactivation of two EcoR1 sites, or the existence of an entirely different erythromycin resistance plasmid.

Strain K60 is a S. faecalis subsp. liquefaciens which is resistant to MLS antibiotics, tetracycline, streptomycin, kanamycin and neomycin. In broth culture, all markers were transferred with high or intermediate

frequencies usually observed in pheromone utilising systems although only erythromycin showed consistently high frequencies. On membrane filters, transfer was slightly more efficient with the frequency of erythromycin transfer reaching a value of 1. The transfer frequencies would suggest the close association of streptomycin, kanamycin and neomycin resistance loci and possibly of tetracycline but not of the erythromycin determinant.

The phenotypes of the transconjugants seemed to confirm the latter with by far the greater percentage of erythromycin selected transconjugants being resistant to that drug only. That the percentage resistant to erythromycin alone was higher on filter probably reflected the higher relative transfer frequencies obtained for erythromycin. As would be expected with transfer frequencies of 1, all transconjugants obtained after filter matings were erythromycin resistant, whatever the selective antibiotic. Although the majority of transconjugants selected on tetracycline were co-resistant to erythromycin and aminoglycosides, a small percentage of those from broth matings were resistant to tetracycline alone indicating that the gene is not closely linked to the aminoglycoside resistance loci, but is capable of independent transfer or is mobilised by a conjugative plasmid. Regardless of the aminoglycoside used for selection, all transconjugants were co-resistant to the other aminoglycosides and to erythromycin. Similar results have been reported in S. faecalis DS16 where the phenotype was related to stable cointegrate formation between pAD2 and the co-resistant plasmid pAD1 (Clewell et al., 1982a) and in S. faecalis strain JH1 in which the resistance genes are located on pJH1 (Banai and LeBlanc, 1983). In the latter case, deletion analysis confirmed that the aminoglycoside resistance genes were located on a 16.5kb segment of the plasmid while the tetracycline resistance gene

was located elsewhere, hence the ability to obtain deletion derivatives resistant to tetracycline alone. Erythromycin, or erythromycin and aminoglycoside co-resistant transconjugants were shown to contain hybrid plasmids consisting of fragments of pJH1 inserted into pJH2 and indeed the erythromycin resistance gene has been found to be located on Tn3871 which is probably identical to Tn917 of S. faecalis DS16 (Banai and LeBlanc, 1984a).

The erythromycin and tetracycline resistance determinants of K60 were tested for the ability to retransfer. No retransfer of tetracycline resistance was detected indicating either a loss of conjugative ability on primary transfer or the necessity of a co-resident conjugative plasmid to mobilise the resistance determinant. Retransfer of erythromycin resistance was observed but at greatly reduced frequencies, comparable in fact to frequencies expected for transfer in the absence of a pheromone system, possibly reflecting the poor aggregation response in a JH2-17 background due to impaired ability of the host either to recognise or to take up exogenous pheromone (Ike and Clewell, 1984).

After storage at room temperature for 12 months, almost half the colonies tested retained the original phenotype while the remainder was susceptible to the aminoglycosides. Neither erythromycin nor tetracycline sensitive derivatives were obtained. On treatment with novobiocin, one derivative was obtained which was resistant to tetracycline only but none was isolated which was tetracycline sensitive. This again reflects the common inability to cure tetracycline resistance in streptococci although the effect could also be due to inability of the technique used here to distinguish between two determinants such as are found in S. faecalis JH1 (LeBlanc and Lee, 1982). The finding that a large percentage of colonies tested either after storage or novobiocin treatment

were susceptible to aminoglycosides is contrary to reports of other S. faecalis strains, for example DS16 and JH1, which have similar resistance phenotypes but in which loss of aminoglycoside resistance is accompanied by simultaneous loss of erythromycin resistance (Tomich et al., 1979; Banai and LeBlanc, 1983). The erythromycin resistance marker also differs from other streptococcal MLS plasmids such as pAMβ1 and pIP501 (Clewell et al., 1974; Horodniceanu et al., 1979a) by the extremely low curing frequency and high transfer frequency in this strain.

Response of cured derivatives to JH2-1 CIA, which cannot be tested with transconjugants in a JH2-2 (Ike and Clewell, 1984) or JH2-17 background, was correlated with phenotype and transfer characteristics. The parental strain had a response titre of 4 which is a common value in clinical isolates (Dunny et al., 1981b). Derivatives which were resistant to erythromycin, responded to CIA but had variable titres while no response was observed with the erythromycin susceptible derivative. These results along with transfer data suggest that the erythromycin resistance determinant is located on a conjugative plasmid which specifies pheromone response.

Attempts made to isolate plasmid DNA from strain K60 were either completely unsuccessful or not reproducible although all bands which were observed, were large (53 to 140kb). The reason for the difficulty in isolation of plasmid DNA from this strain is not clear as DNA other than chromosomal was easily isolated from cured derivatives. Plasmid DNA was also isolated after transfer to another host but there appeared to be no correlation between phenotype and plasmid content. For example, in all transconjugants from matings with JH2-2, bands of approximately the same size were observed regardless of phenotype. A similar situation was seen in transconjugants of JH2-17 matings and cured derivatives

although more bands were observed particularly in those derivatives cured of aminoglycoside resistance.

Since it seemed probable therefore, that there was more than one plasmid of similar size present, samples were digested with restriction endonucleases. As with the other strains in this study, PstI digestion of DNA isolated from transconjugants was unreliable due to the sensitivity of this enzyme to inhibitory substances in the preparations.

Digestion of the K60 sample with EcoRI resulted in sixteen fragments and with PstI, eight fragments. The sum of the PstI fragments was 28kb less than that for EcoRI fragments which indicates that a band or bands in the PstI digest may represent more than one fragment. Comparison of EcoRI fragments obtained after digestion of erythromycin or tetracycline resistant transconjugants would indeed suggest the presence of two plasmids of similar size, the fragments observed in erythromycin resistant transconjugants being complementary to those in the tetracycline resistant transconjugant. The exception to this was fragment F which was present in both phenotypes but it is conceivable that a fragment of this size could have been derived from two different plasmids.

Digestion with EcoRI of derivatives cured of streptomycin resistance revealed the same fragments present as those in the erythromycin resistant transconjugants. It is possible therefore that the erythromycin resistance determinant is located on an approximately 68kb conjugative plasmid, designated pSK601, which can be present as a dimer (140kb) in undigested samples. In addition to those fragments, the cured derivatives which were co-resistant to tetracycline also had novel fragments the sum of which was 10.25kb or 15.65kb depending on whether the strains were obtained after storage or novobiocin treatment respectively. Indeed bands of similar size are seen in corresponding undigested samples from

these strains but no such bands have been observed in the parental strain. The situation was similar with PstI digestion, and although initially it would appear that this is an example of transposition, the fact that none of the pSK601 fragments has been altered in size by an insertion, suggests that the novel fragments are derived instead from separate replicons. The origin of these replicons could be the chromosome, a possibility which is not entirely without precedent in Gram-positive bacteria. For example, elements integrated in the chromosome which confer tetracycline resistance have been shown to be capable of autonomous replication in S. mutans (LeBlanc et al., 1982) and B. subtilis (Shishido et al., 1983). Similarly, autonomous replicons originating from the chromosome have been recognised in Streptomyces (Bibb et al., 1981). Alternatively, since these strains are cured of resistance to aminoglycosides, the novel fragments could be derived from a larger plasmid from which a segment containing the genes conferring aminoglycosides resistance had been deleted but which retains the capability for autonomous replication.

In addition to fragments complementary to pSK601 the tetracycline resistant transconjugant also had novel EcoRI fragments with a total size of 23.60kb and although it is possible that these fragments arise from transposition such as obtained with the 16kb Tn916 (Franke and Clewell, 1981), it is again not clear into which fragment the novel DNA could have transposed. Therefore the same argument for a separate replicon derived either from the chromosome or by deletion of another larger plasmid, can be applied here although an equivalent sized band was not seen in the undigested sample. It is reasonable to assume that the fragments common to K60 and the tetracycline resistant transconjugant represent an approximately 47kb plasmid, pSK602, which determines resistance to tetracycline and which is non-conjugative but can be efficiently mobilised. The single

band of around 65kb seen in undigested samples could be derived from a cointegrate of pSK602 and the novel replicon.

The lack of fragments on digestion of the tetracycline resistant derivative cured of erythromycin and aminoglycosides resistance indicates a chromosomally encoded determinant and would explain the difficulty in obtaining tetracycline cured derivatives. If this were the only tetracycline resistance determinant in strain K60, it must represent an element capable of inordinately high frequency transposition into a conjugative plasmid to allow high frequency transfer. This need not be so however if there is,as seems likely, a second tetracycline resistance determinant located on pSK602 such as has been described in S. faecalis JH1 (LeBlanc and Lee, 1982). The possession of two classes of gene namely tetL and tetM, has been found to be a common phenomenon in S. faecalis (Burdett et al., 1982a,b).

There is no clear evidence to indicate the location of the aminoglycosides resistance genes. In the EcoR1 digests, only fragment C is present in K60 but absent in transconjugants and cured derivatives. The size of this fragment compares favourably with the 16.5kb segment known to be associated with streptomycin and kanamycin resistance in pJH1 (Banai and LeBlanc, 1983) but in pJH1 these resistances are contiguous with and always deleted with erythromycin resistance in cured derivatives. EcoR1 fragment C could encode streptomycin, kanamycin and neomycin resistance and either suffer frequent independent deletion from pSK601 or represent a separate replicon which is mobilised to high frequency transfer by pSK601. It is also feasible that aminoglycosides resistance genes are chromosomally located and capable of transposition to pSK601, although the transposition frequency would have to be excessively high to obtain the transfer frequencies observed.

S. faecalis strain K87 was found to be resistant to MLS antibiotics, tetracycline and the aminoglycosides streptomycin, kanamycin and neomycin. It is also β-haemolytic and bacteriocinogenic and hence an example of the subspecies zymogenes. Transfer to JH2-2 of the β-haemolytic trait was observed in broth culture at high frequency and to JH2-17 at intermediate frequency. High frequency transfer of β-haemolysin production has been demonstrated in many S. faecalis strains (Dunny and Clewell, 1975; Jacob et al., 1975; Borderon et al., 1982) and in all cases, it has been shown that this activity is conferred by conjugative plasmids, of similar size, which share extensive DNA homology (LeBlanc et al., 1983). As expected from the high transfer frequencies, strain K87 responded to JH2-1 CIA with a titre of 16.

Transfer of antibiotic resistance in broth culture was not observed in two of four experiments and could have been due to inhibition of transfer by a haemolysin-bacteriocin plasmid, similar to that of pAMβ1 by pAMγ1 observed in strain DS5 (Clewell et al., 1982b). Alternatively, since the donor is bacteriocinogenic resulting in reduction of viable recipient, viable antibiotic resistant transconjugants may not have been detected if the transfer frequency was low. In the other two broth mating experiments however, antibiotic resistance transfer was observed at intermediate or low frequency. As was the case when anomalous transfer frequencies were obtained in a mating between DS5 and JH2-2, the recipient viable count did not decrease in either mating presumably due to the absence of active bacteriocin. Under normal circumstances, only those cells which had received bacteriocin resistance, encoded by the haemolysin-bacteriocin plasmid, would survive but without active bacteriocin, antibiotic resistant non-haemolytic recipients could be isolated.

Mating on membrane filters had little effect on transfer of β-haemolysin

but greatly improved the reproducibility of antibiotic resistance transfer, because under these conditions, bacteriocins appear to have little effect on the viability of recipient cells (LeBlanc and Lee, 1982). As the transfer frequencies for all resistance markers were similar, it was not possible to infer any linkage between loci. However, analysis of transconjugant phenotypes showed that erythromycin and tetracycline resistances could be transferred independently while transfer of resistance to aminoglycosides always involved concomitant transfer of erythromycin resistance. None of the haemolytic transconjugants was antibiotic resistant. The percentage of antibiotic resistant phenotypes obtained was dependent on the mating method, most noticeably when transconjugants were selected for erythromycin resistance. This indicates that the antibiotic resistance transfer observed in broth culture is not simply contingent on the increase in viable recipients but must be dependent on another unknown variable. If, as is the case in strain DS5 (Clewell et al., 1982b), the presence of a haemolysin plasmid normally inhibits transfer in broth culture of an antibiotic resistance plasmid, it is conceivable that a mutation which affects bacteriocin production could also affect such transfer inhibition.

The conjugative ability of the markers was tested by retransfer to another recipient. Erythromycin resistance retransferred in broth culture at frequencies 10^2- to 10^4-fold higher than in primary transfers suggesting that there is in fact transfer inhibition in the parental strain. Since the transconjugants tested for retransfer were selected from broth matings however, the possibility exists that the resistance loci in these strains could have undergone recombination with DNA specifying conjugative ability from another source before primary transfer. The tetracycline resistant transconjugants were unable to retransfer resistance and so this resistance appears not to be linked to the erythromycin determinant,

despite the similarities in primary transfer frequency values. The initial efficient transfer but subsequent lack of conjugative ability infers that the determinant is located on a non-conjugative plasmid which can be efficiently mobilised either by an erythromycin or haemolysin plasmid. Similar high frequency mobilisation of the non-conjugative pAMα1 has been reported (Dunny et al., 1978). Surprisingly, the retransfer frequencies of haemolysin were reduced 10^3- to 10^4-fold and there was no retransfer observed from one primary haemolytic transconjugant. This impairment of transfer probably reflects the inability of the new host strain to recognise exogenous CIA and a similar effect with the retransfer of the haemolysin plasmid pAD1 has been reported (Ike and Clewell, 1984). Nevertheless the retransfer frequencies may be underestimated due to the difficulty of recognising β-haemolytic colonies when the background concentration of non-haemolytic recipient cells is high and zones of haemolysis around strain K87 consequently are small.

Neither storage at room temperature nor treatment with novobiocin caused loss of erythromycin and tetracycline resistance markers. In the latter case, this was not unexpected in view of the reports describing the inability to cure small, non-conjugative tetracycline resistance plasmids in group B strains (Burdett, 1980) and pAMα1 of S. faecalis strain DS5 (Clewell et al., 1974) possibly due to the high copy number, 50 and 10 copies per cell respectively, of such plasmids (Clewell et al., 1974). In contrast spontaneous loss of plasmid encoded erythromycin resistance from group D hosts can often be demonstrated (Buu-hoi et al., 1984).

Although not as striking as that obtained with DS5, there was a high percentage loss of haemolytic activity after storage, while curing of

haemolysin trait by novobiocin treatment resulted in a frequency comparable to that obtained by Borderon and coworkers (1982) for group D haemolysin plasmids. Resistance to aminoglycosides was eliminated but only at low frequency and after storage. The loss en bloc of resistance to streptomycin, kanamycin and neomycin confirms the close linkage of the genes demonstrated in transfer experiments and since the other markers were not eliminated simultaneously, infers that the aminoglycoside resistance loci are encoded by a separate non-conjugative replicon. Such a non-conjugative plasmid conferring resistance to aminoglycosides alone would be rare among streptococci (Clewell, 1981). However, it is also conceivable that erythromycin and aminoglycoside resistance genes are normally on the same plasmid and that the erythromycin gene is capable of transposition to another co-resident plasmid as is the case with pAD2 of S. faecalis DS16 (Clewell et al., 1982a) and pJH1 of S. faecalis JH1 (Banai and LeBlanc, 1983; 1984a). Both the latter plasmids encode resistance to streptomycin and kanamycin and in the case of pJH1, tetracycline resistance also, and carry erythromycin resistance transposons, Tn917 and Tn3871 respectively (Clewell et al., 1982a; Banai and LeBlanc, 1984a) which can transpose to co-resident haemolysin-bacteriocin plasmids. There are nevertheless several differences between the situation in strain K87 and that in DS16 and JH1 not least being that erythromycin resistance of K87 is constitutive while that of Tn917 and Tn3871 is inducible and that the erythromycin resistance determinant of K87 is capable of high frequency retransfer independent of other markers.

The response of cured derivatives to JH2-1 CIA was greatest by those which were haemolytic. This was anticipated since all haemolysin plasmids of S. faecalis examined determine pheromone response with the possible exception of pPD5 (LeBlanc et al., 1983). The antibiotic resistant, non-

haemolytic derivatives also responded with a low titre suggesting the presence of either an antibiotic resistance specifying plasmid which also encodes pheromone response or another plasmid encoding pheromone response but neither antibiotic resistance nor haemolysin activity such as pAMγ2 and pAMγ3 of S. faecalis DS5 (Clewell et al., 1982b) and pPD1 of S. faecalis 39-5 (Yagi et al., 1983).

In addition to haemolysin production, strain K87 was also bacteriocinogenic when tested against the indicator organism JH2-1. On testing transconjugant strains, only those which were haemolytic also produced bacteriocin against JH2-1 although from the zone diameters there appeared to be quantitatively more bacteriocin produced by transconjugants, which could reflect an increase in gene dosage or a derepression of bacteriocin production. Surprisingly, all the cured derivatives displayed detectable bacteriocin production regardless of whether they were haemolytic which supports the suggestion that there could be plasmids present such as pAMγ2 and pPD1 both of which encode bacteriocin but not haemolysin (Clewell et al., 1982b; Yagi et al., 1983).

With respect to antibiotic resistance and haemolytic activity, indicator strain K87IO is phenotypically identical to K87 and was isolated during a curing experiment from replica plates containing streptomycin on which it appeared very susceptible to bacteriocin of derivatives cured of haemolysin activity. Bacteriocin activity against K87IO in the absence of antibiotic was almost completely the opposite of that observed against JH2-1 with the exception of non-haemolytic derivatives obtained after novobiocin treatment, which were weakly bacteriocinogenic against both indicators. This observation could not be due to loss by K87IO of bacteriocin resistance which is encoded by haemolysin-bacteriocin plasmids since the strains with activity against K87IO were inactive against JH2-1

which has no resistance, but instead suggests that more than one bacteriocin is produced. As strain K87 is inactive against K87IO, it would appear that expression of the bacteriocin with activity against K87IO is repressed in the parental strain, and hence the situation must be very different from that in strains DS5 and 39-5 where both bacteriocins are readily demonstrable.

As K87IO was isolated from agar containing streptomycin, bacteriocin production was tested on antibiotic agar. None of the tetracycline or erythromycin resistant transconjugants, which were bacteriocinogenic without antibiotic, was active when the agar contained streptomycin. Because these strains were susceptible to streptomycin and therefore would be unable to grow, this would have been predicted but for the observation that haemolytic transconjugants, also streptomycin susceptible, not only became bacteriocinogenic to K87IO but, on the basis of zone diameter, appeared to produce an unprecedented level of activity. This observation was repeated with derivatives obtained after storage regardless of whether they were haemolytic or streptomycin resistant and increased production of bacteriocin was obtained from derivatives cured of haemolytic activity by novobiocin treatment. The reason for this high level of bacteriocin on streptomycin agar, particularly since some strains are streptomycin susceptible, is unclear. It is possible that a combination of sufficient cell divisions taking place in the overlay agar coupled with a derepression of bacteriocin production by the action of the drug could produce such an effect. Derepression could also be brought about by streptomycin induced transposition causing mutation analagous to the hyperexpression of haemolysin sometimes observed on transposition of Tn916 into pAD1 (Gawron-Burke and Clewell, 1982). It would be interesting to test bacteriocin production on agar containing other aminoglycosides and against a streptomycin resistant mutant of JH2. On erythromycin and

tetracycline agar, activity was greatly reduced and there was no bacteriocin produced by any transconjugants although K87 showed weak activity.

In addition to the clear zones of growth inhibition around bacteriocin producers, opaque zones like those observed around certain DS5 derivatives were visible and associated only with haemolytic transconjugants and cured derivatives obtained after storage. That opacity was observed around stab inocula of both JH2 and K87 derivatives argues against a strain specific activity. The production of opaque zones also appears independent of phenotype but could nevertheless be related to the presence of one or more plasmid molecules. The reason why none of the novobiocin treated derivatives produce opaque zones and the nature of the opacity itself are obscure.

In undigested plasmid preparations from K87, a band could be seen of a size 59.7kb within the range expected for S. faecalis haemolysin-bacteriocin plasmids (Clewell, 1981; Borderon et al., 1982). As observed in strain DS5, the apparent presence of one band can conceal the existence of more than one similarly sized plasmid molecule (Clewell et al., 1982b) and so K87 band 1 could be composed of a bacteriocin encoding and a haemolysin-bacteriocin encoding plasmid. It was hoped that analysis of the plasmid content of haemolytic transconjugants would verify an association of band 1 with haemolytic activity but while four such transconjugant contained bands 1 and 2, another three strains contained only band 2. Nevertheless, it is possible that the failure to observe band 1 in the latter strains was due to the irreproducibility of band 1 isolation already encountered in repeated preparations of K87. Further discrepancies, however, were found such as the absence of correlation between the presence of band 1 and bacteriocin production, and the presence of

band 1 in antibiotic resistant transconjugants. No correlation of the remaining bands with antibiotic resistance could be made except that the possession of bands 3, 4, 5 and 6 appeared to be unrelated to any phenotypic marker which implies that these bands represent cryptic plasmids.

Restriction endonuclease digestions were carried out to analyse further the plasmid content of K87, its transconjugants and cured derivatives. Unfortunately, digestion patterns did not clarify or support the conclusions based on transfer and curing data. For example, erythromycin resistance was thought to be encoded by a conjugative plasmid. Digestion of samples prepared from transconjugants, while showing the presence of several fragments in common with K87, revealed the presence of eight novel fragments, the origin of which is speculative. Tetracycline resistant transconjugants had digestion patterns resembling those obtained for erythromycin resistant transconjugants but the novel fragments were different in number and size. The generation of novel fragments suggests recombination between different molecules such as can occur on transposition but, if the novel fragments represent erythromycin or tetracycline resistance transposons, they must be structurally quite different from the well characterised streptococcal transposons Tn917 and Tn916 which have no EcoR1 sites (Clewell et al., 1982a; Gawron-Burke and Clewell, 1982). Alternatively, it is possible that during transfer, interaction of different plasmid molecules is producing hybrid plasmids in a process like that described in strain JH1 where recombination between the haemolysin-bacteriocin plasmid and the R plasmid may result in cointegrates composed of both plasmids or hybrids composed of portions of one or both plasmids (Banai and LeBlanc, 1983). Nevertheless, it is difficult to envisage how such recombination could result in the generation of so many novel restriction fragments.

Moreover, in the JH1 system, antibiotic resistant transconjugants were almost exclusively haemolytic, which is clearly not the case in K87 matings although the erythromycin and tetracycline resistant transconjugants whose fragment patterns were similar, all displayed bacteriocin activity against K87IO.

Digestion of haemolytic transconjugants demonstrated two different restriction patterns although the phenotypes with regard to pheromone response and bacteriocin production were indistinguishable, suggesting the co-existence of two plasmids both of which encode haemolysin-bacteriocin but which must be sufficiently different from each other to be compatible within the K87 host. This would be highly unusual among S. faecalis since examination of several plasmids by DNA hybridisation has demonstrated not only homology between haemolysin-bacteriocin genes but extensive plasmid DNA homology extending beyond the genes (LeBlanc et al., 1983). Indeed pAMγ1 and pAD1 have been shown by DNA hybridisation and restriction endonuclease analysis, to be identical and display incompatibility (Clewell et al., 1982b; LeBlanc et al., 1983).

The restriction profiles of cured derivatives were identical regardless of the phenotype with the exception of K87C8 which was without bands 3 and 5, and so no correlation of digestion patterns and the absence of a phenotypic trait could therefore be made. It is probable that bands 3 and 4, and bands 5 and 6 represent open circular and cccDNA forms respectively of two cryptic plasmids, pSK871 and pSK872. pSK871 is not digested by EcoR1 but has one PstI site (fragment B) while pSK872 is not digested by PstI and has at least one EcoR1 site producing fragment G.

The transfer of antibiotic resistance from K88 donors in broth culture was inefficient occurring sporadically and at low frequency. However, the fact that transfer was observed at all in broth culture

suggests the presence of a pheromone system (Clewell, 1981) although, in view of the low frequencies, not necessarily encoded by a resistance plasmid. Indeed, in the presence of JH2-1 pheromone, strain K88 does exhibit an aggregation response, possibly specified by a conjugative cryptic plasmid an example of which is pAMγ3 (Clewell et al., 1982b), and the cell-to-cell contact thus obtained may aid transfer of antibiotic resistance. Alternatively, since strain K88 is a subspecies liquefaciens and produces protease which is known to inactivate CIA (Dunny et al., 1979), destruction of pheromone could lead to fewer mating aggregates and inefficient transfer. No such effect was noted, however, for strain K60 which is another liquefaciens subspecies.

Transfer frequencies of resistance markers after matings on membrane filters were of either intermediate or low frequency but the reproducibility of transfer was markedly superior. This could be due to the increase in incubation time and viable count of donors and recipients but could in part be caused by greater stability of mating aggregates on a solid support allowing the transfer of conjugative elements which do not confer pheromone response (Clewell, 1981). The transfer frequencies observed after filter matings were in general lower than those reported for intraspecific transfer of S. faecalis conjugative resistance plasmids using comparable methods (Malke, 1979; van Embden et al., 1977; Horodniceanu et al., 1982b; Buu-hoi et al., 1984) and were closer to the frequency range expected of chromosome located conjugative transposons (Franke and Clewell, 1981; Horodniceanu et al., 1982c). The low transfer frequencies could alternatively reflect inefficient mobilisation of a non-conjugative resistance plasmid or interaction of plasmids causing transfer inhibition similar to that of pAMβ1 by pAMγ1 (Clewell et al., 1982b).

The percentage of different phenotypes obtained were similar for

broth and filter matings. The streptomycin resistance locus was always transferred independently and therefore is unlinked to the other resistance genes. The majority of tetracycline resistant transconjugants were susceptible to the other resistance markers demonstrating that this locus is also capable of independent transfer. Almost all other transconjugants were co-resistant to erythromycin, chloramphenicol and tetracycline suggesting that erythromycin and chloramphenicol genes are linked and possibly mobilised by a conjugative tetracycline resistance plasmid. One exception was isolated which was susceptible to erythromycin.

Study of the ability to retransfer demonstrated that the tetracycline resistance determinant was indeed capable of self-transfer during filter, but not broth, matings at frequencies comparable to the primary transfers and so is probably located on a conjugative plasmid which does not specify pheromone response. Streptomycin resistance was retransferable in broth culture at frequencies 100-fold higher than those obtained on primary transfer. This increased transfer frequency indicates the location of the resistance gene on a conjugative plasmid which specifies response to CIA. In the new host, pheromone produced by the recipient would not be subject to proteolysis or, alternatively, increased transfer frequency may reflect transposition of the streptomycin resistance gene to a cryptic conjugative plasmid. Such increased transfer of tetracycline resistance is observed on transposition of Tn916 to conjugative haemolysin plasmids (Franke and Clewell, 1981).

Examination of the phenotypes of derivatives obtained after storage or treatment with novobiocin revealed that, like the previous parental strains, tetracycline resistance could not be eliminated. As expected from the transfer experiments, streptomycin resistance could be lost independently of other markers but erythromycin and chloramphenicol

resistances were always eliminated together.

The response of cured derivatives to CIA contrasted with the results of transfer experiments which suggested that streptomycin resistance is encoded by a conjugative plasmid specifying pheromone response. Neither the derivatives resistant to tetracycline and streptomycin nor the derivative resistant to tetracycline alone responded to pheromone but two derivatives cured of streptomycin resistance did respond. This again raises the possibility of there being an independent cryptic plasmid which specifies pheromone response.

Plasmid preparations of K88 revealed the presence of six bands but correlation of the presence of bands with the phenotypes of transconjugants and cured derivatives was difficult. Bands 5 and 6 which were always present in cured derivatives regardless of phenotype, but absent in transconjugants probably represent open circular and cccDNA forms respectively of the same cryptic plasmid, designated pSK881. Further evidence for this was demonstrated by the appearance of bands of topoisomers under band 5 in one undigested plasmid preparation (not shown) and by the fact that both bands appear to have no EcoR1 sites and one PstI site which produces a single fragment of the same size.

The majority of tetracycline resistant transconjugants and derivatives possessed band 4. Both EcoR1 and PstI digestion of DNA from transconjugants and the derivative cured of all but tetracycline resistance resulted in a single fragment of size comparable to band 4. It appears therefore that K88 harbours a tetracycline resistance plasmid of approximately 23.5kb capable of transfer on membrane filters and designated pSK882.

From the transfer characteristics and curing data, it was expected that streptomycin resistance would be specified by a conjugative plasmid with a size of greater than 50kb as all plasmids yet described which

specify pheromone response are at least this size (Clewell, 1981; Dunny et al., 1981b). However, DNA preparations of streptomycin resistant transconjugants contained either no plasmid bands or a band similar in size to K88 band 2. Derivatives cured of erythromycin and chloramphenicol resistances either possessed only pSK881 and pSK882 or band 2, pSK881 and pSK882 or bands 1 and 2, pSK881 and pSK882. Digestion of DNA samples from transconjugants generated novel fragments in addition to some fragments visible also in K88 digests. The presence of novel fragments implies recombination between plasmids or transposition of DNA from the chromosome. As streptomycin resistance can be easily eliminated, however, it is unlikely that the gene is located in the chromosome even although digestion of samples from some cured derivatives resistant to streptomycin and tetracycline revealed the presence of only pSK881 and pSK882. The location of the streptomycin resistance gene therefore remains unclear.

Transconjugants resistant to erythromycin, chloramphenicol and tetracycline all contained K88 band 1 while the derivative cured of streptomycin resistance contained band 1, pSK881 and pSK882. The single transconjugant resistant to tetracycline and chloramphenicol exhibited the same band pattern as those co-resistant to erythromycin suggesting that erythromycin susceptibility in this case may be due to mutation of the methylase gene. While it is possible that band 1 is a cointegrate formed between pSK882 and another non-conjugative plasmid encoding erythromycin and chloramphenicol resistance, similar sized bands were also visible in erythromycin and chloramphenicol susceptible strains. Digestion of samples with EcoR1 and Pst1 did not aid interpretation as most of the fragments generated were also visible in samples prepared from erythromycin and chloramphenicol susceptible strains. The location of the erythromycin and chloramphenicol

determinants is therefore also unknown but as with the streptomycin resistance locus, the relatively high curing efficiency makes a chromosomal site unlikely.

S. faecalis strain SB94, in addition to being antibiotic resistant, was found to be bacteriocinogenic with weak activity against JH2-1 but greater activity against K87IO. With the latter indicator, incorporation of streptomycin in the test agar resulted in increased activity similar, although less striking, to that observed by K87 derivatives. Plasmids encoding bacteriocin alone are not uncommon in S. faecalis for example the conjugative plasmids pAMγ2 and pPD1 (Clewell et al., 1982b; Yagi et al., 1983) and the non-conjugative pOB2 (Oliver et al., 1977) although these plasmids are found in strains which also harbour haemolysin-bacteriocin plasmids.

The transfer of antibiotic resistance at high or intermediate frequency in broth culture was demonstrated. Such transfer frequencies in broth culture suggest that the resistance genes reside on one or more plasmids which either specify pheromone response or are efficiently mobilised by a plasmid encoding pheromone response. Strain SB94 exhibits a response to JH2-1 pheromone typical of antibiotic resistant S. faecalis (Dunny et al., 1981b). Mating on filter membranes had either little effect on transfer frequencies or produced a 10- to 100-fold increase but this increase could be attributed to the longer incubation time and higher cell density.

Analysis of the transconjugant phenotypes indicated that erythromycin, tetracycline and aminoglycosides resistance loci can be unlinked and that erythromycin resistance is probably capable of independent transfer. While tetracycline resistance may be capable of self-transfer, it is possible that mobilisation occurs by an erythromycin resistance plasmid in a process similar to that described for the mobilisation of non-

conjugative tetracycline resistance plasmids by erythromycin resistance sex factors (Burdett, 1980). That attempts to retransfer tetracycline resistance failed, supports this latter proposal. Transconjugants resistant to aminoglycosides were always co-resistant to erythromycin indicating that the aminoglycoside resistance determinants are incapable of independent transfer.

The phenotypes of derivatives obtained after storage or novobiocin treatment confirmed the results of transfer experiments with respect to the separate locations of the resistance genes. After storage, a very high percentage of derivatives were susceptible to erythromycin and a smaller percentage susceptible to aminoglycosides suggesting that the resistance genes were located on discrete plasmid molecules. On treatment with novobiocin, erythromycin and aminoglycoside resistances were lost *en bloc* at low frequency and one derivative was obtained which was also tetracycline susceptible. This was the only instance of elimination of tetracycline resistance among the *S. faecalis* strains examined and it infers that the resistance gene is not located on the chromosome where it would be maintained (Horodniceanu *et al.*, 1982c) but is located on a plasmid. It is conceivable however that the resistance gene could be encoded by a transposable element capable of existence in either location and that apparent curing is due to point mutation which in *S. pneumoniae* can occur at 0.1% frequency (Smith *et al.*, 1981).

The response of cured derivatives to CIA did not correlate with the transfer data in that tetracycline resistant derivatives exhibited the same or greater response than those resistant to erythromycin and tetracycline. Since tetracycline resistance appears to be located on a non-conjugative plasmid, no response would have been expected and this anomaly raises the possibility that these strains may contain a conjugative

plasmid perhaps encoding bacteriocin.

Examination of the plasmid content of strain SB94 revealed the presence of four DNA bands on agarose gels. Bands 3 and 4 were present in none of the erythromycin or tetracycline resistant transconjugants. Like the bands of similar size found also in strains K87 and K88, they have no EcoR1 sites and only one Pst1 site and are probably open circular and cccDNA forms respectively of the same cryptic plasmid, pSK941. It is interesting that strains isolated from very different locations, a hospital and a sewer outlet, contain plasmids with such similarities. Other small cryptic plasmids have been described in several S. faecalis strains (Clewell, 1981). The 5.1kb cryptic plasmid pPD4 of S. faecalis strain 39-5 can be mobilised by conjugative co-resident plasmids and has been found incompatible with the tetracycline resistance plasmid pAMα1 (Yagi et al., 1983). This latter plasmid is known to dissociate into two autonomous replicons the smaller of which, pAMα1Δ2, is capable of replication in S. faecalis (Perkins and Youngman, 1983). pAMα1Δ2 is similar to plasmids pSK871, pSK881 and pSK941 in that it is 5.1kb, cryptic, has one Pst1 site and no EcoR1 sites and such comparisons raise the possibility that the plasmids described in this study represent deleted tetracycline resistance plasmids.

Erythromycin and tetracycline resistant transconjugants all had the same undigested DNA bands present. Digestion with EcoR1 demonstrated that samples from each phenotype contained some fragments in common but also several novel fragments and the digestion patterns were generally dissimilar to the parental strain. The similarities in restriction profiles between transconjugants suggest a molecule in common but with large inserts producing novel fragments. The total of the EcoR1 fragments from tetracycline resistant transconjugants, however, is at least twice the

size of SB94 band 1 and it is possible therefore that there are two plasmid molecules of similar size one encoding tetracycline resistance present in these strains. If this were so, one of these molecules would presumably be present in the parental strain at such a low copy number as to make it impossible to visualise on agarose gels. A non-conjugative plasmid of such a size would not be unprecedented among S. faecalis as pPD6 of strain 39-5 is 53kb (Yagi et al., 1983). This hypothesis would concur with the observation that the derivative cured of tetracycline resistance appeared to contain the same plasmid bands as the parent but the possibility remains that the resistance gene may be located on a transposon capable of integration into the chromosome and that curing represents mutation or deletion.

Likewise, the erythromycin gene could be located on a chromosomal element capable of transposition to a conjugative plasmid producing novel restriction fragments although the high curing efficiency makes this less likely. It is more plausible that the gene is located on a conjugative plasmid with an approximate size of 56kb which may be a third molecule migrating to the position of band 1 in undigested samples with the result that erythromycin susceptible derivatives would appear to retain band 1 and could have similar restriction profiles to the parent.

No evidence was obtained from plasmid preparations on the location of aminoglycosides resistance determinants except that they are not located on bands 2, 3 or 4 of the parent. Since transfer of resistance to aminoglycosides never occurred without co-transfer of erythromycin resistance, it is possible that the aminoglycoside resistance loci are chromosome specified but capable of transposition to the conjugative erythromycin resistance plasmid. Loss of resistance would therefore be due to deletion from the chromosome during storage in non-selective

conditions and would not be linked to erythromycin resistance.

An alternative explanation of the results is that, as in S. faecalis JH1 (Banai and LeBlanc, 1983; 1984a), the antibiotic resistance genes are located on the same conjugative plasmid, hence the high proportion of transconjugants which received all the resistance markers. From this plasmid, the erythromycin determinant could be capable of transposition to another conjugative plasmid of similar size, perhaps encoding bacteriocin production, resulting in transconjugants resistant to this drug alone. Tetracycline resistant transconjugants could arise on transposition of a second chromosomal element to the putative bacteriocin plasmid although this would not explain why the resistance was not retransferable. As has been shown in JH1, the production of hybrid plasmid molecules could give rise to transconjugants resistant to erythromycin and aminoglycosides and deletion of these determinants either together or independently could result in the phenotypes observed on curing. Aside from the similarities in restriction profiles of erythromycin resistant and tetracycline resistant transconjugants, however, there is little visual evidence on gels for such transpositions and deletions.

The lack of transfer of antibiotic resistance in broth culture from the S. faecium strains SB69 and K46 to the S. faecalis recipient was anticipated not only because this would require interspecific transfer which, with the exception of erythromycin resistance plasmids, is rare (Horodniceanu et al., 1982b, c; Buu-hoi et al., 1984) but also because only S. faecalis strains can respond to sex pheromones (Clewell, 1981; Dunny et al., 1981b). On membrane filters however, while no resistance transfer was observed from strain K46, in two experiments very low frequency transfer of tetracycline resistance from SB69 was obtained.

No curing of SB69 antibiotic resistance was observed either after storage or novobiocin treatment and repeated attempts to demonstrate plasmid DNA in the parental strain and tetracycline resistant transconjugants proved negative.

It seems likely therefore that in strain SB69, all resistances are chromosomally encoded but that tetracycline resistance is present on a conjugative transposon. The transfer frequencies obtained were similar to those described for the plasmid free transfer of Tn916 (Franke and Clewell, 1981) and other, chromosomally located, resistance markers from S. faecium strains (Le Bouguenec and Horodnideanu, 1982).

Since plasmid encoded β-lactam resistance has only recently been reported in streptococci and is as yet extremely rare (Murray and Mederski-Samaros, 1983), it was not surprising that the evidence indicated resistance specified by the chromosome presumably involving changes in PBPs (Eliopoulos et al., 1982; Fontana et al., 1983b). Streptomycin resistance of SB69 is also determined by the chromosome.

None of the K46 resistances was transferable in broth culture or on membrane filters. However, the observation that streptomycin resistance was cured on storage, and erythromycin, tetracycline and streptomycin resistances could be cured by novobiocin treatment, suggested that these resistances were plasmid encoded. Loss of these resistance loci occurred independently as would be observed with unlinked genes but resistance to penicillin and ampicillin was never eliminated supporting the conclusion above that resistance is chromosome borne. Since there was some doubt as to the basis of erythromycin and lincomycin resistance in this strain, erythromycin susceptible derivatives were tested and all were found to be lincomycin susceptible also.

Preparation of sufficient plasmid DNA from strain K46 proved extremely difficult but the reason for this is not clear. Although K46 reproducibly contained six DNA bands, it is likely that not all of these bands represent individual plasmids. For example, bands of 34.7kb and 32.9kb may be artefacts produced by the 38.5kb band or topoisomers of the 17.2kb band, the 38.5kb band being the open circular form. That all the derivatives also contain 38.5kb and/or 34.7kb bands as well as the 17.2kb band supports that latter proposal. It is impossible to assign resistance genes to plasmids by comparison of the derivatives of different phenotypes as the band patterns are generally similar. Certain derivatives contained novel bands suggesting the ability of plasmids to undergo deletions although this feature again failed to correlate with phenotype. Several S. faecium isolates have been shown to contain plasmids (Dunny et al., 1981b) some of which may confer antibiotic resistance and be transferable (Le Bouguenec and Horodniceanu, 1982). Recombination between plasmids as well as deletion formation and transposition of resistance genes probably takes place in some of these strains (Le Bouguenec and Horodniceanu, 1982).

Although the resistance phenotypes of the strains in this study are similar i.e. resistance to MLS antibiotics, tetracycline and aminoglycosides, it is clear from comparison of restriction endonuclease digestion patterns that the strains contain structurally different plasmids. The exception seems to be the small cryptic plasmids of strains K87, K88 and SB94. In a number of cases, it is probable that transfer of resistance involves either intermolecular recombination or transposition of chromosomal genes into plasmid molecules which makes interpretation of restriction fragment profiles extremely difficult and this in turn makes allocation of resistance genes to a particular location speculative.

Comparison between the multiple resistant haemolytic S. faecalis

strains JH1 and DS16 have revealed a number of similarities (Banai and LeBlanc, 1983). Results of DNA-DNA hybridisation experiments showed extensive homology between respective haemolysin-bacteriocin plasmids (LeBlanc et al., 1983), and chromosome situated tetracycline resistance (Tn916) as well as plasmid located erythromycin resistance transposons (Tn3871 and Tn917) have been demonstrated in both strains (LeBlanc and Lee, 1982; Banai and LeBlanc, 1984a). Additionally, both strains confer plasmid specified resistance to streptomycin and kanamycin. A preliminary report on twelve group D isolates which have resistance phenotypes resembling JH1, DS16 and the strains in this study, has indicated that the erythromycin resistance genes of five isolates were located on Tn917 and in six strains, hybridisation of plasmid DNA to a pJH1 probe showed significant homology (Lee et al., 1984). It is indeed possible that the strains in this study may contain chromosome specified tetracycline resistance located on a transposon and this could explain the rarity of cured derivatives although, where high primary transfer frequencies have been observed, it is likely that tetracycline resistance is also plasmid specified. Erythromycin resistance encoded by Tn917 is however less likely in these strains as the resistance is expressed constitutively.

Future experiments would therefore be designed to clarify the results already obtained. It is likely that more than one class of tetracycline resistance gene exists in the S. faecalis strains which accounts for the difficulty in obtaining cured derivatives. A comparison of the MICs of parental strains and transconjugants and determination of the MICs of the related antibiotics, minocycline and chelocardin, would help to resolve this question as it is known that different classes of determinant confer different resistance levels (Burdett et al., 1982b). Such comparison of MICs led to the discovery of the two tetracycline loci in S. faecalis JH1

(LeBlanc and Lee, 1982).

In this investigation, transconjugants displaying one phenotypic trait or derivatives cured of one trait were chosen preferentially for study of retransfer ability and for comparison of plasmid content. It would be interesting and informative, however, to examine retransfer from transconjugants of strains K55, K60, K87 and SB94 co-resistant to erythromycin and aminoglycosides to ascertain whether the resistance loci are retransferred in unison from the transconjugants. Curing data from these parental strains indicate that these determinants need not be closely linked and could be present on different molecules but, particularly in the cases of K60, K87 and SB94, the fact that no transconjugants selected on aminoglycosides are erythromycin susceptible demonstrates that co-transfer of erythromycin resistance is a prerequisite for aminoglycoside transfer. This could arise if 1. the resistance loci were on the same plasmid molecule, 2. a non-conjugative aminoglycoside resistance plasmid were mobilised via cointegrate formation with a conjugative MLS resistance plasmid or 3. aminoglycoside resistance genes were capable of transposition to a conjugative MLS plasmid. Comparison of the plasmid content of trans-conjugants resistant to erythromycin alone and those co-resistant to aminoglycosides could give some indication as to which of these alternatives is most likely.

A major problem in interpreting and relating phenotype to the plasmid content of parental strains, transconjugants and cured derivatives, has been the presence and possible transfer of more than one plasmid species and so, in order to resolve this difficulty, the individual plasmids in each parental strain should be isolated. This could be accomplished by sucrose density gradient centrifugation, electroelution from agarose gels or isolation from low melting point agarose, although preliminary results

using the latter method with DS5 plasmids have indicated difficulty in separating plasmids in this agarose. Following isolation, plasmid DNA could be used to transform S. sanguis to antibiotic resistance although it should be noted that certain plasmids do not transform S. sanguis, for example pAMα1 (LeBlanc and Hassel, 1976) and pJH1 (Banai and LeBlanc, 1984b). Comparison of the restriction profiles of purified plasmids with those from transconjugants and cured derivatives would help to identify and clarify possible intermolecular interactions.

Knowing the phenotypes conferred by difficult plasmids, it would be interesting to investigate the precise location and the relationships of the resistance genes to determinants isolated from other sources by DNA-DNA hybridisation. Probes specific for tetL and tetN have been described (Burdett et al., 1982) as well as a probe containing the erythromycin resistance locus of pAMβ1 (Burdett et al., 1982b) which, in contrast to that described by Ounissi and Courvalin (1982), fails to hybridise to Tn917 (Clewell et al., 1982a). The probe containing the haemolysin-bacteriocin determinant of pAD1 has been shown to hybridise to all S. faecalis haemolysin-bacteriocin plasmids studied but not those specifying bacteriocin alone (LeBlanc et al., 1983). It would also be interesting to test by reciprocal DNA-DNA hybridisation experiments the hypothesis that pSK871, pSK881 and pSK941 are similar to pAMα1Δ2.

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