STUDIES ON PLASMIDS OF SOME LACTIC ACID BACTERIA

by

Kiswar Yeasmin

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science, at the University of Leicester.

September 1982

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

S T A T E M E N T

This thesis is based on work conducted by the author in the Department of Microbiology of the University of Leicester during the period between October 1979 and September 1982.

All the work recorded in this thesis is original unless otherwise acknowledged in the text by references. None of the work has been submitted for another degree in this or any other university.

Signed Kiswar Yeasmin Date 20th September, 1982

To my husband

for his patience and understanding

A C K N O W L E D G E M E N T S

I would like to express my gratitude to Dr. Dorothy Jones for her careful supervision and for all the interest and encouragement she has given me throughout the course of my work and in the writing of this thesis.

I am also indebted to all members of the Microbiology Department for their help and co-operation. I thank C. Boyd of the Department of Biochemistry for his technical help with computer work. Anne Freelove and Alison White are thanked for their invaluable help with proof reading. I would also like to thank Mrs. Grace Redfern for the stamina and good humour she has shown in typing this thesis.

This work was carried out during the tenure of a Scholarship from the Commonwealth Scholarship Commission, which is gratefully acknowledged.

A B S T R A C T

A survey was conducted of a number of lactic acid bacteria of the genera *Erysipelothrix*, *Listeria* and *Streptococcus* to investigate the presence of detectable plasmids. When plasmids were detected an attempt was made to correlate their presence with a phenotypic trait. In addition some plasmids were investigated to determine certain physical characteristics, e.g. molecular weight and also the degree of base pair homology between plasmids isolated from different bacteria.

The results indicate that of the strains of *Erysipelothrix* investigated none appeared to contain extrachromosomal DNA. Of the strains of the genus *Listeria*, plasmids were detected only in one representative of *Listeria monocytogenes* serovar 5. This particular serovar is characterized by enhanced haemolytic activity when compared with strains of the various other species in the genus *Listeria*. However, no evidence was obtained to indicate that the haemolysis was plasmid encoded. Plasmid deoxyribonucleic acid was detected in a number of strains representing a variety of species in the genus *Streptococcus*.

The results obtained confirmed earlier reports that the haemolytic activity of "*Streptococcus faecalis* var. *zymogenes*" is plasmid encoded. The results also indicate that the haemolytic activity of haemolytic strains of bacteria previously named "*Streptococcus durans*" was also plasmid encoded. Investigation of the properties of the plasmid DNA from both these sources however did not show a high degree of homology, thus indicating that the plasmids had not been derived from the same source. Plasmids conferring drug resistance were isolated from representatives of the species *Streptococcus faecium*. Pigmentation in some strains of "*Streptococcus faecium* var. *casseliflavus*" appeared to correlate with the presence of plasmid DNA. Strong evidence was also obtained which indicated that lactose fermentation in *Streptococcus bovis* was at least, in part, due to the presence of plasmids.

Although plasmids were detected in representatives of the species

Streptococcus agalactiae, Streptococcus equinus, "Streptococcus faecalis var. liquefaciens", "Streptococcus faecium var. mobilis", their presence could not be correlated with any particular phenotypic trait.

No plasmids were detected in those strains of Str. faecalis and "Str. faecalis var. malodoratus" examined.

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INTRODUCTION

It is now generally recognized that plasmids occur in a wide variety of bacteria (see Broda, 1979; Hardy, 1981). These extrachromosomal elements were first recognized as R factors (Watanabe & Fukasawa, 1960) coding for drug resistance amongst members of the family Enterobacteriaceae. Since that time reports of the presence of such extrachromosomal elements have been made with increasing frequency.

Plasmids have been detected in a wide variety of lactic acid bacteria (Clewell, 1981; Clewell & Franke, 1974; Courvalin et al., 1972, 1974; Dunny et al., 1973). In some cases the plasmids have been shown to encode for phenotypic properties and in other cases the plasmids are cryptic, i.e. they are not known to encode for any phenotypic property.

The term "lactic acid bacteria" is used to refer to a number of Gram-positive, non-sporeforming, facultatively anaerobic genera which produce lactic acid as the sole or main end product of the fermentation of carbohydrates. The bacteria generally referred to by this term are the members of the families Lactobacillaceae and Streptococcaceae. In addition, bacteria such as those of the taxa Erysipelothrix and Listeria may also be included.

The purposes of this study were to survey a number of lactic acid bacteria for the presence of plasmids and, if plasmids were detected, to attempt to link their presence to a phenotypic trait.

LACTIC ACID BACTERIA STUDIED

Of the many taxa referred to as the lactic acid bacteria, representatives of the genera Erysipelothrix, Listeria and Streptococcus were included in the study reported here. The names of all taxa which are not included in the Approved Lists of Bacterial Names (Skerman et al., 1980), or for which names have not been formally proposed since the Lists were compiled, are placed in quotation marks.

ERYSIPELOTHRIX

The genus Erysipelothrix was named by Koch (1880) for an organism called by him "Erysipelothrix muriseptica" isolated from the blood of mice injected subcutaneously with putrefying blood. Pasteur & Dumas (1882) described a similar organism from swine erysipelas and Pasteur & Thuiller (1883) used cultures of this organism to demonstrate the use of live attenuated bacteria in the artificial immunization of pigs.

Loeffler (1886) first observed a bacillus similar to "E. muriseptica" (Koch, 1880) in blood vessels of the skin of a pig that had died from swine erysipelas. Rosenbach (1909) as a result of a comparative study of some of these organisms isolated from various sources, suggested the species names, "E. porci", "E. muriseptica" and "E. erysipeloides" for isolates from the pig, the mouse and humans respectively.

In contrast, Rickmann (1909) using morphological, cultural and serological data concluded that the differences between the three species proposed by Rosenbach (1909) were neither distinct nor constant and were due to host variation. Rickmann (1909) therefore concluded that the three were just variants of a single species (see Woodbine, 1950).

The name "Erysipelothrix porci" (Rosenbach, 1909) was antedated by "Bacterium rhusiopathiae" (Migula, 1900), and the new combination Erysipelothrix rhusiopathiae (Migula) was proposed by Buchanan (1918). During the next 50 years the name Erysipelothrix rhusiopathiae became firmly established in the literature. However, Langford & Hansen (1953) challenged the validity of this name and proposed the name "Erysipelothrix insidiosa" (Trevisan) comb.nov; because of the use of the name "Bacillus insidiosa" by Trevisan (1885) to describe an organism recognized as being identical with E. rhusiopathiae. However, the name "E. insidiosa" was not generally recognized until the seventh edition of Bergey's Manual of Determinative Bacteriology (1957) when Langford & Hansen were the authors of the section which included the genus Erysipelothrix. They recognized one species only under the name "E. insidiosa" (Trevisan)(Langford & Hansen 1953). Shuman & Wellmann (1966)

requested an opinion from the Judicial Commission on Bacterial Nomenclature on the conservation of the specific epithet rhusiopathiae in the species name Erysipelothrix rhusiopathiae because the specific epithet "insidiosa" had not been used in the literature for more than 50 years. The Judicial Commission (Judicial Commission, 1967) recommended the conservation of the species epithet rhusiopathiae in the name E. rhusiopathiae (Buchanan, 1918; Migula, 1900) rather than the specific name "insidiosa" or other previously used specific epithets (Opinion 32 - Judicial Commission, 1970).

Erysipelothrix is described by Seeliger (1974) as a group of Gram-positive, non-sporing,non-acidfast, non-motile rods, with a tendency to form long filaments. Members of the genus Erysipelothrix are catalase-negative, facultatively anaerobic and fermentative. Acid is produced from few sugars, but like Listeria species, no gas is produced. Alpha but no beta haemolysis is produced on blood agar.

The cell wall of E. rhusiopathiae contains lysine, glycine, serine, glutamic acid and alanine, but not meso-diaminopimelic acid. Furthermore, Schleifer & Kandler (1972) showed that the peptidoglycan exhibited the B1δ linkage.

E. rhusiopathiae has no isoprenoid quinones. This is in accord with the absence of catalase and cytochromes in this genus (Collins et al., 1979).

Fatty acid analysis studies have shown that E. rhusiopathiae contains mainly straight chain, even-numbered fatty acids, from C_{10} to C_{18} (Tadayon & Carroll, 1971).

Erysipelothrix rhusiopathiae has a mol % G+C value of 36 which is similar to that of L. monocytogenes, L. grayi and L. murrayi (38-40%) (Stuart & Welshimer, 1973, 1974). However, Stuart & Welshimer (1974) found that the two strains of E. rhusiopathiae included in their DNA reassociation studies only bound 10% of labelled L. monocytogenes DNA with 3 to 6% of the bases imperfectly matched.

At present the genus Erysipelothrix contains only one species, E. rhusiopathiae.

LISTERIA

Listeria monocytogenes, the type species of the genus *Listeria*, was first adequately described by Murray, Webb & Swann in 1926, during an epizootic amongst the laboratory breeding stock of rabbits in the Department of Pathology at Cambridge. These authors named the organism "*Bacterium monocytogenes*" in recognition of the monocytosis observed in the infected rabbits.

Murray *et al*. (1926) could not classify their organism in any of the genera described at that time (Bergey, 1925). Consequently, they proposed the temporary use of "the indefinite Bacterium" (Bergey, 1925, p.28) as a genus name until the taxonomic position of the organism was clarified.

There is now little doubt that many earlier workers, as early as Hülphers (1911), had probably isolated and described the same bacterium. However, a year after Murray *et al*. (1926) published their findings, Pirie (1927) described what was soon recognized (by the Curator of the National Collection of Type Cultures in London) to be the same bacterium. Pirie (1927) isolated the organism from the livers of the African jumping mouse (Tatera lobengulae) and named it "*Listerella hepatolytica*" in honour of Lord Lister. However, because the generic name "*bacterium*" as applied by Murray *et al*. (1926) was undesirable in the long term and "*Listerella*" (Pirie, 1927) had already been applied to a group of slime moulds (Mycetozoaire), Pirie (1940) suggested the name *Listeria monocytogenes*. This name was approved by the Judicial Commission on Bacterial Nomenclature and Taxonomy (Opinion, 12, 1954). It had in fact already been adopted in the Sixth edition of Bergey's Manual of Determinative Bacteriology (Breed *et al*., 1948).

As implied by the specific epithet *monocytogenes*, all sero and biovars of the species *L. monocytogenes* as then described possessed the ability to provoke a monocytosis in appropriate animals (see Seeliger, 1961). The relevance of monocytosis to the pathogenicity of *L. monocytogenes* is not yet understood (see Carroll *et al*., 1966; Tadayon *et al*., 1969; 1970).

Listeria monocytogenes is pathogenic for a wide range of animal species including man. The racing pigeon is the only animal known to resist heavy experimental infection, but this bird sometimes remains a carrier for a long period of time and might play a role in the spread of the bacterium (Donker-Voet, 1965).

The clinical symptoms of disease show no fundamental differences between animals and man (see Seeliger, 1961). Listeriosis is the term which is used for all forms of disease caused by L. monocytogenes. The symptoms can be manifested as meningoencephalitis, septicemia, abortion, stillbirth or neonatal death.

Gray & Killinger (1966) suggested that in animals L. monocytogenes usually attacks the strongest of the herd or flock but there is no other evidence to support this view. In man, L. monocytogenes appears to be an opportunistic pathogen, attacking the very young and the very old; people whose immune response has been lowered due, for example, to viral infections, leukemia, diabetes, pregnancy as well as malnutrition. Prolonged use of corticosteroids has also been associated with a number of instances of clinical listeria infections. Rappaport et al. (1960) established a relationship between L.monocytogenes and repeated abortion. Sword (1966) found that increased iron levels enhanced development of listeriosis. Fortunately, early recognized clinical cases in humans can be dealt with adequately by a number of antibiotics.

Although it is well established that L. monocytogenes can attack a wide range of animal species, very little is known about its epizotiology and epidemiology. Its main reservoir in nature has never been established. The wide host range suggests the occurrence of symptomless carriers, and that predisposing factors and stress promote the chance of infection (Bojsen-Møller & Jessen, 1966; Blenden, 1974; Kwantes & Isaac, 1975).

Almost as soon as listeria infections were first recognized in domestic animals, especially sheep, it was suggested that there appeared to be a

relationship between silage feeding and the disease. Blenden et al. (1966) noted that though clinical listeriosis in farm animals occurred during any season, it occurred much more frequently in late winter and spring. Gray (1960a,b) demonstrated L. monocytogenes in samples of poor silage (i.e., silage with a high pH). Kruger (1963) and Palsson (1963) showed that the occurrence of L. monocytogenes in silage in appreciable numbers was dependent on the pH of the silage which had to be more than pH 5.6 for multiplication of the bacterium to occur. Blenden et al. (1968) confirmed this finding and furthermore demonstrated that if pH values were adjusted to the reverse in good and poor quality silage, the degrees of growth of L. monocytogenes were also reversed.

Although there have been many studies on the characterization of L. monocytogenes and some other members of the genus Listeria (Gray & Killinger, 1966; Jones, 1975b; Seeliger, 1961), there have to date been few on the classification and differentiation of all the members of the genus Listeria and related bacteria. Exceptions are the studies of Wilkinson (1973), Wilkinson & Jones (1977), and Feresu (1980).

More recently, new species of the genus Listeria have been described. All, with the exception of those referred to as "Ivanov serological Group 5" (Ivanov, 1957), are not known to be pathogenic. However, since many of them, together with such organisms as the atypical lactobacilli of Thornley & Sharpe (1959) resemble L. monocytogenes, they have to be considered in any study of the genus Listeria.

For many years the genus was monospecific containing only L. monocytogenes (Breed et al., 1957). However, over the years further species have been proposed. In the latest edition of Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974), the genus contains four species, namely L. monocytogenes, L. grayi, L. murrayi and L. denitrificans. More recently, two further groups have been described. One, a non-haemolytic group, has been awarded species status by Seeliger & Schoofs (1979) and named

Listeria innocua. The other comprises very haemolytic strains and is presently referred to as Ivanov Group 5 (Cooper et al., 1973; Hunter, 1973; Ivanov, 1957, 1962), or "L. bulgarica" (Ivanov, 1975).

Listeria monocytogenes

There is a great deal of evidence based on a number of different criteria, e.g. numerical taxonomic, serological and chemical studies (Collins et al., 1979 ; Da Silva & Holt, 1965; Davis & Newton, 1969 ; Davis et al., 1969; Jones, 1975a,b; Jones et al., 1979; Sneath & Cowan, 1958; Stuart & Welshimer, 1973, 1974; Wilkinson, 1973; Wilkinson & Jones, 1975, 1977), that L. monocytogenes is a homogeneous species. However, the DNA homology studies of Stuart & Welshimer (1973, 1974) indicate two groups within the species but, as these authors pointed out, the two homology groups cannot be distinguished by any other characteristics.

The morphological, biochemical and serological characters of L. monocytogenes have been well documented (Gray & Killinger, 1966; Seeliger, 1961; Wilkinson, 1973; Wilkinson & Jones, 1977). L. monocytogenes, as defined in the latest edition of Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974), is a Gram-positive, small, coccoid rod with rounded ends in smooth cultures and elongated rods and long filaments up to 100 µm, in rough cultures. It is motile by means of a few, rarely more than four, peritrichous flagella. It grows well on blood agar with a narrow zone of beta haemolysis around the colonies. The degree of haemolysis varies between strains and with the species of blood used. In some cases it can only be detected if the colony is removed from the agar plate. L. monocytogenes produces acid but no gas from glucose and a few other carbohydrates. Hydrogen sulphide is not produced and nitrate is not reduced to nitrite.

L. monocytogenes requires carbohydrate for growth and glucose is commonly used to meet this requirement. The catabolism of glucose under both aerobic and anaerobic conditions is essentially homofermentative (Miller & Silverman, 1959). This suggests that L. monocytogenes could be related to

the family Lactobacillaceae. Friedman & Alm (1962) however showed that small quantities of pyruvate were also produced when low concentrations of glucose (0.2%) were used.

The studies of Trivett & Meyer (1971) indicate that some enzymes of the citric acid cycle are present in L. monocytogenes. On the basis of their results, these authors suggested that the organism utilizes a split noncyclic citrate pathway which is probably important in biosynthesis but plays no part in energy production.

Seeliger (1961) and Seeliger & Welshimer (1974) reported L. monocytogenes as invariably catalase positive, but Jones (1975b) and Jones et al. (1979) observed that the catalase activity of L. monocytogenes was very weak or negative when the bacterium was grown on media low in yeast extract. There have also been reports that the catalase activity of L. monocytogenes is depressed in media containing higher concentrations (1%) of glucose (Friedman & Alm, 1962) but Jones (1975b) could not repeat this observation. Friedman & Alm (1962) reported that the catalase appears to be of the haem containing type because it is inhibited by potassium cyanide and sodium azide.

Different results have been obtained in attempts to investigate the cytochrome content of L. monocytogenes. Although they were unable to demonstrate cytochromes in L. monocytogenes, Keeler & Gray (1960) could detect iron in fragments of cell walls contaminated with membranes. This led them to believe that low levels of cytochrome were present. However, Trivett & Meyer (1971) on the basis of spectrophotometer readings of dense whole cell suspensions and cell extracts, concluded that no cytochromes were present but added a note to their paper that J.H. Jackson (Thesis, University of Kansas, 1969) had demonstrated the presence of cytochromes in some strains of L. monocytogenes after the bacteria had been stored for several days in the presence of a reducing agent.

Jones (1975b) and Jones et al. (1979) found that the cytochrome content of L. monocytogenes depended on the iron content of the medium on which the

bacterium was grown. Negative or just detectable results were obtained on unsupplemented medium and definite but low amounts of cytochromes were detected on iron supplemented medium. These authors were able to detect cytochromes a, b and b_1 (Jones, 1975b; Jones et al., 1979).

Listeria monocytogenes serovar 5

Listeria monocytogenes serovar 5 was first described by Ivanov (1957) after he had isolated the bacterium from aborted lambs in Bulgaria. Ivanov (1962) further reported on the cultural and biochemical characteristics of 20 strains of this serovar. More recently, further strains similar to L. monocytogenes serovar 5 have been isolated in Holland, New Zealand and Australia (Dennis, 1975; Donker-Voet, 1972; Hunter, 1973).

This serovar of L. monocytogenes differs from the other serovars of the species in a number of properties. It has more fastidious growth requirements, a low lethal activity but high abortifacient activity for sheep (Cooper et al., 1973; Ivanov, 1957, 1962). The main difference from other serovars of L. monocytogenes is the marked degree of haemolysis which results in an unusually large haemolytic zone, with a distinctive pattern of multiple zone of haemolysis on bovine, sheep and human blood agar plates (Cooper & Dennis, 1978; Cooper et al., 1973; Hunter, 1973), but not on horse blood (Hunter, 1973). Cooper et al. (1973) noted that serovar 5 strains produced double, large zones of haemolysis, with the inner complete (i.e., beta) zone 3-4 mm and the outer incomplete (alpha) zone 6-8 mm in diameter at 37°C. At 25°C the double zone was not produced nor was complete haemolysis as pronounced as at 37°C. Cooper et al. (1973) also reported that the degree of haemolysis was affected by the oxygen tension. A narrow zone of haemolysis was observed when cultures were incubated in 10% CO_2 compared with that observed in the normal atmosphere. Bovine blood was recommended by Hunter (1973) because sheep blood may possess antibodies to Listeria spp.

Unlike other serovars of L. monocytogenes which show a CAMP-like reaction, L. monocytogenes serovar 5 is completely CAMP-negative (Brzin, 1979).

It even slightly inhibits the production of staphylococcal beta toxin and conversely staphylococci inhibit haemolysis in serovar 5 strains. Ivanov (1975) noted that cultures of L. monocytogenes serovar 5 intensify the haemolysis of "Corynebacterium equi" when grown near it on 5% sheep blood agar and Brzin (1979) proposed that this should be called "Ivanov's Test". Brzin (1979) obtained similar results to those obtained with Staphylococcus aureus in the CAMP-test (Christie et al., 1944) on substituting Staph. aureus with L. monocytogenes serovar 5. However, with streptococci of serological Group B, the so-called half-moon zone of haemolysis was narrower because of the narrow zone of listerial haemolysis. All bacteria which were CAMP-positive with Staph. aureus were also positive with L. monocytogenes serovar 5 (Brzin, 1979).

Different workers have observed differences between the carbohydrate fermentation of serovar 5 strains and other serovars of L. monocytogenes (Cooper et al., 1973, 1978; Donker-Voet, 1972; Hunter, 1973). Although their results disagree with regard to certain sugar fermentations, all the workers agree that fewer carbohydrates are fermented by serovar 5 strains. Ivanov (1975) described 36 strains which were very similar to L. monocytogenes serovar 5 except that they were non-motile. On this basis he suggested that this group be classified as a subtype of L. monocytogenes serovar 5. He designated the motile strains as subserovar 5a and the non-motile strains as subserovar 5b. Ivanov (1975) further suggested that serovar 5 strains (subserovars a and b) be assigned to a separate species for which he proposed the name "Listeria bulgarica".

Later work in Ivanov's laboratory using disc electrophoresis in polyacrylamide gels showed that the electrographs of the protein spectra of subserovars 5a and 5b were almost indistinguishable (Ivanov & Massalski, 1979).

Julák & Mára (1973) compared the fatty acid composition of one strain of serovar 5 with other L. monocytogenes serovars cultured in glucose medium and observed much lower ratios of branched to saturated fatty acids and anteiso- to iso-branched acids in the serovar 5 strains.

Non-haemolytic *Listeria monocytogenes* strains

Many non-haemolytic strains of *L. monocytogenes* have been isolated from vegetation, soil, human faeces and many other sources. These strains are very similar in all phenotypic aspects to *L. monocytogenes* except that they are non-haemolytic on sheep and horse blood, lack lipase activity on egg-yolk agar, are not pathogenic to experimental animals, do not provoke a mononucleosis and possess extra and/or different somatic antigenic factors (Bojsen-Møller, 1967; Donker-Voet, 1972; Ralovich *et al.*, 1972 ; Weis & Seeliger, 1975; Welshimer, 1968, 1975; Welshimer & Donker-Voet, 1971).

Groves & Welshimer (1979) observed a correlation between xylose-positive, rhamnose-negative, CAMP-negative isolants and their non-pathogenicity. However, Kampelmacher & van Noorle-Jansen (1979) could not distinguish haemolytic and non-haemolytic strains on the basis of acid production on twenty sugars including rhamnose and xylose.

The taxonomic position of these strains in not clear and opinions differ on their relationship to *L. monocytogenes* (Bojsen-Møller, 1967; Welshimer, 1968, 1975; Welshimer & Donker-Voet, 1971).

Wilkinson (1973) examined three strains of non-haemolytic *L. monocytogenes*, two of which were isolated from cultures of haemolytic strains of this species, but whether they were stable mutants or contaminants is not known. The other strain was received as a *Listeria* sp. (Murray 50) isolated from cow brain. These three strains formed a separate cluster showing a similar numerical taxonomic relationship to *L. monocytogenes* as that shown by the *L. grayi*-*L. murrayi* taxon (Wilkinson, 1973; Wilkinson & Jones, 1977).

Other non-haemolytic strains have been noted by Weis (1975) and Weis & Seeliger (1975). At that time these workers did not make any recommendations about the taxonomic position of these strains. However, in his opening speech at the 6th International Symposium of Problems of Listeriosis (1975), Seeliger pointed out that since the non-haemolytic

strains of L. monocytogenes did not cause monocytosis in either experimental animals or humans, the specific name monocytogenes was inappropriate for them. Subsequently, Seeliger & Schoofs (1979) proposed a new species name for these strains: Listeria innocua. This species, as described by Seeliger & Schoofs (1979), would comprise all strains currently assigned to L. monocytogenes serovars 4f and 4g (or serovar 6) and other non-haemolytic strains closely related to these serological types by partially common somatic antigens. Seeliger and co-workers (Patocka et al., 1979) have, however, since demonstrated that the type strain of L. innocua which they previously considered non-pathogenic, induces encephalitis in suckling mice. The proposal for the new species L. innocua therefore seems rather premature, and there is need for more work on these non-haemolytic strains.

Listeria grayi and Listeria murrayi

Errebo-Larsen & Seeliger (1966) proposed that a bacterium isolated from chinchilla faeces, which differed from L. monocytogenes mainly on the basis of its fermentation of mannitol and serologically, be assigned to a new species, Listeria grayi.

Later, strains differing from L. grayi mainly in their ability to reduce nitrate were isolated from vegetation by Welshimer & Meredith (1971). These strains had very similar carbohydrate reactions to L. grayi except that most of them acidified rhamnose whereas L. grayi strains did not, and there was also a slight delay in their acidification of D-galactose, up to 72 hours (Welshimer & Meredith, 1971). On this basis Welshimer & Meredith assigned these strains to a new species, Listeria murrayi.

All the present evidence from numerical taxonomic (Jones, 1975a,b; Stuart & Pease, 1972; Stuart & Welshimer, 1974; Wilkinson, 1973; Wilkinson & Jones, 1977); serological (Errebo-Larsen & Seeliger, 1966; Welshimer & Meredith, 1971; Wilkinson & Jones, 1975); DNA base ratio (Stuart & Welshimer, 1973, 1974) and DNA base pairing studies (Stuart & Welshimer, 1973, 1974) show a reasonably close taxonomic relationship between L. monocytogenes, L. grayi and L. murrayi. Deoxyribonucleic acid from all these species

contains 38-40 moles percent guanine plus cytosine (% G+C). Numerical taxonomic studies indicate a relationship of greater than 75%S(percent similarity) between them (Jones, 1975a,b; Stuart & Pease, 1972; Stuart & Welshimer, 1974; Wilkinson, 1973; Wilkinson & Jones, 1977). In addition some serological relationship between either or both the species L. grayi and L. murrayi, and the species L. monocytogenes, has been reported (Errebo-Larsen & Seeliger, 1966; Welshimer & Meredith, 1971). However, Wilkinson & Jones (1975) detected no common somatic antigens between L. monocytogenes and L. grayi and L. murrayi.

Wilkinson (1973) in a numerical taxonomic study, found that strains of L. grayi and L. murrayi were very similar in many characters and formed a tight cluster at 90%S. Her serological results supported this conclusion and she was therefore of the opinion that the two species did not differ sufficiently from each other to warrant separate species status. Since L. grayi was named before L. murrayi, Wilkinson (1973) suggested that strains presently designated L. grayi and L. murrayi should all be included in one species, L. grayi. This opinion is supported by the gel electrophoresis study of Ivanov & Massalski (1979). In contrast, Stuart & Welshimer (1973, 1974) mainly on the basis of their DNA studies, maintained that the taxa L. grayi and L. murrayi are sufficiently distinct from L. monocytogenes to warrant separate genus status. For this genus they proposed the name "Murraya". Within the genus "Murraya" two taxa were recognized, "Murraya gravi subsp. grayi", and "Murraya grayi subsp.murrayi" (Stuart & Welshimer, 1973, 1974).

Listeria denitrificans

Listeria denitrificans, first isolated by Sohier et al. (1948) was assigned to the genus Listeria by Prévot (1961) because it is a Gram-positive, catalase-positive, non-sporing, rod-shaped bacterium. However, because it differed from L. monocytogenes by being non-pathogenic, non-haemolytic and by the ability to reduce nitrate to nitrite, it was assigned to a new species

Listeria denitrificans (Prévot, 1961). Chatelain & Second (1966) and Wilkinson (1973) reported that L. denitrificans was cellulolytic. However, Jones (1975a) in her coryneform study did not find this to be so. From other evidence, we know that cellulose digestion by members of the genus Cellulomonas requires a fairly rich medium. Jones (1975a) employed a relatively poor medium.

Listeria denitrificans also differs from the other listeriae in numerous taxonomically important reactions such as production of acid from various sugars (Stuart & Welshimer, 1973), oxygen requirements and dye sensitivity (Kramer & Jones, 1969), protein pattern (Ivanov & Massalski, 1979) and menaquinone content (Collins et al., 1979).

STREPTOCOCCUS

The term "streptococcos" was first used by Billroth (1874) to describe chain-forming, coccoid shaped bacteria which he noted in wounds and discharges from the animal body. He also noted the presence of 'streptococcos' in about one half of the cases of erysipelas which he examined. The terms "streptococcos' and 'streptococcus' were subsequently used by various authors to designate a particular kind of cell congregation and were not used in the generic sense. The generic name Streptococcus was first used by Rosenbach (1884) to describe a coccus, growing in chains, which he had isolated from suppurative lesions in man. To this organism he gave the name Streptococcus pyogenes. Rosenbach (1884) was of the opinion that Streptococcus pyogenes was different from the organism isolated from cases of erysipelas (designated "Streptococcus erysipelatos") mainly on the basis of the appearance of the two organisms in gelatin and agar cultures, but subsequent workers did not find these criteria, or additional ones, adequate for distinguishing between the two forms.

In the next twenty years the association between streptococci and a large number of diseases in man and animals was established, as was the importance of these organisms in the dairy industry. As a result, numerous bacteriological investigations were carried out in an attempt to classify

and identify the various forms. These early classifications were based on pathogenicity, cultural appearances on gelatin and agar, cellular morphology, reactions on blood and in milk culture and growth temperature. The early literature is well documented by Sherman (1937) and Wilson & Miles (1975).

Streptococcus faecalis and taxa considered to be related.

The first definitive classification of streptococci was that of Andrewes & Horder (1906). Streptococcus faecalis was the name applied by them to the most common streptococcus isolated from human faeces. Andrewes & Horder (1906) noted the active fermentation of many carbohydrates by Str. faecalis. These streptococci were later shown by Dible (1921) to be almost certainly the same as those studied by Thiercelin (1899) and called by him "enterococcus". The work of Andrewes & Horder was verified by a number of subsequent workers on intestinal streptococci (Broadhurst, 1915; Fuller & Armstrong, 1913; Winslow & Palmer, 1910; and others).

In later classifications the circumscription of Str. faecalis became less distinct, as bacteriologists came to depend more on a smaller number of tests, and attention to some of the important physiological reactions was largely discontinued. In one such classification (Gordon, 1922), Str. faecalis or the "enterococcus", was identified merely as a non-haemolytic streptococcus which fermented mannitol but not raffinose.

Orla-Jensen (1919) in his classical investigations on the lactic acid bacteria, described two mannitol-fermenting, heat-resistant diplococci of intestinal origin which he named Streptococcus faecium and "Streptococcus glycerinaceus", without relating either to Str. faecalis or to the "enterococcus". In contrast to the taxon he called "Str. glycerinaceus", Orla-Jensen (1919) noted that Str. faecium fermented arabinose and seldom fermented glycerol or sorbitol. In addition, Orla-Jensen (1919) described the occurrence of streptococci from bovine faeces that possessed unique physiological characteristics which afforded their differentiation from other faecal streptococci. For these strains, Orla-Jensen (1919) proposed the name Streptococcus bovis.

Sherman (1937, 1938) endeavoured to relate Orla-Jensen's "Str. glycerinaceus" and Str. faecium to Andrewes & Horder's Str. faecalis. He concluded that there were no grounds for distinguishing between "Str. glycerinaceus" and Str. faecium and since"Str. glycerinaceus" (Orla-Jensen) was identical with Str. faecalis (Andrewes & Horder) all should be included in the species Str. faecalis (Andrewes & Horder). In a review of the streptococci, Sherman (1937) divided these bacteria into four physiological groups which he designated the "pyogenic", "lactic", "viridans" and "enterococcus" groups. The "enterococcus" group included Str. faecalis and its varieities, and "Str. durans". The "viridans" group included, amongst others, Str. bovis and Str. equinus.

The term "enterococcus" was chosen because it was used by Thiercelin, (1899) to describe some streptococci of intestinal origin. The "enterococcus group" as described by Sherman (1937, 1938) comprised Str. faecalis and its varieties, "Str. faecalis var. liquefaciens" and "Str. faecalis var. zymogenes", and "Str. durans" (Sherman & Wing, 1937). The last species being a haemolytic member of the genus Streptococcus, first isolated from dried milk but also found in human faeces (Smith & Sherman, 1938). The "enterococcus" group was characterized by growth at both 10 and 45°C; ability to grow in the presence of 6.5% NaCl; initiation of growth at pH 9.6; growth in relatively strong solutions of methylene blue (0.1 per cent in skimmed milk); withstand heating at 60°C for 30 min and growth on 40% bile.

In 1943 Gunsalus & Sherman observed that all enterococci tested fermented glycerol aerobically. However, two types of enterococci could be discerned by the ability to ferment this polyol under anaerobic conditions. Later, Gunsalus (1947) reported that those strains fermenting glycerol anaerobically required an exogenous hydrogen acceptor which occurred naturally in yeast extract. This hydrogen acceptor could be replaced by fumarate. In view of the differential activity with glycerol under anaerobic conditions, Gunsalus suggested a reconsideration of the speciation along the lines proposed by Orla-Jensen. Orla-Jensen (1943)

had recognized that Str. faecalis (Andrewes & Horder) was identical with his "Str. glycerinaceus". However, he maintained the distinction of Str. faecium and extended the description of this species by noting that Str. faecium fermented melibiose but not melezitose nor inositol. In addition, he reported that Str. faecium grew characteristically at 50°C (Orla-Jensen, 1943).

Skadhauge (1950) noted the ability of strains of Str. faecalis and its varieties to grow on a medium containing potassium tellurite (1/2500) and to reduce this substance to the black coloured tellurium. Strains of "Str. durans" and Str. faecium did not grow in a medium containing this concentration of potassium tellurite. Further distinctions between Str. faecalis and its varieties and "Str. durans" and Str. faecium were noted by Barnes (1956) (tetrazolium reduction) and Deibel et al. (1963) (utilization of citrate and gluconate and anaerobic fermentation of glycerol). The latter authors noted that "Str. durans" was similar physiologically to Str. faecium, and suggested that "Str. durans" was but a varietal form of Str. faecium. The serological studies of Lancefield (1933, 1934); Shattock (1949), Sharpe & Shattock (1952) and Shattock (1955) indicated that Str. faecalis and its varieties together with Str. faecium and "Str. durans" belonged to Lancefield's serological Group D. During this time it was also noted that other streptococci notably Str. bovis and Str. equinus (Andrewes & Horder) which Sherman (1937) had placed in his "viridans" group also contained the Group D antigen.

Later, taxa such as "Streptoooccus avium" isolated from chickens and other sources (Nowlan & Deibel, 1967); "Streptococcus faecium var. casseliflavus" isolated from vegetation (Mundt, 1963a,1963b);"Str. faecalis var. malodoratus" isolated from cheese (Pette, 1955) and some others, were shown to contain the Group D antigen. Many, but not all, of these and the taxa listed previously were referred to as faecal streptococci although not all were isolated from faeces. Thus, the terms enterococci, faecal streptococci and streptococci of serological Group D were, and are still used

inter-changeably and by implication synonymously. Streptococcus faecalis, Streptococcus faecium, "Streptococcus avium", Streptococcus bovis and Streptococcus equinus are found as part of the normal gut flora of animals and man. However, on physiological and biochemical grounds, Str. faecalis and its varieties,"Str. durans", Str. faecium and "Str. avium" are quite distinct from Str. bovis and Str. equinus. The first four taxa have characteristics typical of the "enterococcus" division of Sherman (1937), although of course Sherman (1937) did not recognize Str. faecium (Orla-Jensen) nor "Str. avium" (Nowlan & Deibel, 1967) as separate species.

In view of the confusion caused by the use of terms such as faecal streptococci to refer to two distinct physiological groups, and the further confusion caused by the fact that the taxon "Str. faecalis var. casseliflavus" has never been reported to be present as a gut organism but occurs on vegetation, the use of the term faecal streptococci should be dropped except when it is necessary to use the term in ecological studies of gut flora.

Streptococcus faecalis.

The name Streptococcus faecalis was given by Andrewes & Horder (1906) to a group of streptococci characteristic of the human intestine. They described it as a Gram-positive coccus occurring as short-chains; non-haemolytic, growing well on gelatin at 20°C, forming H_2S and being chemically very active. They regarded the fermentation of mannitol as particularly characteristic.

Subsequent studies on Str. faecalis (Abrams, 1958; Armstrong et al., 1959; Barnes et al.,1961; Bibb & Straughn, 1962; Cummins & Harris, 1956; Hartsell & Caldwell, 1961; Ikawa, 1961; Jones & Shattock, 1960; Orla-Jensen, 1919; Salton, 1958; Shattock, 1955; Sherman, 1937, 1938; Sherman et al.,1937; Shockman et al., 1961a,b; Shockman et al., 1963; Smith et al., 1961, 1962; Tsung et al., 1962) have resulted in the description of the species which appears in the current edition of Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974). As described by

Andrewes & Horder (1906), Str. faecalis was one species with no named varieties or subspecies. As a result of the studies of Sherman et al. (1937) four varieties were recognized; "Str. faecalis var. faecalis" (non-haemolytic and non-proteolytic), "Str. faecalis var. haemolyticus" (haemolytic and non-proteolytic), "Str. faecalis var. liquefaciens" (non-haemolytic but proteolytic) and "Str. faecalis var. zymogenes" (haemolytic and proteolytic). The variety "Str. faecalis var. haemolyticus" was never generally accepted but the other three varieties were recognized and referred to in the literature either as varieties of Str. faecalis or sometimes as separate species (i.e. Str. faecalis, "Str. liquefaciens", "Str. zymogenes"). However, several workers over the years cast doubt on the distinction between the various varieties of Str. faecalis (e.g. Deibel, 1964; Jones et al., 1972; Jones, 1978).

Currently, the species Str. faecalis is considered to comprise ovoids cells, 0.5-1.0 µm in diameter, occurs mostly in pairs or short chains, generally nonmotile. The peptide subunit of the peptidoglycan consists of L-alanine, D-glutamine, L-lysine and D-alanine. A bridge tripeptide of L-analine joining peptide subunits through L-lysine and D-alanine was described by Kandler et al. (1968) who obtained an excellent correlation between peptidoglycan structure and physiological characteristics.

Group D antigen is present in Str. faecalis and glycerol teichoic acid is the group specific antigenic determinant (Elliott, 1962), in which the glycerol phosphate contains glucose in glucosidic linkage to glycerol,and D-alanine is esterified to hydroxyl groups of glucose (Wicken & Baddiley, 1963, Wicken et al., 1963).

Lactic acid is the primary fermentation product of glucose in this organism; however, if neutrality of culture is maintained, greater amounts of formate, acetate and ethanol are formed (Gunsalus & Niven, 1942). Gluconate fermentation involves a mixed pathway (Goddard & Sokatch, 1964). Aerobically, glycerol is phosphorylated and oxidized to lactate with oxygen serving as hydrogen acceptor. Anaerobically, a pyridine-linked glycerol

dehydrogenase couples with fumarate as hydrogen acceptor (Jacobs & VanDemark, 1960). Areobic incubation alters the end-products of carbohydrate metabolism (London & Appleman, 1962).

Streptococcus faecalis utilizes pyruvate as an energy source by fermentation via the phosphoroclastic and dismutation pathways. Citrate, serine and malate are also fermented and the energy-yielding processes are linked apparently to pyruvate metabolism as lipoate is required for the fermentation of these energy sources (Deibel, 1964; Deibel & Niven, 1964). Arginine (and often agmatine) is utilized as an energy source but the mechanism has not been elucidated. Similar or identical hydrolytic reactions are effected by Str. faecium, but the energy released is not available for growth (Deibel, 1964).

Rarely, strains of Str. faecalis possess catalase activity maintained and enhanced by aerobic serial transfer and cation fortification of the medium (Jones et al., 1964). The catalase activity is not associated with iron-porphyrin compounds. Some strains, when grown aerobically, possess a potent, adaptive peroxidase which precludes demonstrable peroxide formation in the culture (Seeley & VanDemark, 1951).

In glucose broth final pH values of 4.4 to 4.0 are obtained by Str. faecalis. Most strains produce acid from glucose, sucrose, mannose, fructose, galactose, maltose, cellobiose, trehalose, lactose, melezitoze, sorbitol, glycerol and mannitol (polyol fermentation is enhanced by fumarate addition). Generally, arabinose, inulin, melibiose and raffinose are not fermented.

The organism characteristically grows in the presence of 0.04% tellurite reducing it to tellurium (Skadhauge, 1950). Growth occurs at pH 10-10.5 in carbonate-buffered media; the use of glycine-buffered media for this tolerance test is not recommended (Chesbro & Evans, 1959). The heat-tolerance test (60°C for 30 min) is markedly affected by the pH value of the menstruum (White, 1963). The strains grow at 47°C but not at 50°C.

Most strains decarboxylate tyrosine to tyramine plus carbon dioxide. Gutalin is not hydrolysed. Streptococcus faecalis grows in the presence of 0.1% thallous acetate, or 0.02% sodium azide or 0.5-1.0 units penicillin/ml. A gamma-reaction is observed on blood agar (Deibel et al., 1963). However, some strains called "Str. faecalis var. zymogenes" produce beta haemolysis. Some strains produce hyaluronidase (Rosan & Williams, 1966).

Variations occur depending upon the strain but generally 7 to 13 amino acids and five of the B vitamins are necessary for growth in synthetic media. Folic acid is not required for the growth of Str. faecalis (Deibel et al., 1963). Lipoate is required when the energy source consists of pyruvate, serine, citrate or malate (Deibel, 1964).

The most common source of Str. faecalis is faeces of humans and warm-blooded animals. They are found occasionally in urinary tract infections and subacute endocarditis. Str. faecalis has been shown to be common in many food products, often unrelated to direct faecal contamination (Deibel, 1964). Relationship of the organisms with food poisoning is questionable (Deibel & Silliker, 1963). Streptococcus faecalis occurs frequently in plants where an epiphytic relationship exists (Mundt et al., 1962).

The mol % G+C content of DNA ranges from 33.5 (chemical analysis) to 38 (buoyant density).

"Streptococcus faecalis var. zymogenes".

MacCollum & Hastings (1899) described under the name of "Micrococcus zymogenes" an organism obtained from a case of acute endocarditis. They noted the characteristic grouping in pairs, less frequently in short chains, and stated that the organism showed points of resemblance to Streptococcus pyogenes and the pneumococci on the one hand, and to the pyogenic staphylococci on the other. These workers indicated that their organism was probably a common intestinal form, as evidenced by the fact that it had also

been isolated from sewage by their associate, Dr. Norman MacLead Harris. Shortly after the work of MacCollum & Hastings, workers from other laboratories reported the same organism in specimens from human autopsies, and since that time it has been isolated intermittently from clinical and faecal sources (Sherman *et al.*, 1937). Although given the generic name *Micrococcus* by MacCollum & Hastings, this organism was early recognized as belonging to the genus *Streptococcus* (Winslow & Winslow, 1908).

Although it cannot be certain that all cultures that have been called "*Streptococcus zymogenes*" by subsequent investigators have in fact been the organism of MacCollum & Hastings, there would appear to be little room for error in its identification. MacCollum & Hastings's description is an extraordinary, beautiful example of the accuracy with which the characteristics of bacteria were sometimes portrayed by the earlier bacteriologists, whose working tools were so limited in comparison with the methods of today. The liquefaction of gelatin and the proteolysis of casein were noted; attention was called to the strong reducing action of the organism in litmus milk, the especially significant point being observed that complete reduction of the litmus took place before acidulation and curdling; it was noted that the organism grew more profusely on glycerol agar than on ordinary nutrient agar, plainly indicating that glycerol was utilized.

The haemolysis of blood as a test in the study of the streptococci was of course not in use at that time. Within the limits of present knowledge of the streptococci, the only adequately described organisms which fulfil entirely the combination of characteristics given by MacCollum & Hastings are the haemolytic "*Str. zymogenes*" and the non-haemolytic types which have been frequently designated as "*Str. liquefaciens*" and *Str. faecalis* (Sherman, 1937). With the exception of the ability to haemolyse blood, Sherman *et al.* (1937) recognized that "*Str. zymogenes*" was very similar to *Str. faecalis* and proposed that haemolytic strains be designated "*Str. faecalis* var. *zymogene*

Other than the ability of "*Str. faecalis* var. *zymogenes*" to haemolyse

blood, its characters are identical with Str. faecalis. However, Deibel (1964) and Jones et al. (1972) have cast doubt upon the validity of the subspecies. In their opinion all such strains should be included in the species Streptococcus faecalis.

It has been known for many years that the ability to haemolyse blood is not a stable character. Jacob et al. (1975) have shown that the haemolytic ability of "Streptococcus faecalis var. zymogenes" is plasmid borne and can be lost easily.

"Streptococcus faecalis var. zymogenes" is the most characteristic haemolytic Streptococcus of the normal human intestine. It has little or no virulence for laboratory animals, and in spite of its clinical history as an occasional invader of the human body, "Str. faecalis var. zymogenes", in common with other enterococci, is to be considered as essentially non-pathogenic.

"Streptococcus faecalis var. liquefaciens"

The occurrence of streptococci which liquefy gelatin has been known since the early days of bacteriology, and the older literature records the names of many streptococci which were supposed to have this property. One of the first such types described was "Streptococcus coli-gracilis" (Escherich). However, it is impossible now to identify any of these organisms from their recorded descriptions.

The name "Streptococcus liquefaciens" was first used by Sternberg (1892) for a streptococcus which cannot now be recognized from his description. Orla-Jensen (1919) revived the name and applied it to a group of proteolytic streptococci which he studied. It is of interest to note that Orla-Jensen established this species around a type culture which von Freudenreich (1894) had described many years before under the name of "Micrococcus casei-amari".

"Streptococcus liquefaciens" was later recognized as a variety of Streptococcus faecalis which exhibited proteolytic activity and differed

from the organism called "Streptococcus zymogenes", by its inability to haemolyse blood (Sherman et al., 1937). There was, therefore, only a slender basis for considering "Str. liquefaciens" as a species independent of its closely related forms, "Str. faecalis var. faecalis" and "Str. faecalis var. zymogenes", because in most phenetic characters all three are very close. Consequently, some workers have long looked upon such gelatin liquefying streptococci simply as proteolytic varieties of Str. faecalis, while others have considered them to be non-haemolytic strains of "Str. zymogenes".

There is little or no evidence on whether or not "Str. liquefaciens". like Str. faecalis, may occasionally be the cause of human infections because non-haemolytic streptococci from such sources are not routinely listed for gelatin liquefaction. However, Elser & Thomas (1936) found that gelatin liquefying streptococci recovered in pure culture from blood in cases of subacute endocarditis were characteristically non-haemolytic.

The primary source of "Streptococcus liquefaciens" is probably the intestines of man and other animals. It is widely distributed and, like Str. faecalis, its hardy nature equips it for growth under diverse conditions. It is frequently found in dairy and other food products in which it is able to grow vigorously. The organism has been isolated from plants and some of the strains obtained from this source are much more actively proteolytic than those obtained from stools and milk. In view of the apparent variability in haemolytic and proteolytic properties of organisms belonging to this taxon, Sherman et al. (1937) suggested that "Str. liquefaciens" like "Str. zymogenes" should be considered simply as a variety of Str. faecalis. However, it is now generally accepted that neither "Str. faecalis var. liquefaciens" nor "Str. faecalis var. zymogenes" are sufficiently distinct from "Str. faecalis var. faecalis" to warrant even varietal or subspecies status. Most workers agree that all should be included in the species Str. faecalis (Deibel, 1964, Jones et al.,1972, Jones, 1978).

"Streptococcus faecalis var. malodoratus"

The species "Str. faecalis var. malodoratus" was named by Pette (1955) for an organism isolated from Gouda cheese which resembled Str. faecalis. A taxonomic study by Jones et al. (1972) which included two strains of "Str. faecalis var. malodoratus" did not resolve their taxonomic relationships. They were more closely related to Str. faecium than to Str. faecalis, but were not related closely enough to Str. faecium to be included in that species (Jones, 1978). Production of H_2S gas is the characteristic property of this group of organisms. In view of the report in the literature that H_2S gas production by strains of Escherichia coli (Magalhães & Veras, 1977; Ørskov & Ørskov, 1973) and Shigella sonnei (Farmer et al., 1976) is a plasmid encoded property, there is the possibility that H_2S production in "Str. faecalis var. malodoratus" is also determined by a plasmid.

Streptococcus faecium

Orla-Jensen in 1919 described two mannitol fermenting, heat-resistant diplococci of intestinal origin, which he named Streptococcus faecium and "Streptococcus glycerinaceus" without relating either to Streptococcus faecalis as described by Andrewes & Horder (1906). He noted that Streptococcus faecium fermented arabinose but seldom fermented glycerol or sorbitol, in contrast to the organism he called "Str. glycerinaceus". As noted earlier, "Str. glycerinaceus" (Orla-Jensen) has been shown to be synonymous with Str. faecalis (see page 16).

Streptococcus faecium grows at 10 and 45°C, survives heat treatment at 60°C for 30 min, growth takes place in media having an initial pH value of 9.6 and in the presence of 6.5% NaCl. The organism differs from Str. faecalis mainly in its weak reduction of litmus milk; non-fermentation of sorbitol and fermentation of arabinose. Orla-Jensen (1943) extended the description of Str. faecium by noting its ability to ferment melibiose and its inability to ferment melezitose and inositol. He also noted that strains of

Str. faecium grew characteristically at 50°C. Later, Skadhauge (1950) reported that other characters which distinguish Str. faecium from Str. faecalis are its inability to grow on a nutrient medium containing potassium tellurite (1/2500) and Barnes (1956) noted its inability to reduce tetrazolium salts. Both of these features distinguish Str. faecium from Str. faecalis. Deibel et al (1963) noted that the growth of Str. faecium in a semi-synthetic medium required supplementation with folic acid.

However, like Str. faecalis, Str. faecium fulfils all the criteria of Sherman's "enterococcus" division (resistance to heat at 60°C for 30 min, initiation of growth at pH 9.6, tolerance of methylene blue, ability to grow at 10 and 45°C and tolerance of 6.5% NaCl),and was placed in the "enterococcus" group of Sherman (1937) although Sherman (1937 and 1938) did not consider Str. faecium sufficiently distinct from Str. faecalis to warrant a separate species (see page 16).

Str. faecium reacts with Group D antisera and is thus, like the original members of the "enterococcus" group (Sherman, 1937), designated serological Group D (Sharpe & Shattock, 1952; Sherman, 1938; Skadhauge, 1950; Smith et al.,1938).

"Streptococcus durans"

The bacterium known as "Streptococcus durans" was first isolated from a baby food which contained powdered milk and the bacterium was at first considered to be Streptococcus pyogenes, or - because of its haemolytic activity - a pyogenic streptococcus of probable clinical importance (Sherman & Wing, 1937). Although cursory studies of this streptococcus showed that it was physiologically clearly different from the 'pyogenic' group, and had no virulence for laboratory animals, the confusion caused by its occasional isolation from dairy products led Sherman & Wing (1935, 1937) to study these organisms in detail. As a result of this study they considered that these bacteria comprised a new species for which they proposed the name "Streptococcus durans" because of its marked tolerance to heat and desiccation.

On the basis of its physiological characteristics, "Str. durans" was placed by these workers in the "enterococcus" group. Subsequent studies of the haemolytic streptococci of the human intestine (Smith & Sherman, 1938) showed that "Str. durans" commonly occurred in human faeces.

Although "Streptococcus durans" showed all the characteristics of the "enterococcus" group of Sherman (1937) and contained the Group D antigen, Sherman (1937 and 1938)and Smith & Sherman (1938) were of the opinion that "Str. durans" was distinct from Str. faecalis. However, these workers did not study the possible relationship between "Str. durans" and Str. faecium because they had concluded that Str. faecium was synonymous with Str. faecalis (see page 16).

More recently the distinction between "Str. durans" and Str. faecium has been called into question. Many workers now consider that "Str. durans" strains are less vigorous forms of Str. faecium and in addition some "Str. durans" strains are haemolytic variants (Collins & Jones, 1979; Deibel,1964; Deibel & Seeley, 1974; Jones et al.,1972; Jones, 1978). The name is not included in the Approved Lists of Bacterial Names (Skerman et al., 1980).

"Streptococcus faecium var. casseliflavus"

Mundt & Graham (1968) proposed the name "Streptococcus faecium var. casseliflavus" for some yellow-pigmented streptococci isolated from plants by Mundt et al. (1967). These bacteria showed some morphological and biochemical similarities to both Str. faecalis and Str. faecium, and also reacted with Group D antiserum. Mundt & Graham (1968) considered them to be more phenotypically similar to Str. faecium than to Str. faecalis but sufficiently different from Str. faecium to warrant at least subspecies rank.

Graudal (1952) had encountered yellow-pigmented strains among his collection of motile streptococci, but he did not specify the sources of these strains. Although none of his strains came from plant material, it is not known whether the yellow-pigmented strains originated from human or animal sources or both.

According to the work of Mundt & Graham (1968), cultures of "Streptococcus faecium var. casseliflavus" with few exceptions, grow readily on 5% bile salt-agar; in broth at 10°C; in broth adjusted to pH 9.6 or containing 6.5% NaCl. These strains reduce litmus rapidly prior to formation of an acid curd. Nearly all cultures survive heating in broth for 30 min at 60°C. Most of the strains grow at 45°C. Graudal (1952) reported that all of his strains released ammonia from arginine. However, only 14% of the cultures of Mundt & Graham (1968) did so. However, some of the cultures of Mundt & Graham (1968) released ammonia from serine. Strains of "Str. faecium var. casseliflavus" produce grey colonies on potassium tellurite (1/2500), reduce 2,3,5-triphenyltetrazolium-HCl to a pink colour and ferment cellobiose, dextrin, maltose, mannose, and sorbitol, thus resembling Str. faecalis (Mundt & Graham, 1968). These workers also noted that, like Str. faecium, "Str. faecaium var. casseliflavus" produces peroxidase but not catalase on heated blood media, dissimilates malate, and ferments arabinose, melibiose and salicin, but not melezitose. Like both Str. faecalis and Str. faecium, "Str. faecium var. casseliflavus" ferments glucose, galactose, lactose, mannitol, sucrose, trehalose and citrate, without production of gas. None produces H_2S, or acetylmethylcarbinol, digests casein or gelatin, hydrolyzes ureae, reduces nitrate or decarboxylates arginine, ornithine or tyrosine.

"Streptococcus faecium var. casseliflavus" can be distinguished from Str. faecalis and Str. faecium mainly on the basis of the fermentation of raffinose, a sugar also fermented by Streptococcus bovis but rarely attacked by enterococci (Papavassiliou, 1962) and of course by the production of yellow pigment. It was the production of the pigment which drew the attention of Mundt et al. (1967) to these bacteria. The most commonly occurring yellow bacterial pigments are usually the carotenoids. Mundt & Graham (1968) suggested that the pigments of "Str. faecium var. casseliflavus" were flavanoid in nature, while others (Cosenza & Girrard, 1970, Taylor et al. 1971)

indicated that they were carotenoids.

The colour of the colony of "Str. faecium var. casseliflavus" on solid media is a very pale lemon yellow. Within the cell and upon extraction, the pigment is stable to oxygen and to treatment with 3 and 30% H_2O_2. It differs, therefore, from the pigment noted by Hannay (1950) in Str. faecalis, which developed into a primrose colour and then turned brown upon exposure to oxygen. The absorption of light by the extracted pigment from the cells begins at 365 mμ and is complete at 377 mμ(Mundt & Graham, 1968). Mundt & Graham(1968) also showed that the supernatant of the lysozyme treated cells gave a positive Molisch test. They discounted the possibility that the pigment was a xanthophyll or carotenoid on the basis of the above properties and suggested its possible identity as a glycosidic flavanol or flavanone (Mundt & Graham, 1968).

However, Taylor et al. (1971) confirmed the findings of Cosenza & Girrard (1970) by showing that pigments extracted from three strains of yellow enterococci showed the spectral and solvent partition characteristics of carotenoids, and found that an unusual C_{32} carotenoid aldehyde appeared to predominate in the pigment extract.

Since carotenoid pigments may protect membrane-bound functions from photodynamic damage (Liaaen-Jensen, 1965), it is reasonable to suppose that the possession of pigment confers survival value on those enterococci which are most often found in soil, insects (Cosenza & Girrard, 1970) and as plant epiphytes (Mundt et al., 1967).

Taylor et al. (1971) observed that the yellow pigmentation in the Group D streptococci with which they worked seemed to vary from organism to organism independent of other characteristics, thus rendering pigmentation of questionable value for taxonomic purposes.

Later chemical and nucleic acid studies have suggested that "Str. faecium var. casseliflavus" is distinct from both Str. faecalis and Str. faecium.

Amstein & Hartman (1973) used gas chromatography to compare the relative fatty acid composition of various strains of enterococci, including Str. faecalis, Str. faecium, "Str. faecium var. durans" and three strains of "Str. faecium var. casseliflavus". Based on differences between the fatty acid composition of the strains of "Str. faecium var. casseliflavus" and that of the other strains, these workers concluded that "Str. faecium var. casseliflavus" deserved varietal, if not separate species status.

Roop et al. (1974) and Vaughan et al. (1979) using deoxyribonucleic acid (DNA)-DNA hybridization, showed that an authentic strain of "Str. faecium var. casseliflavus" exhibited little homology with reference strains of Str. faecium, Str. faecalis or Str. lactis. They found that the DNA of the yellow-pigmented strains hybridized with the DNA of Str. faecium and Str. faecalis at a level of less than 25%. On the basis of their studies and the previous work-up of Roop et al. (1974), Vaughan et al. (1979) suggested that "Str. faecium var. casseliflavus" be elevated to the rank of species and be designated "Streptococcus casseliflavus" (Mundt & Graham) comb.nov. However, the species name is not in the Approved Lists of Bacterial Names (Skerman et al., 1980).

"Streptococcus faecium var. mobilis"

Langston et al.(1960) coined the name "Str. faecium var mobilis" for motile strains of enterococci-like bacteria isolated by them from grass silage. Similar streptococci have also been isolated from animal and plant material but most frequently from vegetation and silage (see Graudal, 1957; Hugh, 1959; Lund, 1967). Although the bacteria named "Str. faecium var. mobilis" by Langston et al. (1960) showed some morphological and biochemical similarities to both Str. faecalis and Str. faecium, most workers agree that these strains are more phenotypically similar to Str. faecium than to Str. faecalis (see Graudal, 1957; Langston et al., 1960; Lund, 1967). Graudal (1957) divided his motile strains on the basis of whether or not they produced a yellow pigment. On the basis of esterase and protein patterns of

"Str. faecium var. mobilis" by gel electrophoresis, Lund (1967) was of the opinion that they were distinct from both Str. faecalis and Str. faecium. Collins & Jones (1979) concluded from the menaquinone studies that "Str. faecium var. casseliflavus" and "Str. faecium var. mobilis" probably constitute one taxon but are sufficiently distinct from both Str. faecalis and Str. faecium to be excluded from both these species. However, it is still not generally agreed whether "Str. faecium var. casseliflavus", "Str. faecium var. mobilis" and Str. faecium are all members of the same taxon or whether the former two taxa constitute a group distinct from Str. faecium. It is worth mentioning that the species name is not in the Approved Lists of Bacterial Names (Skerman et al.,1980).

Streptococcus bovis

Streptococcus bovis was described as a new species and named by Orla-Jensen (1919), who isolated it from cow faeces and from milks which had been heated or incubated at high temperatures. Ayers & Mudge (1923) found Str. bovis to be the predominating streptococcal form in the mouths and intestines of cows. Although obtained from a number of sources, this streptococcus is generally recognized as having its habitat in the bovine alimentary tract.

Str. bovis does have some characteristics in common with the enterococci such as the ability to grow at 45°C, and bile tolerance. In addition, a substantial proportion of strains of Str. bovis ferment mannitol, though the majority do not attack this substance.

Sherman & Stark (1931) noted that a particular characteristic of Str. bovis is its ability to hydrolyze starch actively when tested by the starch agar method.

Winslow & Palmer (1910) and Fuller & Armstrong (1913) had observed that the prevailing type of streptococcus in the faeces of cows fermented raffinose, while the predominating forms from the intestines of man and the horse did not exhibit this property. Str. bovis has been isolated from human faeces, and what appear to be simply varieties of this organism, which do not hydrolyze

starch, are commonly found in the human intestine (Sherman, 1937).

In his classification of the streptococci, Sherman (1937) excluded Str. bovis and Str. equinus from his "enterococcus" division because these organisms do not initiate growth at 10°C, grow in media containing 6.5% sodium chloride nor survive heating at 60°C for 30 min. Str. bovis and Str. equinus were grouped in Sherman's "viridans" division. Str. bovis was found to be an active fermenter of arabinose, raffinose, starch and usually inulin (Fuller & Armstrong,1913; Orla-Jensen, 1919; Sherman, 1937; Sherman & Stark, 1931; Winslow & Palmer, 1910). Ayers & Mudge (1923) reported the inability of Str. bovis to produce ammonia from peptone, its weak reducing action and its failure to hydrolyze sodium hippurate. However, all of the enterococci as defined by Sherman (1937), as well as Str. bovis and Str. equinus, possess the Group D antigen (see Jones, 1978). This fact, as touched on earlier, has resulted in the view that Str. bovis is related to Str. faecalis. The two taxa are quite distinct, however, by a number of criteria.

Streptococcus equinus

Andrewes & Horder (1906) described and named Streptococcus equinus. They noted that at the time of their study this streptococcus, characterized by its inability to ferment lactose, formed the predominant streptococcal flora in the streets of London. As horse dung made up a large part of the organic pollution of London air at that time, they suspected this material as the source of the organism. An investigation of fresh horse dung confirmed their suspicion.

Andrewes & Horder (1906) noted that Str. equinus was not pathogenic and non-haemolytic. They, and later workers (see Jones 1978) described the characteristic properties of Str. equinus as follows: milk is not coagulated; there is little or no reducing action on neutral red; growth does not occur at 10°C; sucrose, salicin and coniferin are usually fermented; lactose and mannitol are not fermented; raffinose and inulin are not attacked as a rule,

but a number of variant types which ferment these substances have been noted. The study of Winslow & Palmer (1910) confirmed the findings of Andrewes & Horder (1906) and the former workers also reported the isolation of Str. equinus from the intestines of cows and man.

Although the only constant difference between the species Str. bovis and Str. equinus is the inability of the latter species to produce acid from lactose, Sherman (1937) thought that Str. equinus merited separate species status because "the general pattern" of reactions given by Str. equinus, aside from its inability to ferment lactose, was sufficiently distinct. This view has been upheld by Smith & Shattock (1962), Jones et al.(1972), and Deibel & Seeley (1974). However, Seeley & Dain (1960) were of the opinion that Str. equinus was probably a lactose negative variant of Str. bovis,or Str. bovis a lactose positive variant of Str. equinus.

It has not yet been shown whether the production of acid from lactose by Str. bovis is a stable character. If the property is unstable, then it can be suspected that it is a plasmid coded property. If this could be shown to be the case then Str. bovis and Str. equinus would be merely varieties of one taxon. Like Str. bovis, Str. equinus is a member of serological Group D.

Streptococcus agalactiae

The species Streptococcus aglactiae was described and named by Lehmann & Neumann (1896), for some streptococci isolated from cases of bovine mastitis. Migula (1900) had previously given the name "Streptococcus mastitidis" to what are now recognized as the same streptococci as those described and named Str. agalactiae by Lehmann & Neumann (1896).

In the classification of Sherman (1937) the species Str. agalactiae was allocated to the "pyogenic" group which included the streptococcal species recognized at that time as being pathogenic for man and animals. Lancefield

(1933), as a result of her serological study of the genus Streptococcus, allocated the species Str. agalactiae to serological Group B on the basis of the presence of a particular cell wall carbohydrate antigen.

Brown (1939) reported that the streptococci of serological Group B studied by him could be divided into three biotypes on the basis of the fermentation of lactose and salicin, as follows: 1) acid produced from salicin but not from lactose (these were saprophytic strains from human sources); 2) acid produced from lactose and salicin (this group included strains isolated from human sources and bovine mastitis); 3) acid produced from lactose but not from salicin (this group comprised isolates from bovine mastitis only).

Plummer (1934) noted that the human and bovine strains of Group B streptococci resembled each other in their fermentative characters. He suggested that all were bovine in origin, and were transferred to humans through contact with cows and/or cows' milk. Pomales-Lebran et al. (1947) also concluded that despite minor differences, the taxonomic relationship between Group B streptococci of human and bovine origin was close.

McDonald et al. (1975) injected Group B streptococci of human origin into the mammary glands of cows. As noted earlier by Little (1938), infection of the udder developed and in the opinion of McDonald et al. (1975), was more acute than that produced by bovine strains. McDonald et al.(1975) concluded that human strains were more pathogenic for cows than bovine strains, and further suggest that the strains from the different sources were biochemically different.

In the 1930's little attention was given to streptococci of serological Group B associated with humans, although the bacteria had been isolated from humans on a number of occasions. Lancefield and Hare (1935) isolated Group B streptococci from the female vagina before and after delivery. Colebrook & Purdie (1937) recovered Group B streptococci from the blood of a case of puerperal endocarditis and Fry (1938) isolated Group B streptococci from three fatal human infections.

In the 1940's and 1950's there were few reported cases of human infections caused by Group B streptococci, or reports of human carriers. Indeed, Lancefield & Hare (1935) recognized Group B streptococci as commensals among the normal flora of the human upper respiratory tract and of the female urogenital tract. However, within the last two decades, increasing attention has been given to the role of these organisms in the etiology of early and late onset neonatal infections and other human pathological conditions.

The majority of streptococci of serological Group B are haemolytic but non-haemolytic strains have been isolated from human, animal and fish sources (Wilkinson et al., 1973a). Christie et al. (1944) noted that most Group B strains would lyse sheep or ox blood cells when grown in the proximity of beta-lysin producing Staphylococcus aureus strains under anaerobic conditions (CAMP test).

Str. agalactiae hydrolyses sodium hippurate and produces a low pH in glucose broth. Acid is produced from glucose, maltose, sucrose and trehalose by this organism. It ferments glycerol aerobically. Xylose, arabinose, raffinose, inulin, mannitol and sorbitol are not fermented by Str. agalactiae.

Orla-Jensen (1919) first reported the production of an orange to red pigment by some Group B streptococci in a medium of casein peptone broth with added soluble starch. The production of pigment was also noticed on blood agar when incubated anaerobically (Wilson & Miles, 1975). Pigment production appears to be more frequent among human than bovine strains (Islam, 1977; Jewes, 1980; Merrit & Jacobs, 1976; Mhalu, 1976), and among haemolytic as compared with non-haemolytic strains (Fallon, 1974; Islam, 1977).

The relationship of Str. agalactiae strains from human and bovine sources is still unclear as is the basis of their pathogenicity.

BIOLOGY OF PLASMIDS

The terminology used in this section is based on Novick et al. (1976).

A plasmid may be defined as a replicon that is stably inherited in an extrachromosomal state (Novick et al., 1976). Implicit in this definition is the idea that a plasmid is readily maintained in the cell without specific selection. In prokaryotes, naturally occurring plasmids are generally dispensable, i.e. their loss is not detrimental to the host in the absence of selective pressure.

All plasmids fall into one of two categories: conjugative or non-conjugative. A conjugative plasmid is one that can bring about the transfer of DNA (its own and other) by bacterial conjugation (Novick et al., 1976). A non-conjugative plasmid is one that is unable to do this. A non-conjugative plasmid may, however, be mobilised by a conjugative plasmid and in this way be transferred from one cell to another.

Many plasmids have been named according to the phenotype they bestow upon their host. The most common of these are resistance (R) plasmids, which carry information for resistance to antibiotics and/or other antibacterial drugs (Novick et al., 1976) and col plasmids which carry genetic information for the production of a colicin (Novick et al., 1976). To a large extent these names are arbitrary as a plasmid initially identified as, for example, an R plasmid, may later be found also to carry genes for other characters affecting the host's phenotype.

Functions encoded by Plasmids

(i) Maintenance genes

All plasmids carry a set of genes which direct self replication and segregation (essential properties of a replicon).

One group of genes which appears to be associated with replication, but may not necessarily be essential genes, are the incompatibility genes. The character which these determine is plasmid incompatibility, i.e. the inability of two different plasmids to co-exist stably in the

same host cell in the absence of continued selection pressure (Novick et al., 1976). Incompatibility is always seen between isogenic plasmids (e.g. two copies of F or of col Ib-P9) and homogenic ones (e.g. two F' plasmids) and is also seen between certain non-homogenic plasmids. The phenomenon is quite distinct from other processes which prevent one plasmid superinfecting a cell in which another is resident (e.g. entry exclusion, restriction) in that the two plasmids must be present together in the cell before the incompatibility mechanism can act. Indeed, the resident plasmid is often the one displaced and not the incoming one as is the case with other superinfection phenomena.

By testing pairs of plasmids together it can be determined if they can exist together (compatible) or if they cannot (incompatible). In this way every plasmid can be placed in an incompatibility group, each member within a group being incompatible with the other members of it, but compatible with members of other groups (Novick et al., 1976).

Incompatibility appears to act by one plasmid preventing the replication of another; the plasmid not replicated is thus absent from progeny cells. Incompatible plasmids therefore probably share an identical replication control system. Incompatibility (inc) genes are generally located with the genes for plasmid replication, indicating perhaps a functional relationship (Andres et al., 1979; Crosa et al., 1978).

(ii) Transfer genes

These are the genes which allow conjugation and DNA transfer to occur, i.e. they determine fertility. They are thus only found on conjugative plasmids. The transfer (tra) genes are generally arranged in a single operonic structure which is under repressive control (Helmuth & Achtmon, 1975; Ippen-Ihler et al., 1972; Sakanyan et al., 1978). The pilus genes determine the synthesis of the conjugative pili - the presence of which is required for conjugation to occur (Brinton

et al., 1964; Hirota et al., 1966). Pili are appendages present in many bacteria; they may be as long as the cell itself (see Ottow, 1975). The two most likely functions of pili are to provide a hollow tube through which DNA passes from donor to recipient and to bring donor and recipient together by retracting, once the tip of the pilus has become attached to the recipient. Of the remaining tra genes, some are involved directly in the process of DNA transference, and others have been identified as the genes responsible for the entry exclusion phenomenon (Achtman et al., 1977).

As yet, pili have not been demonstrated for all groups of conjugative plasmids, although there is no reason to suspect that they do not determine them. The main problem in demonstrating the presence of pili arises because only a small proportion of the cells in a culture are effective donors (and hence produce pili) at any time because of plasmid repression (Meynell et al., 1968). Consequently, the titre of pili in a culture is low and difficult to determine.

(iii) Somatic gene function

This term is used to embrace all functions which a plasmid may encode, which are not directly concerned with its own propagation and survival. They are usually those genes which confer some novel phenotype on the host cell. It is these genes, therefore, that are important taxonomically as by alteration of the host's phenotype, they could influence the classification and therefore also the identification of that bacterium. This is particularly true for those plasmids which carry genes for characters routinely used in clinical identification, e.g. colicin production and serological reactions, sugar fermentation, H_2S production, antibiotic resistances, etc. To date a wide variety of these characters have been found to be plasmid borne (see Table 1).

TABLE 1
Characters reported to be Plasmid-borne*

Antibiotic Resistances

Ampicillin

Carbenicillin

Cephaloridine

Cephalosporin

Chloramphenicol

Cloxacillin

Erythromycin

Fusidic acid

Gentamycin

Kanamycin

Levomycin

Methicillin

Neomycin

Penicillin

Streptomycin

Sulphonamide

Tetracycline

Trimethoprim

Carbon Source Utilization

Camphor

Naphthalene

Octanol

Salicylates

Toluene

Heavy Metal Resistances, etc.

Acridine

Antimony salts

Arsenate

Arsenite

TABLE 1 (Cont'd)

Bismuth salts

Cadmium salts

Cobalt salts

Ethidium bromide

Lead salts

Mercury salts

Nickel

Silicate

Sugar Fermentation

Citrate

Lactose

Raffinose

Sucrose

Others

Colicin production and Immunity

Enterotoxin production

Haemolysin production

Hydrogen sulphide production

Interference with phage production (*pif*)

K88 antigen production

Nitrogen fixation genes (*nif*)

Protease production

Restriction and modification system

Tumour induction

Ultraviolet resistance (*uvr*)

Urease production

* Based on various reports in the literature (see Broda, 1979; Hardy, 1981, Novick, 1969; Novick *et al.*, 1976).

Cryptic Plasmids

In many bacteria, plasmid DNA has been found by physical techniques, but no distinguishing phenotypic characters have been shown to be associated with their presence (Christiansen et al., 1973; Rush et al., 1969). The functions these plasmids carry are thus unknown and they are termed cryptic plasmids. Such plasmids and plasmid regions may play a significant and unsuspected role in determining phenotype and hence could prove to be important for identification.

Chromosomal Integration

This is an important property of some conjugative plasmids.

Most (if not all) conjugative plasmids may transfer bacterial genes during conjugation by the process of mobilisation (the transfer of DNA to which the plasmid is not stably and covalently linked; Novick et al., 1976). Plasmids from a number of incompatibility groups, notably F and F-like plasmids, can stably integrate into the bacterial chromosome (Datta & Barth, 1976; Moody & Runge, 1972; Sotomura & Yashikawa, 1975), and consequently transfer bacterial genes at a high frequency during conjugation. The amount of DNA transferred is variable as it depends on the length of time a mating pair stays together. Normally during transfer only part of the plasmid is transferred and so this DNA cannot replicate autonomously. To survive, it must be incorporated into the recipient's chromosome by reciprocal recombination. The recipient thereby becomes a hybrid containing some of its original genes and some of the donor's.

Transposons

A transposon is a genetic element comprising a gene or genes conferring a trait such as drug-resistance or a toxin, together with sequences which enable the element as a whole to transpose copies of itself (Broda, 1979). Transposition involves the transfer of a copy of the transposon to another DNA molecule; a copy of the transposon is retained at the original site. Transposons can insert at many different positions within a DNA molecule, although they insert preferentially at particular sequences or regions.

The transposition is a rec A-independent event by which genetically and physically discrete sequences of DNA move from one site to another (Kleckner, 1977), implying that it depends on other host enzymes and/or on transposon-encoded enzymes. Recombination events occur at the termini of the transposable element. The sequences at each end of the transposon are similar except that they are inverted with respect to each other.

Early Observations of Drug-Resistance in Bacteria

Bacterial resistance to antibiotics has been observed in strains isolated before the clinical use of antibiotics (Watanabe, 1971). Smith (1967) discovered an R factor conferring tetracycline and streptomycin resistance in a strain which had been lyophilized in 1946. Today it is difficult to find an area which has never been exposed to clinical use of antibiotics. However, some studies have been carried out in remote areas. Maré (1968) conducted a study of organisms from faecal specimens of Kalahari bushmen and of wild animals. Some microbial species were found which were resistant to drugs. Davis & Anadam (1970) also found transferable R factors in enteric flora from natives of Borneo. Gardner et al., (1969) found R factors in Gram-negative bacteria isolated in the Solomon Islands. One of these resistant strains was from a soil sample which raises some interesting questions about the primary reasons for micro-organisms encoding drug resistance. The enormous selective pressure because of the use of antibiotics, has led to consideration of drug resistance as a clinical phenomenon. However, many antibiotics are produced by soil organisms so it is likely that in some circumstances drug resistance may confer a selective advantage on other organisms in the soil. Antibiotic resistance is probably a very "old" property of organisms which has recently come to our attention because of selective pressures imposed by the widespread use of antibiotics.

Multiple drug resistant strains were first described by Kitamoto et al., (1956). A strain of Shigella flexneri serovar 4a, isolated during a dysentery

outbreak in Japan, was found to be resistant to streptomycin, tetracycline, chloramphenicol and sulphonamide. Soon afterwards, many similar isolates were made. The transferability of resistance was first formally demonstrated by Akiba et al. (1960).

Plasmid Mediated Antibiotic Resistance

Resistance (R) plasmids conferring resistance to one, two or several antibiotics, have become prevalent in the last twenty years following the widespread use of antibiotics. Much work has been done both on the plasmids themselves and on the ways in which they confer resistance. Unlike chromosomal antibiotic resistance which normally arises by a change in permeability of the bacterial cell wall or by mutation of the site of action of the drug (e.g. ribosomal proteins), most plasmid-borne antibiotic resistance is mediated by specific enzymes capable of inactivating the drug. For example, chloramphenicol in inactivated by acetyltransferase (Shaw, 1967); ampicillin, carbenicillin, cephaloridine and penicillin are all inactivated by specific β lactamases (Richmond et al., 1971). Erythromycin and lincomycin are inactivated by methylase enzyme, and kanamycin, neomycin and gentamycin are inactivated by N-acetyl transferases, O-phosphotransferases and O-nucleotidyltransferases (see Davies & Smith, 1978).

Tetracycline resistance is one of the few not brought about in this way. Resistance in this case is by reduction of the bacteria's ability to accumulate the drug (Benveniste & Davies, 1973).

Extrachromosomal elements in the genera Streptococcus, Listeria and Erysipelothrix

STREPTOCOCCUS

The frequency of appearance of drug resistance among members of the genus Streptococcus has been steadily increasing in recent years (Jones & Finland, 1957; McCormack et al., 1962; Toala et al., 1969). Since 1953, the streptococci of serological Group D have become increasingly resistant to many commonly used antibiotics (Jones & Finland, 1957; Toala et al., 1969), especially to tetracycline (Tc) and to a lesser degree erythromycin (Em) and chloramphenicol (Cm) (Jones & Finland, 1957; Marder & Kayser, 1974; Toala et al., 1969). A comparison of Group D streptococci isolated in the Netherlands before 1964 and in 1974-1976 showed an increase in Tc resistance from 37% to 58% and in Em and Cm resistance from <2% to 14% and 12% respectively (Van Embden et al., 1977).

Amongst streptococci of serological Group A prevalences of Tc resistance of from 4% to 44% have been recorded in various countries (see Engel et al., 1978). In the Netherlands the frequency of Tc resistance rose from 1% before 1960 to 16% in 1974. The prevalence of Em and Cm resistance has remained low (<1%) in most countries except Japan (Mitsuhashi et al., 1974; Nakae et al., 1977).

The frequency of Tc resistance in streptococci of serological Group B is rather similar to that in Group D streptococci (37% to 85%) (Anthony & Concepcion, 1975; Eikhoff et al., 1964; Finch et al., 1976; Jokipii & Jokipii, 1976; Prakash et al., 1973; Rousset et al., 1977). In the Netherlands it increased from 6% before 1964 to 29% in 1974. In most countries, the prevalences of Em and Cm resistance are reported to be still <2%, although a single report from India mentioned a frequency of Cm resistance as high as 15% (Prakash et al., 1973).

Jones & Finland (1957) first observed penicillin resistance among clinical enterococcal strains. Later, Toala et al.(1969) confirmed their findings. The higher resistance of enterococci to penicillin led to the use

of drug combinations, e.g. penicillin plus streptomycin against enterococcal infections. In recent years, increasing numbers of enterococci have been found to be resistant to the synergistic effect of penicillin plus streptomycin (Moellering et al., 1971a).

Fortunately, the combination of penicillin plus gentamycin has been shown to produce enhanced killing against virtually all strains of enterococci invitro (Moellering et al., 1971a; Watanakunakorn, 1971; Wilkowske et al., 1971). Clinical studies have confirmed the in vivo efficacy of this combination, against infections due to enterococci (Carrizosa & Kaye, 1974; Weinstein & Schlesinger, 1973).

Resistance to penicillin is perhaps the best known example of intrinsic resistance to antimicrobial agents found among enterococci. This is supported by the fact that the penicillin susceptibility of faecal enterococci from isolated primitive populations such as Solomon Islanders is similar to that of strains currently isolated in the United States (Moellering & Krogstad, 1979). The mechanism of resistance to penicillin among enterococci has not been elucidated to date, but limited studies suggest that Str. faecalis does not produce an extracellular β-lactamase (Weinstein & Moellering, 1975).

Low-level resistance to various aminoglycosidic aminocyclitol antibiotics is also a feature of enterococci (Calderwood et al., 1977; Toala et al., 1969). Although the molecular basis for this resistance remains to be worked out, there is convincing evidence that it is due to decreased uptake through the cell wall via transport mechanisms for aminoglycosides (Moellering & Weinberg, 1971). However, entry through the cell wall appears to be the basis for the well-known synergistic interaction of inhibitors of cell wall synthesis such as penicillin with streptomycin or gentamycin and/or other aminoglycosides against enterococci (Calderwood et al., 1977; Hunter, 1947; Moellering & Weinberg, 1971; Moellering et al., 1971a,b,c; Watanakunakorn, 1971). Low-level resistance to clindamycin and lincomycin is also a characteristic feature of some species of enterococci (Str. faecalis

and Str. faecium) (Karchmer et al., 1975). Resistance to this antibiotic was found among isolates of Str. faecalis and Str. faecium obtained from Solomon Islanders who had had no previous clinical exposure to antibiotics in general; and also from the clinical isolates of enterococci in the United States (Moellering & Krogstad, 1979). The molecular basis of low-level resistance to clindamycin and lincomycin among enterococci is currently unknown.

Kojima & Yuki (1968) first gave an explanation of penicillin resistance in Streptococcus faecalis. On the basis of frequent loss of resistance to to the antibiotic, it was suggested that penicillin resistance in Str. faecalis is controlled by cytoplasmic factors.

Conjugation

Since the discovery in Shigella (Akiba et al., 1960; Ochiai et al., 1959) of extrachromosomal elements responsible for multiple drug resistance, such plasmids have been found in many other genera such as Salmonella (Datta, 1962) and Staphylococcus (Novick, 1963; Novick & Richmond, 1965). Even though a number of drug-resistance determinants in the genus Staphylococcus and the family Enterobacteriaceae have long been known to reside on plasmid DNA molecules, the existence of plasmids in streptococcal species has been reported only fairly recently (Courvalin et al., 1972; Dunny et al., 1973).

It is now generally accepted that resistance to various antimicrobial drugs in streptococci is mediated by plasmids (Courvalin et al., 1972; Dunny et al., 1973; Jacob & Hobbs, 1974). Many of these plasmids have been shown to be self-transferable (Dunny & Clewell, 1975; Horodniceanu et al., 1976,1979; Jacob & Hobbs, 1974; Jacob et al., 1975; LeBlanc et al., 1978; Malke, 1979; Tomich et al., 1979; Van Embden et al., 1977), by a process that needs cell to cell contact between donor and recipient and which resembles conjugation in several aspects (Jacob & Hobbs, 1974). Non-self-transferable plasmids can be mobilised by some self-transferable ones (Dunny & Clewell, 1975) Several workers have reported that conjugal transfer of

streptococcal plasmids involves intraspecific matings between isolates of Group D streptococci (Dunny & Clewell, 1975; Jacob & Hobbs, 1974; Marder & Kayser, 1977). Interspecific plasmid transfer between streptococci of serogroups B and D has also been reported (Hershfield, 1979; Horodniceanu et al., 1979; Van Embden et al., 1977).

The first definitive studies which provided a possible explanation for the prevalence of resistance to erythromycin among clinical isolates of enterococci were conducted by Courvalin et al. (1972). These investigators found a strain of Streptococcus faecalis with high level resistance to erythromycin and lincomycin which could be cured of resistances to these antimicrobial agents with acriflavine or ethidium bromide; moreover, they demonstrated that the presence or absence of these resistances was associated with the presence or absence of a plasmid. Second, a small plasmid in Str. mutans was identified by Dunny et al. (1973). Since then plasmids have been identified in numerous strains of streptococci; a list of the plasmids that have been at least partially characterized and named is given in Table 2 .

Table 2 lists the plasmid encoded features so far noted amongst streptococci. These include self transfer by conjugation (Jacob & Hobbs, 1974), mobilization of non-conjugative plasmids (Dunny & Clewell, 1975), drug resistances, haemolysins, proteases, bacteriocins, bacteriocin sensitivity, resistance to ultraviolet light, resistance to arsenite, arsenate and chromate and utilization of lactose, sucrose and citrate. Although specific plasmids have not been characterized yet, the involvement of plasmids has also been suggested in streptococci A-FF22 production (Tagg & Wannamaker, 1976), nisin production (Fuchs et al., 1975; LeBlanc et al., 1980), galactose and xylose fermentations (LeBlanc et al., 1980) and production of M proteins and serum opacity factor (Cleary et al., 1975).

In a few cases workers have found that plasmids are responsible for properties which relate to species nomenclature. For example, "Str. faecalis var. zymogenes" is so named because of its production of haemolysin; in fact most of these organisms are simply Str. faecalis strains bearing plasmids

Table 2 List of streptococcal plasmids reported in the literature*

Species	Plasmid	Mol wt ($\times 10^6$)	Related phenotype(s)*	Conjugative	Original host	References
S. faecalis	pAMα1	6.0	Tc^r(amplifiable)	No	DS5	Clewell *et al.*, 1975; Yagi & Clewell, 1976, 1977.
	pAMβ1	17.	Em^r	Yes	DS5	Clewell *et al.*, 1974; Hershfield, 1979.
	pAMγ1	35	Hly-Bac,UV^r,PR	Yes	DS5	Clewell *et al.*, 1974; Dunny & Clewell, 1975; Yagi, unpublished data.
	pAMγ2	35	Bac,PR	Yes	DS5	Yagi *et al.*, unpublished data.
	pAMγ3	35	PR	Yes	DS5	Yagi *et al.*, unpublished data.
	pAD1	35	Hly-Bac,UV^r,PR	Yes	DS16	Dunny *et al.*, 1979; Tomich *et al.*, 1979.
	pAD2	15	Em^r,Sm^r,Km^r	No	DS16	Tomich *et al.*, 1979.
	pOB1	46	Hly-Bac,PR	Yes	5952	Oliver *et al.*, 1977.
	pOB2	28	Bac(streptocin 101)	No	5952	Oliver *et al.*, 1977.
	pPD1	35	Bac,UV^r,PR	Yes	39-5	Clewell *et al.*, 1979; Brown *et al.*, unpublished data.
	pPD2	10	Cryptic	?	39-5	Clewell *et al.*, 1979
	pPD3	5	Cryptic	?	39-5	Clewell *et al.*, 1979.
	pPD4	3	Cryptic	?	39-5	Clewell *et al.*, 1979.
	pPD5	35	Hly-Bac	Yes	39-5	Brown *et al.*, unpublished data.

Table 2 (Cont'd).

pAM539	26	Sensitivity to streptocin 101	?	ND539	Clewell et al., 1976; Oliver et al., 1977.
pAM547	9	Cryptic	?	ND547	Clewell et al., 1976.
pAM81	16	Em^r	Yes	DU81	Yagi & Clewell, unpublished data.
pIP613	18	Em^r	Yes	BM6201	Courvalin et al., 1974; Hershfield, 1979.
pIP614	65	Tc^r	Yes	BM6201	Courvalin et al., 1974; Hershfield, 1979.
pJH1	50	Em^r,Tc^r,Km^r,Nm^r	Yes	JH1	Jacob & Hobbs, 1974.
pJH2	38	Hly-Bac,PR	Yes	JH1	Jacob et al., 1975; Brown & Clewell unpublished data.
pJH3	38	Hly-Bac	Yes	JH3	Jacob et al., 1975.
pIP800	70	Km^r,Gm^r,Cm^r	Yes	BM4100	Courvalin et al., 1980.
pIP801	53	Em^r,Hly-Bac	Yes	BM4100	Courvalin et al., 1980.
pIP802	49	Cryptic	Yes	BM4100	Courvalin et al., 1980.
pIP803	2	Tc^r(?)	No	BM4100	Courvalin et al., 1980
pJH4	26	EM^r,Km^r,Sm^r	Yes	JH7 (S.faecium)	Courvalin et al., 1978.
pJH5	76	Em^r,Tc^r,Km^r, Sm^r,Cm^r	Yes	JH6	Courvalin et al., 1978.
pFK14	26	Em^r,Sm^r,Cm^r	Yes	HK187	Marder & Kayser, 1977.
pl	36	Cryptic	?	HK187	Marder & Kayser, 1977.

Table 2 (Cont'd).

p2	31	Cryptic	?	HK187	Marder & Kayser, 1977.
p4	4	Cryptic	?	HK187	Marder & Kayser, 1977.
pDR1	45	Sm^r,Km^r	Yes	EBC-22	Krogstad et al., 1978.
pCS2	18	Em^r	?	CS14	Corb & Murray, 1977.
pCS3	19	Em^r	?	CS19	Corb & Murray, 1977.
pCS4	13	Em^r	?	CS20	Corb & Murray, 1977.
pCS8	13	Cryptic	?	CS29	Corb & Murray, 1977.
pCS9	26	Em^r	?	CS29	Corb & Murray, 1977.
pRI401	30	Tc^r	Yes	M439	Van Embden et al., 1977.
pRI402	41	Em^r	Yes	M439	Van Embden et al., 1977.
pRI404	37	Tc^r	Yes	M403	Van Embden et al., 1977.
pRI405	17	Em^r	Yes	M440	Engel et al., 1980; Van Embden et al., 1978.
pX14-1	39	Hly-Bac,UV^r	Yes	X-14	Frazier & Zimmerman, 1977, 1980; Miehl et al., 1980.
pX14-2	3.6	Cryptic	?	X-14	Frazier & Zimmerman, 1977, 1980.
pIP683	44	Cm^r,Gm^r,Km^r	Yes	D366	Horodniceanu et al., 1979.
pIP685	20	Tc^r	Yes	D366	Horodniceanu et al., 1979.
pCF10	35	Tc^r,PR	Yes	SF-7	Dunny et al., unpublished data.

Table 2 Cont'd).

S. agalactiae	pIP501	20	Em^r,Cm^r	Yes	B96(B6101)	Hershfield, 1979; Horodniceanu *et al.*, 1976; 1979.
	pIP612	23	Em^r,Cm^r	Yes	B97(B6105)	El-Solh *et al.*, 1978; Horodniceanu *et al.*, 1979.
	pIP635	20	Em^r,Cm^r	Yes	B98	Horodniceanu *et al.*, 1979.
	pIP639	18	Em^r	Yes	B110	Horodniceanu *et al.*, 1979.
	pIP640	18	Em^r	Yes	B113	Horodniceanu *et al.*, 1979.
	pIP642	18	Em^r	Yes	B115	Horodniceanu *et al.*, 1979.
	pMV103	18	Em^r	Yes	D25303	Hershfield, 1979.
	pMV141	17	Em^r	Yes	MV141	Hershfield, 1979.
	pMV158	3.5	Tc^r	No	MV158	Burdett, 1980.
	pMV163	3.6	Tc^r	No	MV163	Burdett, 1980.
	pMV120	30	Tc^r	Yes	MV120	Burdett, 1980.
	pPB2	16	Em^r,Cm^r	?	PB2	Lutticken & Laufs, 1979.
	pB96	17	Em^r,Cm^r	?	B96	Lutticken & Laufs, 1979.
S. pyogenes	pAC1 (pDC10535)	17	Em^r	Yes	AC1(10535)	Clewell & Franke, 1974; Malke, 1979.
	ERL1	19	Em^r	Yes	13234	Burdett, 1980; Malke, 1974; Malke *et al.*, 1976

	pSM19035	18	Em^r	No	19035	Behnke et al., 1979; Boitsov et al., 1979; Malke, 1974.
	pSM22095	18	Em^r	No	22095	Behnke et al., 1979; Boitsov et al. 1979. Malke, 1974.
	pSM15346	19	Em^r	Yes	15346	Malke, 1974; 1979.
	pSM10419	15	Em^r	?	10416	Malke et al., 1981.
S. mutans	pAM7	3	Cryptic	?	LM7	Dunny et al., 1973.
	pVA380	2.4	Cryptic	?	V380(S. ferus)	Macrina et al., 1978; 1980.
	pVA380-1	2.9	Cryptic	?	V380(S. ferus)	Macrina et al., 1978; 1980.
	pVA310	3.6	Cryptic	?	V310	Macrina et al., 1977; 1978.
	pVA318	3.6	Cryptic	?	V318	Macrina et al., 1977; 1978.
	pVA403	3.6	Cryptic	?	V403	Marina et al., 1978.
S. sanguis	pAM77	4.5	Em^r	No	A1	Yagi et al., 1978.
S. pneumoniae	pDP1	2.0	Cryptic	?	D39S	Smith & Guild, 1979.
S. lactis	pLM3001	30	Lac,Prt,Asa^r, Asi^r,Cr^r	?	C2	Efstathiou & McKay, 1977.
	pSK04	40	Lac,Prt	?	C10	Kuhl et al., 1979.
	pSK08	33	Lac,Prt	?	ML3	Kuhl et al., 1979.

Table 2 (Cont'd).

	pSK13	45	Lac,Prt	?	M18	Kuhl et al., 1979.
	pDR1	32	Lac	?	DR1215	Le Blanc et al., 1979.
	pDR2	29	Suc	?	DR1215	Le Blanc et al., 1980.
S. cremoris	pLM3601	36	Lac	?	B1	Anderson & McKay, 1977
S. diacetylactis	pGK0551	5.5	Cit	?	18-16	Kempler & McKay, 1979.
	pGK4101	41	Lac	Yes	18-16	Kempler & McKay, 1979.
	pGK0552	5.5	Cit	?	DRC1	Kempler & McKay, 1979.
	pGK4102	41	Lac,Prt	?	DRC1	Kempler & McKay, 1979.

*Abbreviations: Tc, tetracycline; Em, erythromycin; Sm, streptomycin; Km, kanamycin; Nm, neomycin; Gm, gentamycin; Cm,chloramphenicol; Asa,arsenate;Asi,arsenite; Cr, chromate; UV, ultraviolet light; Hly-Bac, haemolysin-bacteriocin; Bac, bacteriocin; PR, pheromone response; Lac, lactose utilization; Prt, protease production; Suc, sucrose utilization; Cit, citrate utilization.

* Taken from Clewell, (1981).

which determine haemolysin expression. Similarly, plasmids determining citrate utilization appear to be responsible for the name Str. lactis subsp. diacetylactis (or Str. diacetylactis) (Kempler & McKay, 1979).

However, Clewell et al. (1974) described the occurrence of three plasmids (designated α, β and γ, with molecular weights of 6 x 10^6, 17 x 10^6 and 34 x 10^6, respectively),in a strain of Str. faecalis, highly resistant to erythromycin and lincomycin. They showed that loss of erythromycin resistance is due to the loss of the β-plasmid in the strain.

At about the same time, Courvalin and his co-workers (1974) showed that strains of Str. faecalis resistant to tetracycline and erythromycin could also be cured, either independently or simultaneously, by acriflavine. Loss of tetracycline resistance was associated with loss of resistance to minocycline, and loss of erythromycin resistance was associated with loss of resistance to oleandomycin, spiramycin, lincomycin, to factor I of pristinamycin and to factor S of virginiamycin. Two plasmid DNA molecules were identified, distinguishable by their molecular weights and their guanine plus cytosine content in a Str. faecalis strain resistant to tetracycline and erythromycin (Courvalin et al., 1974).

Involvement of erythromycin and lincomycin resistance with plasmid DNA has been shown by many workers in streptococci from widely separated parts of the world (Clewell & Franke, 1974; Clewell et al., 1974; Corb & Murray, 1977; Courvalin et al., 1974; Jacob & Hobbs, 1974; Malke, 1974; Marder & Kayser, 1977).

Jacob & Hobbs (1974) found that the high level resistance to erythromycin, kanamycin, neomycin, streptomycin and tetracycline in "Str. faecalis var. zymogenes" is determined by a self-transferable plasmid. They showed the transfer of drug resistance plasmid to an antibiotic-sensitive strain of Str. faecalis in liquid culture at 37°C. Accordingly, the suggestion was made that plasmid transfer takes place in Str. faecalis by conjugation (Jacob & Hobbs, 1974). Later, Marder & Kayser (1977) demonstrated the

presence of four plasmids in a strain of "Str. faecalis var. liquefaciens" which was resistant to erythromycin and chloramphenicol. They showed that one of the plasmids which determined drug resistance, could be transferred at high frequency to an antibiotic-sensitive strain of Str. faecalis. Unlike the situation with Escherichia coli (Cook & Ewins, 1975; Dudgeon et al., 1921; Minshew et al., 1978) there is no evidence that the plasmid determined haemolytic activity of "Str. faecalis var. zymogenes" contributes to pathogenicity in human infections. In fact, the haemolysin of these bacteria has been shown to possess bacteriocin activity (Basinger & Jackson, 1968; Brock & Davie, 1963; Granato & Jackson, 1969).

It was reported by Brock & Davie (1963) that all "Str. zymogenes" ("Str. faecalis var. zymognes") strains produced a characteristic bacteriocin which was active against lactic acid bacteria and most other Gram-positive bacteria. Mutants which had lost the haemolytic characteristic simultaneously lost their bacteriocin-producing ability. Moreover, Brock & Davie (1963) showed that the two characteristics were generated or lost together. Both activities were destroyed by chloroform and were antagonized by lecithin. Both activities were destroyed at the same rate by treatment at 45°C under mildly acid conditions, but both remained stable when heated in agar. Later, Davie & Brock (1966) demonstrated that a ribitol teichoic acid, associated with the cellular membrane of "Str. faecalis var zymogenes" inhibited the lysin, and prevented "Str. faecalis var zymogenes" from killing itself. Removal of the teichoic acid from "Str. faecalis var zymogenes" spheroplasts rendered them susceptible to this lysin. The inhibitory capacity of isolated teichoic acid depends upon the presence of a D-alanyl ester. This fact seems to be reflected in partially resistant mutants which contain smaller amounts of base labile D-alanine than wild type "Str. faecalis var. zymogenes". In each of the major groups of Gram-positive organisms there are strains that are resistant to "Str. faecalis var. zymogenes" lysin. Whether the resistance of these organisms is also due to a teichoic acid or related substance, or not,

has not been examined. However, the evidence presented by Davie & Brock, (1966) suggests that the Group D bacteriocin (haemolysin) may be a general membrane-lytic agent and thus may be useful in examining membrane structure.

The sensitivity of Str. faecalis strains to "Str. faecalis var. zymogenes" bacteriocin depends greatly on their phsyiological age. Sensitivity decreases from the mid-log phase on, and is completely lost in the stationary phase.

The sensitivity of erythrocytes to the haemolytic activity of the bacteriocin shows considerable species variation. Basinger & Jackson (1968) demonstrated the order of increasing sensitivity to be goose<sheep<dog<horse<human<rabbit. However, when red cell stromato were used as indicators of haemolysis in a standard system employing rabbit erythrocytes, the order of increasing effectiveness was sheep<rabbit<human<horse<goose. When rabbit red blood cells were used in varying concentrations with a constant haemolysin concentration, there was a lag of about 30 min, which for a given haemolysin preparation was constant for all red cell concentrations. If red cells are held constant and lysin concentrations varied, the time taken to reach half-maximal lysis varied directly with lysin concentration, but was not strictly proportional. Basinger & Jackson (1968) showed that bacterial membranes were one to three orders of magnitude more effective as inhibitors than were red cell stromato.

The order of increasing effectiveness seemed to be Escherichia coli< Bacillus megaterium<Streptococcus faecalis<Micrococcus lysodeikticus. In addition to membranes, a D-alanine containing glycerol teichoic acid, trypsin in high concentration, and deoxyribonuclease also inhibited haemolysis. Ribonuclease, D-alanine, L-alanine, DL-alanyl DL-alanine, N-acetyl D-alanine and N-acetyl L-alanine did not inhibit haemolysis.

Tomura et al. (1973) observed the transfer of a haemolysin-bacteriocin determinant at a relatively high frequency (up to 5.8×10^{-2} per donor) and

although no direct evidence was obtained to indicate conclusively that the property was plasmid coded, it is likely that this was indeed the case. At about the same time, Jacob et al. (1975) presented evidence that the haemolytic activity of "Str. faecalis var. zymogenes" strains JH1 and JH3 was associated with a plasmid. These authors found that haemolytic activity was lost from a low percentage of cells grown in broth at either 37 or 45°C. All non-haemolytic variants (Hly^-) had lost bacteriocin activity (Bcn^-), and those from strain JH3 had also lost resistance to the bacteriocin (Bnr^-). However, the majority of Hly^-, Bcn^- variants from JH1 had retained bacteriocin resistance (Bnr^+). Both strains, JH1 and JH3, were shown to contain a plasmid of molecular weight 38×10^6. In addition, as noted earlier, strain JH1 also contained a plasmid (pJH1) which carried the genes determining resistance to various antibiotics. Hly^-, Bcn^-, Bnr^- variants of strain JH3 had completely lost the plasmid pJH3; whereas Hly^-, Bcn^-, Bnr^- variants of strain JH1 had completely lost pJH2 but retained plasmid pJH1. It was also shown that the Hly^+, Bcn^+, Bnr^+ traits from both parental strains were transferable to non-haemolytic Str. faecalis strains during mixed incubation in broth at 37°C, and haemolytic recipient strains were found to have received plasmid pJH2 from strain JH1 and pJH3 from JH3 by conjugation (Jacob et al., 1975). Beta-haemolytic activity is the only property distinguishing the "zymogenes" variety from Str. faecalis. Since Jacob et al. (1975) showed that this activity is plasmid borne in strains JH1 and JH3, they endorsed the growing taxonomic view that the varietal status of the haemolytic strains of Str. faecalis (i.e. "Str. faecalis var. zymogenes") should be dropped.

Recently, there have been several other reports which confirm that the genes coding for haemolysis-bacteriocin production in enterococci are borne on plasmids (Dunny & Clewell, 1975; Tomich et al., 1979; Tomura et al., 1973).

Frazier & Zimmerman (1980) first reported that the plasmid which codes

for haemolysin production in "Str. faecalis var. zymogenes" also carries genes for resistance to bacteriophage as well as to ultraviolet light. At about the same time, Miehl et al. (1980) reported the presence of a plasmid in Str. faecalis which confers resistance to ultraviolet light.

The conjugation phenomenon in the genus Streptococcus appears to have been noted as early as 1964 by Raycroft & Zimmerman, when they found that chloramphenicol resistance could be transferred from one strain of Streptococcus faecalis to another. This observation was subsequently confirmed by other investigators (Courvalin et al., 1978; Marder & Kayser, 1977; Van Embden et al., 1977) who also found transferable plasmids associated with resistance to chloramphenicol in enterococci. Several groups of workers have demonstrated that resistance to chlorampenicol in Str. faecalis is due to the inducible production of chloramphenicol acetyl transferase in such strains (Courvalin et al., 1978; Miyamura et al., 1977). Not surprisingly, the enzymes obtained from strains of Str. faecalis have been shown to be similar to an enzyme from Staphylococcus aureus (Courvalin et al., 1978) and identical (in molecular weight, pH optimum and heat stability) to enzymes derived from chloramphenicol resistant strains of "Streptococcus haemolyticus" (Group A Streptococcus) and Streptococcus pneumoniae (Miyamura et al., 1977).

During the last 10-15 years, centres in various parts of the United States (Iannini et al., 1976; Standiford et al., 1970) and overseas (Ruhen & Darrell, 1973) have found 25-50% of clinical enterococcal isolates resistant to high levels of streptomycin and/or kanamycin. However, it has been found that enterococci with high level aminoglycoside resistance is refractory to synergism when penicillin and aminoglycoside are used together (Calderwood et al., 1977). It has been shown that high level resistance to streptomycin and kanamycin and resistance to penicillin-streptomycin and penicillin-kanamycin is coded for by self-transferable plasmids in resistant strains (Courvalin et al.,1980; Krogstad et al., 1978a; Slocombe, 1978).

Krogstad et al (1978) reported that, like the plasmid-borne resistances in the Gram-negative bacteria and staphylococci, plasmid-linked resistances to aminoglycoside antibiotics in Group D streptococci are also mediated by enzymatic modification of the antibiotic. They found streptomycin adenylyl transferase and neomycin phosphotransferase enzymes in the antibiotic resistant strain. The latter enzyme was found to phosphorylate amikacin as well as its normal substrates, such as kanamycin, and the phosphorylated drug failed to produce synergism when combined with penicillin against a strain sensitive to penicillin-amikacin synergism (Krogstad et al., 1978). They showed that phosphorylation occurred at the 3'-hydroxyl position.

Courvalin et al. (1978) demonstrated that the genes conferring resistance to aminoglycoside-aminocyclitol antibiotics in Str. faecalis and Str. faecium are carried by transferable plasmids, and the resistance was mediated by constitutively synthesized phosphotransferase enzyme and acetyl-transferase enzyme (Courvalin et al., 1980).

Interspecific plasmid transfer was first reported by Van Embden et al., (1977). They transferred an erythromycin resistant plasmid from a Group D Streptococcus to a Group B Streptococcus. The plasmid which determines resistance to the antibiotic has been shown to be transferred from Group D Streptococcus to different species of streptococci and also to Lactobacillus casei by a number of workers (Gasson & Davies, 1980; Gibson et al, 1979; LeBlanc et al., 1978; Malke, 1979). Hershfield (1979) showed the transfer of erythromycin resistant plasmid from Group B streptococci to other Group B strains and to streptococci of serological Groups D, F and H.

El-Solh et al. (1978), Engel et al. (1980) and Weisblum et al. (1979) suggested a common evolutionary origin of erythromycin resistance plasmids present in different groups of streptococci and staphylococci by deoxyribonucleic acid (DNA)-DNA hybridization studies and by restriction enzyme analysis of plasmid DNA.

Erythromycin resistance in clinical isolates of pathogenic bacteria is

generally associated with a chemical alteration of ribosomal structure (Weisblum, 1975). A specific N^6-dimethylation of adenine in 23S rRNA constitutes the biochemical basis for resistances.

Evidence of conjugative R plasmids in Group B streptococci was first reported by Horodniceanu et al. (1979). They found that 10 out of 21 drug-resistant Str. agalactiae strains carried conjugative R plasmids. All of them were transferred into a Group B recipient but seven were also transferred into a Group D Str. faecalis recipient. Since no phages were detected in donor or recipient strains, and because in cultures mixed with donor cell-free supernatants no recipient cells acquired resistance markers, and as the transfer of plasmid DNA was not affected by the presence of deoxyribonuclease I, Horodniceanu et al. (1979) inferred that the mechanism of transfer of plasmid carried by Str. agalactiae strains might be a conjugation-like phenomenon.

Burdett (1980) examined 30 tetracycline resistant clinical isolates of Group B streptococci to assess the extent to which tetracycline resistance is plasmid mediated. Of these, 27 strains showed no physical or genetic evidence of plasmid-mediated resistance; however, one conjugative and two small multicopy non-selftransmissible tetracycline resistance plasmids were identified. The conjugative plasmid was transmissible to Str. faecalis as well as to Str. agalactiae. The two non-conjugative plasmids were readily mobilized by a number of sex factors into these same two backgrounds and, in addition, readily transformed Str. sanguis (Challis) to tetracycline resistance.

Dunny & Clewell (1975) showed that the plasmid designated as pAMα1 (mol wt 6×10^6) which codes for tetracycline resistance in Str. faecalis is non-selftransferable but can be mobilized by a selftransferable haemolysin-bacteriocin determining plasmid. Later, Yagi & Clewell (1976) demonstrated that the tetracycline resistant plasmid in the Str. faecalis strain undergoes gene amplification (formation of plasmids of increased size, presumably due to the inclusion of repeated units of a 2.65-megadalton

segment of DNA containing the tetracycline resistance determinant) as a result of extended growth in subinhibitory concentrations of tetracyclines. The gene amplification has been shown to be associated with an increase in resistance to tetracycline. It was also found that the 2.65 Mdal segment corresponds to the amount of DNA deleted from the plasmid in easily obtainable tetracycline sensitive variants (Yagi & Clewell, 1976).

Subsequently, Yagi & Clewell (1980) confirmed that the plasmid which determines tetracycline resistance in Str. faecalis is a non-conjugative plasmid and is present to the extent of about 10 copies per chromosomal genome equivalent. By restriction enzyme analysis,as well as by electron microscope studies, the tetracycline resistance determinant was found to be flanked by small direct repeats defining a 2.65 Mdal segment on the plasmid. The presence of these direct repeats suggested a recombinational mechanism for amplification,and these recombinational sequences were assumed to be responsible for the rather high frequency of appearance of tetracycline sensitive variants having a 2.65 Mdal deletion (Clewell et al., 1975; Yagi & Clewell, 1976). Further studies by Yagi & Clewell (1980) showed that amplification of the tetracycline resistance determinant of plasmid pAMα1 in Str. faecalis depends on the host's recombination machinery (Yagi & Clewell, 1980).

Evidence for plasmid-promoted conjugational transfer of chromosomal determinants in Streptococcus faecalis was first reported by Franke et al., (1978). The authors showed the mobilization of the tetracycline resistance determinant which is located on the chromosome by certain transmissible plasmids. Franke & Clewell (1980, 1981) have reported that a transferable tetracycline resistance determinant located on the chromosome of Str. faecalis strain DS16 is located on a 10-Mdal transposon, designated Tn 916. This element is inserted at multiple sites into several different conjugative plasmids. Transposition of Tn 916 from the chromosome to the conjugative plasmid pAD1 is Rec independent, as is its ability to transfer in the absence

of plasmid DNA (at a frequency of c 10^{-8}) (Franke & Clewell, 1980, 1981).

Sex Pheromones in Streptococcus faecalis

Brinton's demonstration of pili connecting donor and recipient strains of Escherichia coli was generally accepted as the morphological correlate of conjugal transfer in Gram-negative bacteria (Brinton, 1965). However, a morphological correlate of conjugal transfer in Gram-positive bacteria has been more difficult to demonstrate, probably because the frequency of transfer among the Gram-positive organisms studied thus far has been low (i.e. a frequency of 10^{-4} to 10^{-6}).

A number of Str. faecalis and Str. agalactiae strains have been shown to transfer plasmid DNA by a process resembling conjugation. Genes determining haemolysins, bacteriocins, bacteriocin sensitivity and drug resistances are transferable (Dunny & Clewell, 1975; Jacob et al., 1975; Jacob & Hobbs, 1974; Marder & Kayser, 1977; Oliver et al., 1977; Tomura et al., 1973; Van Embden et al., 1977). Contact between donor and recipient cells appears necessary for the transfer of DNA. Gene transfer by transformation was excluded because DNAase did not decrease the frequency of transfer, and transduction was excluded because filtrates prepared from donor strains were incapable of producing recombinants.

Clewell et al. (1978) and Dunny et al. (1978) observed that mating mixtures of Str. faecalis strains formed aggregates, or "clumps", not usually observed during growth of donors or recipients alone. In addition, Dunny et al. (1978) also noted that recipient strains of Str. faecalis produced a trypsin sensitive, heat resistant, nuclease resistant factor, designated clumping inducing agent (CIA) which caused strains carrying certain conjugative plasmids to aggregate. Ribonucleic acid and protein synthesis but not DNA synthesis were required for aggregation to occur. Recipient filtrates that contained CIA activity also induced donors to mate at high frequencies. Introduction of a transferable plasmid into strains

producing CIA dramatically reduced the amount of CIA activity produced by the strain, but allowed the strain to respond to exogenously added CIA. The most obvious function of CIA in its induction of aggregate formation is the facilitation of mating. Thus CIA would appear to represent a sex pheromone (Dunny et al., 1978).

Interestingly, donors harboring different conjugative plasmids respond to different CIAs (Dunny et al., 1979). A given recipient actually produces multiple pheromones. The acquisition of a given plasmid shuts off production of only the related pheromone, whereas the cell continues to produce other pheromones which can induce other donors with different conjugative plasmids. The pheromones have now been identified by relating them to the plasmids, originally used to detect them (Dunny et al., 1979).

Studies have shown that, in addition to an aggregation response, a pheromone induces a function that is related more directly to plasmid transfer (Clewell & Brown, 1980). Krogstad et al. (1980) presented electron micrographs of mating mixtures of Str. faecalis, showing what appear to be intercellular connections between chains of streptococci in the apparent absence of pili. Similar connections have been observed in pheromone-induced aggregates of cells harboring pPD1. Moreover, preparations of uninduced cells have also shown such intercellular connections. Thus, in this case at least, it is not clear whether the observed connections were an actual reflection of conjugal contact or an artifact of the preparation (Krogstad et al., 1980).

It is noteworthy that there have been a few reports of the occurrence of pili in streptococci (Cole et al.,1976; Handley & Carter, 1979; Handley & Hannan, 1977; Henriksen & Henrichsen, 1975), and, although they did not comment, Tomura et al. (1973) displayed an electron micrograph of a strain of Str. faecalis which appears to possess pili.

The chemical nature of the sex pheromones is currently being examined. The sensitivity of the pheromones to proteases and their heat stability and

dialyzability suggests that these compounds are small peptides (Clewell et al., 1979; Dunny et al., 1978).

A model has been proposed to explain the relationship between plasmids, pheromones, and the aggregation phenomenon (Dunny et al., 1979). The model schematically shows a plasmid-free recipient strain that produces two different pheromones, cA and cB; two isogenic donor strains harboring the conjugative plasmids pA and pB are also present. All three strains have the chromosomally determined binding substance. Plasmid pA determines the ability to respond to cA and, at the same time, through an IcA (inhibitor of cA) gene, prevents production of endogeneous cA. Similarly, plasmid pB allows its host to respond to cB and prevents the production of endogeneous cB via gene IcB. The response of the donor cell to the pheromone is shown as an interaction (direct or indirect) of the latter with a responding substance (repressor or activator) determined by gene RcA or RcB which, in turn, activates aggregation substance synthesis. Aggregation substance, which could be either plasmid determined or chromosomally determined, locates itself on the cell surface, where it can recognize binding substance (Dunny et al., 1979).

It should be possible to test this model genetically by obtaining mutations in the proposed determinants, and such efforts are currently under way (Clewell, 1981).

Transduction

There are, to my knowledge, no reports of transduction amongst streptococci of serological Group D. Bacteriophage-mediated transduction occurs however in serological Groups A, C, G (Colon et al., 1972; Hyder & Streitfeld, 1978; Leonard et al., 1968; Malke, 1972; Stuart & Ferretti, 1973, 1978; Ubukata et al., 1975; Wannamaker et al., 1973) and N (Allen et al., 1963; McKay & Baldwin, 1974; McKay et al., 1973; Sandine et al.,1962), as well as in pneumococci (Porter et al., 1979). The erythrogenic toxin associated with Group A strains causing scarlet fever has long been known to

be associated with a phage genome (see Zabriskie et al., 1972). Recently, Totolian (1979) reported the transduction of Str. pyogenes determinants of M protein and serum opacity factor. Transducing phages have never been reported in Group B or D streptococci, but it is not clear whether this simply reflects the absence of a search for such systems.

Transformation

Transformation has been reported in streptococci of serological Groups F, H, N and O (Dobrzanski, 1972; Perry & Slade, 1962; Westergren, 1978), Str. pneumoniae (Lacks, 1979) and in certain strains of Str. mutans (Perry & Kuramitsu, 1981). Interspecies and intergeneric transformation has also been reported (Bracco et al., 1957; Davidson et al., 1976; Perry & Slade, 1962; Westergren & Emilson, 1977).

Plasmid transformation was also reported by LeBlanc and co-workers (1976 and 1978a), who introduced erythromycin resistance plasmid from Str. faecalis into strains of Group H and Group F streptococci. Several other plasmids from different species and genera have now been shown to transform the streptococcus strain Challis (Behnke et al., 1979; Burdett, 1980; Macrina et al., 1980; Malke et al., 1981), as well as Str. pneumoniae (Barany & Tomasz, 1980; Saunders & Guild, 1980; Shoemaker et al., 1979). Two streptococcal plasmids (pAMα1 and pAM77) have been shown to transform Bacillus subtilis (see Clewell, 1981).

Resistance Transposons in Streptococcus faecalis

Two resistance transposons have been characterized in Str. faecalis. Both of these, Tn 916 (Franke & Clewell, 1980, 1981) and Tn 917 (Clewell et al., 1980; Tomich et al., 1978, 1980), were identified originally in the clinical isolate DS16. Resistance transposon Tn 916 has a size of 10 Mdal and confers tetracycline resistance. It is chromosome borne and appears to have fertility properties (Franke & Clewell, 1980, 1981). This transposon has been shown to insert into several sites in different conjugative haemolysin plasmids (pAD1, pAMγ1, and pOB1) at a frequency of c 10^{-5} to 10^{-6}.

Some insertions of Tn 916 into the plasmid pAD1 result in the inactivation of haemolysin expression whereas others result in a hyperexpression of haemolysin, giving rise to zones of haemolysis three to four times the normal diameter on horse blood agar (Franke & Clewell, 1980, 1981). With certain insertions, the appearance of hyperexpression of haemolysin is stimulated by the presence of tetracycline in the blood agar; the absence of the drug results in nonhaemolytic colonies. In the latter types, if a blood agar plate lacking tetracycline with mature colonies is allowed to incubate for an additional few days, a wave of haemolysis appears to spread through the plate. The expression of one colony seems to trigger the expression of nearby colonies. Similar observations have been made by LeBlanc in connection with a tetracycline resistance element originating in Str. faecalis, which inserts into the haemolysin plasmid pJH2 (see Clewell, 1981). The basis of hyperexpression of haemolysin and its stimulation in some cases by tetracycline remains a mystery (Clewell, 1981).

Transposon Tn 917 is a 3.3-Mdal transposon that carries an inducible erythromycin resistance determinant in Str. faecalis. Originally identified on the non-conjugative plasmid pAD2 of strain DS16, this transposon has been shown to transpose to a co-resident plasmid pAD1 at a frequency of c 10^{-6} (Tomich et al., 1980). Tn 917 is flanked by 0.28-kilobase repeated sequences which are inverted with respect to each other (Tomich et al.,1980). Insertion occurs into multiple sites in pAD1 and, in some cases, results in inactivation of haemolysin expression. Unlike Tn 916 insertions, Tn 917 insertions in pAD1 so far have not been observed to give rise to hyper-expression.

Tn 917 has the interesting feature that transposition is inducible upon exposure of cells to low concentrations of erythromycin (Tomich et al.,1978; 1980). Studies have shown that transposition involves a pAD1 : pAD2 co-integrated structure which forms during the first 20 to 30 min of induction; resolution of the co-integrated structure into pAD1 : Tn 917 and pAD2 takes longer, requiring as much as 30 min or more (Tomich et al.,1980). It is

conceivable that the determinants of erythromycin resistance (possibly encoding an enzyme which methylates an adenine residue in 23S ribosomal ribonucleic acid) and a transposase are linked together on a single operon or are controlled by a common regulatory function (Clewell, 1981).

Plasmid Maintenance and Incompatibility

Very little is known about plasmid incompatibility and replication (maintenance) functions in streptococci. Although numerous plasmids have been identified, attempts to categorize them into specific compatibility groups have been limited by the lack of convenient combinations of selectable plasmid markers. In the case of the R plasmids, many bear only macrolides, lincosamides and streptogramin B (MLS) resistance markers. In other cases, conjugative plasmids harbor haemolysin determinants or bacteriocin determinants or both, which are not amenable to positive selection. It is likely that, as in the Gram-negative species, many compatibility groups will be found eventually. This is certainly supported by the observation that some strains of Str. faecalis harbor as many as five plasmids.

Evidence recently reported by Romero et al. (1979) placed members of a collection of Str. faecalis R plasmids into three groups. Recent studies (see Clewell, 1981) have also shown that the haemolytic plasmids pAD1 and pAMγ1 are incompatible; that is, these plasmids are members of the same group. Although these two plasmids both determine haemolysins, the construction of derivatives with different resistance transposons allowed compatibility testing. Two other haemolysin plasmids (pOB1 and pPD5) tested by this approach were found to be compatible with pAD1 and pAMγ1 (see Clewell, 1981).

As in other genera, in streptococci there are examples of multicopy plasmids and plasmids that are present to the extent of only one to two copies per chromosomal genome equivalent. Usually, the smaller plasmids are present as multicopies, although there are exceptions, such as the 4.5-Mdal pAM77 plasmid in Str. sanguis (Yagi et al.,1978) and the 2.0-Mdal

pDP1 in Str. pneumoniae (Smith & Guild, 1979) which occurs at only one to two copies per chromosomal genome equivalent. An interesting copy number control process has been found in the tetracycline resistance gene amplification of pAMα1, a phenomenon whereby the mass of plasmid DNA taken as a percentage of chromosomal mass remains constant. Although the basis of this phenomenon is unknown the possible interpretations given by Clewell (1981) are as follows:

i) Due to a limited number of replication sites (e.g. one to two sites for the 10 copies per chromosomal genome equivalent in the unamplified state), the initiation of a round of replication requires that the previous round be completed. Thus, assuming that completion of a round of replication requires at least several minutes, replication of the larger molecules occupies a greater fraction of the division cycle, and initiation of new rounds is less frequent (Yagi & Clewell, 1976). Fewer initiations resulted in fewer copies.

ii) The amplified segment on pAMα1 includes a determinant that encodes a function which affects initiation negatively. The increased gene dosage arising because of amplification results in fewer copies.

LISTERIA

Perez-Diaz et al. (1981) reported the presence of a cryptic plasmid in strains of L. murrayi, L. grayi and L. monocytogenes. All plasmids were found to have the same molecular weight (40-Mdal). No plasmids were detected in strains of clinical origin.

ERYSIPELOTHRIX

There has been no report so far about the presence of plasmids in the genus Erysipelothrix.

MATERIALS AND METHODS

Bacterial strains

The strains used in the study are listed in the Tables. All the strains except those altered by experimental methods, were received from other workers or from culture collections. Type strains were included where possible.

Str. agalactiae strains were chosen for study depending on their source of isolation, ability to produce pigment, fermentation of lactose and starch hydrolyzing ability (see Table 5).

The Listeria strains were selected so that haemolytic, markedly haemolytic, and non-haemolytic strains were included in the study.

Streptococci of serological group D were chosen because of their ability to haemolyse blood, resistance to antibiotics, production of yellow pigment, production of acid from lactose, production of H_2S and their ability to liquefy gelatin (see Table 3).

Maintenance of cultures

On receipt, all bacteria were subcultured and tested for purity.

All bacteria were maintained in Petri dishes on Blood Agar Base No. 2 (Difco) and stored at 4°C after overnight incubation at 35°C. Strains of haemolytic streptococci of serological group D were maintained on BAB2 containing 7.5% (v/v) defibrinated horse blood; whereas 5% (v/v) defibrinated sheep blood (Difco) was used with BAB2 to maintain haemolytic Listeria monoctogenes. Antibiotic resistant strains of Str. faecalis and Str. faecium were maintained on BAB2 containing kanamycin (200 µg/ml). Working stocks were subcultered monthly.

At the same time broth cultures were stored frozen in Blood Base broth (BAB broth) plus 15% glycerol at -76°C in small vials containing glass beads (Feltham et al., 1978). These were set up in duplicate as a safeguard against contamination of one vial.

TABLE 3 STRAINS USED IN STUDY

Study No.	Species	Collection number as received	Source	Additional Information
C50	Erysipelothrix rhusiopathiae	NCTC 8163*	Spleen of pig with endocarditis	-
C334	Erysipelothrix sp.	Sneath 176 (Barrett K7)	Human erysipeloid	-
C335	"	Sneath 185 (Gledhill strain MEW 22)	Mouse	-
C337	"	Sneath 193 (Riding strain PV 174)	Swine erysipelas	-
C338	"	Sneath 182 (Gledhill strain D)	Duck	-
C524	"	Leicester Cattle Market	Pig skin	-
C212	Listeria denitrificans	L26* Pasteur Institute 55134	Cooked blood of beef	-
C670	" "	ATCC 14870*	Cooked blood of beef	-
C214	A/B Listeria grayi	Seeliger L332/64*	Faeces of chinchilla	-
C360	" "	Seeliger		-
C644	Listeria innocua serovar 6a	Seeliger		Non-haemolytic
C645	Listeria innocua serovar 6b	Seeliger		Non-haemolytic
C52	A/B Listeria monocytogenes	NCTC 7973 serovar 1a	Guinea-pig mesenteric lymph node	β-haemolytic
C201	" "	NCTC 10357* serovar 1a	Rabbit	Non-haemolytic
C259	" "	Kahn 560		-
C260	" "	Kahn 611		-
C659	" " serovar 5	Pritchard L242	Placenta-ovine abortion	β-haemolytic
C661	" "	Pritchard L102A		β-haemolytic
C662	" "	Pritchard L133	Ovine gall bladder	β-haemolytic

TABLE 3 (Cont'd)

C663	A/B *Listeria monocytogenes* serovar 5		Pritchard L72		β-haemolytic
C664	"	"	Pritchard L173		β-haemolytic
C666	"	"	Pritchard L234	Foetal-abomasum ovine abortion	β-haemolytic
C667	"	"	Pritchard L102B		β-haemolytic
C668	"	"	Pritchard L236	Ovine gall bladder	β-haemolytic
C261	*Listeria* sp.		Kahn 611		Non-haemolytic
C345	"		Seeliger NR 4206 serovar 4f		"
C383	"		Ortel 372/73 via Ralovich serovar 4g		"
C454	"		Leicester Royal Infirmary B480(S)		"
G44	*Listeria murrayi*		ATCC 25401*	Standing corn stalks and leaves	-
G45	"	"	ATCC 25402	"	-
		Serological Group			
B14	*Streptococcus agalactiae*	B	NCTC 8181*	Bovine	(see Table 5)
B32	" "	"	NCDO 1352	Bovine	"
B33	" "	"	NCDO 1334	Bovine	"
B34	" "	B	NCDO 1520	Bovine	"
B1	*Streptococcus* sp.	"	NCTC 8017	Human	"
B2	"	"	NCTC 9409	Human	"
B3	"	"	NCTC 9412	Human	"
B4	"	"	RGM 4192	Human	"
B6	"	"	RGM 61040	Human	"
B7	"	"	K575	Human	"
B8	"	"	K577	Human	"

TABLE 3 (Cont'd)

Study No.	Species	Serological Group	Collection No. as received	Source	Additional Information
B9	Streptococcus sp.	B	K578	Human	(see Table 5)
B10	"	"	K590	Human	"
B35	"	"	R78/3791	Human A.S.	"
B38	"	"	R78/4078	Human A.S.	"
B39	"	"	R78/4056	Human N.E.O.S.	"
B41	"	"	R78/4105	Human N.E.O.S.	"
B42	"	"	R78/4143	Human A.S.	"
B43	"	"	R78/4154	Human N.E.O.S.	"
B46	"	"	R78/3443	Mother (rectal)	"
B47	"	"	R78/3445	Mother (vaginal)	"
B48	"	"	R78/3452	Baby (N/T)	"
B49	"	"	R78/3456	Baby (N/T)	"
B50	"	"	R78/3458	Mother (rectal)	"
B51	"	"	R78/3464	Baby (N/T)	"
B54	"	"	R78/4158	Baby (skin)	"
B72	"	"	RGM 62730	Human	"
B84	"	"	R77/2413	Bovine	"
B87	"	"	R77/2435	Bovine	"
B90	"	"	R77/2452	Bovine	"
B94	"	"	R77/2469	Bovine	"
B96	"	"	R77/2475	Bovine	"
B97	"	"	R77/2480	Bovine	"
B98	"	"	R77/2481	Bovine	"
B99	"	"	R77/2483	Bovine	"
B105	"	"	R77/2518	Bovine	"
B108	"	"	R77/2521	Bovine	"
B112	"	"	KO 415	L.R.I.	"
B130	"	"	R78/4830	Human N.L.O. Mls.	"

TABLE 3 (Cont'd)

B16	*Streptococcus bovis*	D	NCTC 8177	Cow dung	Lactose fermentation
PB78	" "	"	NCDO 964		"
PB80	" "	"	NCDO 1251		"
D598	" "	"	NCTC 8140	Cow dung	"
D599	" "	"	NCDO 599	Babies' faeces	"
B17	"*Streptococcus durans*"	D	NCTC 8307*		β-haemolytic
W12	" "	"			β-haemolytic
W22	" "	"			Non-haemlytic
W31	" "	"			"
PB11	*Streptococcus equinus*	D	W14		-
PB12	" "	"	W15		-
PB13	" "	"	W16		-
PB83	" "	"	NCDO 1037	Horse faeces	-
D1090	" "	"	NCDO 1090		-
D1091	" "	"	NCDO 1091		-
B18	*Streptococcus faecalis*	D	NCTC 775*		-
PB77	" "	"	NCIB 6783		-
PB125	" "	"	K523		-
W1	" "	"			-
JH2-1	" "	"	from Dr.A.Jacob		Fus
JH2-25	" "	"	" "		Km,Nm,Sm,Em,Tc.
B20	"*Streptococcus faecalis* var *liquefaciens*"	D	NCTC 8175		Gelatin liquefaction
PB85	" "	"	NCDO 611		"
W2	" "	"	W2		"
D846	"*Streptococcus faecalis* var *malodoratus*"	"	NCDO 846		H_2S production

TABLE 3 (Cont'd)

D847	"*Streptococcus faecalis* var *malodoratus*"	D	NCDO 847		H_2S production
B21	"*Streptococcus faecalis* var *zymogenes*"	D	NCTC 8176		β-haemolytic
PB4	" " "	"	W3		"
PB79	" " "	"	NCDO 1595		"
JH2-27	" " "	"	From Dr.A.Jacob		"
B19	*Streptococcus faecium*	D	NCTC 7171*		-
W4	" " "	"			-
W19	" " "	"			-
W100	" " "	"	MUTK 1		Km,Nm,Sm,Em
W86	"*Streptococcus faecium* var *casseliflavus*"	D	ATCC 25788		Pigmentation
W87	" " "	"	ATCC 25789		"
W98	" " "	"	MUTK 21	From Dr.Mundt	"
W99	" " "	"	MUTK 20	"	"
W101	" " "	"	MUTK 564	"	"
W102	" " "	"	MUTK 559	"	"
W103	" " "	"	MUTK 562	"	"
W104	" " "	"	MUTK 6	"	"
W105	" " "	"	MUTK 61	"	"
W106	" " "	"	MUTK 31	"	"
W107	" " "	"	MUTK 442	"	"
W88	"*Streptococcus faecium* var *mobilis*"	D	NCIB 9645		"
W92	" " "	"	ATCC 14436		-

TABLE 4 List of altered strains

Study number	Species	Source	Additional Information
B16-1	Streptococcus bovis	B16	Lac$^-$ variant isolated after incubation at 42°C
B16-2	" "	"	"
B16-3	" "	"	"
B16-4	" "	"	"
B16-5	" "	"	"
B16-6	" "	"	"
B16-7	" "	"	"
B16-8	" "	"	"
B16-9	" "	"	"
B16-10	" "	"	"
B16-11	" "	"	"
B16-12	" "	"	"
B16-21	" "	"	Lac$^-$ variant isolated after incubation with AO
B16-22	" "	"	"
B16-23	" "	"	"
B16-24	" "	"	"
B16-25	" "	"	"
B16-26	" "	"	"
B16-27	" "	"	"
B16-28	" "	"	"
B16-29	" "	"	"
B16-30	" "	"	"
B16-31	" "	"	"
B16-32	" "	"	"
B16-33	" "	"	"
B16-34	" "	"	"
B16-35	" "	"	"
B16-36	" "	"	"

TABLE 4 (Cont'd)

B16-37	Streptococcus bovis	B16	Lac^- variant isolated after incubation with AO
B16-38	" "	"	"
B16-39	" "	"	"
B16-40	" "	"	"
B17R	"Streptococcus durans"	B17	Hly^+, Rif
B17-1	" "	"	Hly^- variant isolated after incubation at 45°C
B17-2	" "	"	"
B17-3	" "	"	"
B17-4	" "	"	"
B17-5	" "	"	"
B17-6	" "	"	"
B17-7	" "	"	"
B17-8	" "	"	"
B17-9	" "	"	"
B17-11	" "	"	Hly^- variant isolated after incubation with AF
B17-12	" "	"	Hly^- variant isolated after incubation with EB
B17-21	" "	"	"
B17-22	" "	"	"
B17-23	" "	"	"
W12-R	" "	W12	Hly^+, Rif
W12-1	" "	"	Hly^- variant isolated after incubation at 45°C
W12-2	" "	"	"
W12-3	" "	"	"
W12-4	" "	"	"
W12-5	" "	"	"
W12-6	" "	"	"
W12-7	" "	"	"
W12-8	" "	"	"

TABLE 4 (Cont'd)

W12-9	"*Streptococcus durans*"	W12	Hly^- variant isolated after incubation at 45°C
W12-10	"	"	"	"
W12-11	"	"	"	"
W12-12	"	"	"	"
W12-21	"	"	"	Hly^- variant isolated after incubation with AF
W12-22	"	"	"	"
W12-31	"	"	"	Hly^- variant isolated after incubation with EB
W12-32	"	"	"	"
B17-4F	"	"	B17-4	Hly^- Fus
W12-1F	"	"	W12-1	Hly^- Fus
JH2-21	*Streptococcus faecalis*		JH2-1	JH2-1 Transconjugant Hly^+, Fus
JH2-22	"	"	"	"
JH2-23	"	"	"	"
JH2-24	"	"	"	"
JH2-4	"	"	"	"
JH2-5	"	"	"	"
JH2-6	"	"	"	"
JH2-7	"	"	"	"
JH2-79	"	"	"	"
JH2-80	"	"	"	"
JH2-81	"	"	"	"
JH2-82	"	"	"	"
JH2-12	"	"	"	"
JH2-13	"	"	"	"
JH2-14	"	"	"	"
JH2-15	"	"	"	"
JH2-17	"	"	"	"
JH2-18	"	"	"	"
JH2-19	"	"	"	"

TABLE 4 (Cont'd)

JH2-20	*Streptococcus faecalis*	JH2-1	Hly^+, Fus
JH2-100	"	"	Km, Nm, Sm, Em, Fus
JH2-101	"	"	"
JH2-102	"	"	"
JH2-104	"	"	Em, Fus
B21R	"*Streptococcus faecalis* var *zymogenes*"	B21	Hly^+, Rif
PB4R	"	PB4	Hly^+, Rif
PB79R	"	PB79	Hly^+, Rif
B21-1	"	B21	Hly^- variant isolated after incubation at 45°C
B21-2	"	"	"
B21-3	"	"	"
B21-4	"	"	"
B21-5	"	"	"
B21-6	"	"	"
B21-7	"	"	"
B21-8	"	"	"
B21-9	"	"	"
B21-10	"	"	"
B21-11	"	"	"
B21-12	"	"	"
B21-13	"	"	"
B21-14	"	"	"
B21-15	"	"	"
B21-16	"	"	"
B21-17	"	"	"
B21-18	"	"	"
B21-19	"	"	"
B21-20	"	"	"
B21-21	"	"	"

TABLE 4 (Cont'd)

B21-22	"Streptococcus faecalis var zymogenes"	B21	Hly^- variant isolated after incubation at 45°C
B21-23	"	"	"
B21-24	"	"	"
B21-25	"	"	"
B21-26	"	"	"
B21-27	"	"	"
B21-28	"	"	"
B21-29	"	"	"
B21-30	"	"	"
B21-31	"	"	Hly^- variant isolated after incubation with AF
B21-32	"	"	"
B21-41	"	"	Hly^- variant isolated after incubation with AO
B21-42	"	"	"
B21-51	"	"	Hly^- variant isolated after incubation with EB
B21-52	"	"	"
B21-53	"	"	"
B21-54	"	"	"
B21-55	"	"	"
B21-56	"	"	"
B21-57	"	"	"
B21-58	"	"	"
PB4-1	"	PB4	Hly^- variant isolated after incubation at 45°C
PB4-2	"	"	"
PB4-3	"	"	"
PB4-4	"	"	"
PB4-5	"	"	"
PB4-6	"	"	"
PB4-7	"	"	"

TABLE 4 (Cont'd)

PB4-8	"Streptococcus faecalis var zymogenes"	PB4	Hly^- variant isolated after incubation at 45°C
PB4-9	"	"	"
PB4-10	"	"	"
PB4-11	"	"	"
PB4-12	"	"	"
PB4-13	"	"	"
PB4-14	"	"	"
PB4-21	"	"	Hly^- variant isolated after incubation with AF
PB4-31	"	"	Hly^- variant isolated after incubation with AO
PB4-32	"	"	"
PB4-41	"	"	Hly^- variant isolated after incubation with EB
PB4-42	"	"	"
PB4-43	"	"	"
PB4-44	"	"	"
PB4-45	"	"	"
PB79-1	"	PB79	Hly^- variant isolated after incubation at 45°C
PB79-2	"	"	"
PB79-3	"	"	"
PB79-4	"	"	"
PB79-5	"	"	"
PB79-6	"	"	"
PB79-7	"	"	"
PB79-8	"	"	"
PB79-9	"	"	"
PB79-10	"	"	"
PB79-11	"	"	"
PB79-12	"	"	"

TABLE 4 (Cont'd)

PB79-13	"Streptococcus faecalis var zymogenes"	PB79	Hly^- variant isolated after incubation at 45°C
PB79-14	" "	"	"
PB79-15	" "	"	"
PB79-21	" "	"	Hly^- variant isolated after incubation with AF
PB79-22	" "	"	"
PB79-31	" "	"	Hly^- variant isolated after incubation with AO
PB79-41	" "	"	Hly^- variant isolated after incubation with EB
PB79-42	" "	"	"
PB79-43	" "	"	"
PB79-44	" "	"	"
PB79-45	" "	"	"
W100R	Streptococcus faecium	W100	Km, Nm, Sm, Em, Rif.
W100-1	" "	"	Antibiotic sensitive variant isolated after incubation at 45°C
W100-2	" "	"	"
W100-3	" "	"	"
W100-4	" "	"	"
W100-5	" "	"	Antibiotic sensitive strain isolated after incubation with AF
W100-6	" "	"	"
W100-7	" "	"	Antibiotic sensitive strain isolated after incubation with AO
W100-8	" "	"	Antibiotic sensitive strain isolated after incubation with AF
W100-9	" "	"	"
B19-1	" "	B19	Fus
B19-100	" "	"	B19 Transconjugant Km, Nm, Sm, Em, Fus

TABLE 4 (Cont'd)

W99-1	"Streptococcus faecium var casseliflavus"	W99	Non-pigmented strain isolated after incubation at 45°C
W99-2	" "	"	Non-pigmented strain isolated after incubation with AF
W99-3	" "	"	"
W99-4	" "	"	Non-pigmented strain isolated after incubation with EB
W99-5	" "	"	"
W99-6	" "	"	"
W99-7	" "	"	"
W106-1	" "	W106	Non-pigmented strain isolated after incubation at 45°C
W106-2	" "	"	"
W106-3	" "	"	"
W106-4	" "	"	Non-pigmented strain isolated after incubation with AF
W106-5	" "	"	"
W106-6	" "	"	Non-pigmented strain isolated after incubation with EB

Abbreviations are on the next page.

Abbreviations

*	Type strain
ATCC	American Type Culture Collection
NCDO	National Collection of Dairy Organisms
NCIB	National Collection of Industrial Bacteria
NCTC	National Collection of Type Cultures
K	Dr. R.K.A. Feltham
W	Dr. Dorothy Jones, Leicester University
R	Public Health Laboratory Services (PHLS)
KO	Leicester Royal Infirmary
RGM	Dr. R.G. Mitchell, Oxford
A.S.	Adult Septicaemia
N.E.O.S.	Neonatal Early-onset Septicaemia
N.E.O.M.	Neonatal Early-onset Meningitis
N.L.O.M.	Neonatal Late-onset Meningitis
N/T	Nose and Throat

Dr. A. Jacob, Dept. of Bacteriology & Virology, University of Manchester

Hly^+	β-Haemolytic strain
Hly^-	Non-haemolytic strain
Lac^-	Lactose, non-fermenting strain
AO	Acridine Orange
AF	Acriflavine
EB	Ethidium Bromide
Fus	Resistance to Fusidic Acid
Rif	Resistance to Rifampicin
Km	Resistance to Kanamycin
Nm	Resistance to Neomycin
Sm	Resistance to Streptomycin
Em	Resistance to Erythromycin

TABLE 5 Salient characteristics of the streptococci of serological Group B used in study

Strain numbers	Species	Source	Lactose fermentation	Pigment production	Starch hydrolysis
B14	Str. agalactiae	Bovine	+	-	+
B32	" "	"	+	-	+
B33	" "	"	+	+	+
B34	" "	"	+	-	+
B1	Str. species	Human	+	+	+
B2	" "	"	+	+	+
B3	" "	"	+	+	+
B4	" "	"	-	+	+
B6	" "	"	-	+	+
B7	" "	"	-	+	+
B8	" "	"	-	+	+
B9	" "	"	-	+	+
B10	" "	"	-	+	+
B35	" "	"	-	+	+
B38	" "	"	-	+	+
B39	" "	"	-	+	+
B41	" "	"	-	+	+
B42	" "	"	-	+	+
B43	" "	"	-	+	-
B46	" "	"	-	+	+
B47	" "	"	-	+	+
B48	" "	"	-	+	+
B49	" "	"	+	+	+
B50	" "	"	-	+	+
B51	" "	"	-	+	+
B54	" "	"	-	+	+
B72	" "	"	-	+	+

TABLE 5 (Cont'd)

B84	Str. species	Bovine	-	-	+
B87	"	"	-	+	+
B90	"	"	-	+	+
B94	"	"	-	+	+
B96	"	"	-	+	+
B97	"	"	-	+	+
B98	"	"	-	+	+
B99	"	"	-	+	+
B105	"	"	-	+	+
B108	"	"	+	+	+
B112	"	"	+	-	+
B130	"	"	-	+	+

Solid media

1) Blood Agar Base

Blood Agar Base No. 2 (BAB2) (Difco) was used as the general medium for maintenance of cultures and as basal medium for a number of tests. The composition of the medium was as follows:-

	Grams per litre
Proteose Peptone	15.0
Liver Digest	2.5
Bacto-Yeast Extract	5.0
Sodium Chloride	5.0
Bacto-Agar	12.0

The medium was sterilized by autoclaving at 121°C for 15 min., cooled to 60°C and dispensed into Petri dishes.

When BAB broth was required, the same ingredients minus agar were used; and the resulting broth was sterilized as described above.

2) Blood media

Horse blood agar plates were prepared by adding 7.5% (v/v) defibrinated horse blood (Difco) to the sterile molten BAB2 preparation (pre-cooled to about 50°C) and dispensed into Petri dishes. Instead of horse blood, 5% defibrinated sheep blood (Difco) was used in exactly the same way as described above to prepare sheep blood agar plates.

3) Lactose fermentation assay medium

Assays for lactose fermentation were carried out on MacConkey agar (Oxoid) plates. The composition of the medium was as follows:-

	Grams per litre
Peptone	20.0
Lactose	10.0
Bile Salts	5.0
Sodium Chloride	5.0
Neutral red	0.075
Agar No. 3	12.0

The medium was sterilized by autoclaving at 121°C for 15 min.

4) Antibiotic media

Antibiotic plates were prepared by adding filter sterilized antibiotics to the sterile molten BAB2 preparation at about 50°C.

Antibiotics used

Erythromycin (Em) (Sigma)
Fusidic acid (Fus) (Leo Pharmaceutical Product)
Kanamycin sulphate (Km) (Sigma)
Neomycin sulphate (Nm) (Sigma)
Streptomycin (Sm) (Sigma)
Rifampicin (Rif) (Sigma)

All the antibiotics used except rifampicin were dissolved in a minimal volume of distilled water and sterilization was achieved by filtering through a sterile millipore filter (Millipore Corporation) of pore size 0.45 µm. Rifampicin (5 mg) was dissolved in 1 ml of 0.1M HCl and filter sterilized.

General liquid medium

Brain Heart Infusion (BHI) (Oxoid) broth was routinely used to grow cells. The medium has the following composition:-

	Grams per litre
Calf Brain Infusion Solids	12.5
Beef Heart Infusion Solids	5.0
Proteose Peptone	10.0
Sodium Chloride	5.0
Dextrose	2.0
Disodium Phosphate	2.5

The medium was sterilized by autoclaving at 121°C for 15 min.

Viable Counts

Viable counts were performed on broth cultures to determine the dilutions needed to produce the required inoculum containing a known number of cells.

A 100 ml BHI culture of the organism was grown statically at 35°C for 18 hr. Tenfold serial dilutions were made in 0.9% (w/v) saline to

give dilutions of 10^{-1} to 10^{-8}. Aliquots (0.1 ml) of each dilution were spread inoculated with a bent glass rod onto BAB2 plates, which were examined after 48 hr incubation at 35°C. For viable counts three plates were inoculated for each dilution. Those plates giving colonies sufficiently discrete to be countable (generally where there were no more than 250 colonies per plate) were counted using a colony counter.

Curing experiments

The concentration of curing agent used must fulfil two requirements.

1) It must be a sub-inhibitory concentration, i.e. produce 1-99% inhibition of growth.

2) It must produce loss of the requisite character at a readily detectable rate.

To determine the correct concentration of the agent to be used, the following were estimated:-

A) Survival rate of organism in curing mixture.

B) Efficiency with which characters were cured.

A) Survival rate of organisms

i) At varying concentrations of Ethidium Bromide (EB) (Sigma).

The method used was based on that of Bouanchaud *et al.* (1969). An ethidium bromide solution (2.4 mg/ml) was prepared and autoclaved (121°C for 15 min) on the day of use. Appropriate volumes of this were added to 10 ml amounts of sterile BHI broth to give final concentrations of 0 to 4.0 µg/ml of the curing agent.

Each 10 ml of broth was inoculated with 10^4 cells and shaken at 35°C for 18 hr. A control broth containing no ethidium bromide was similarly treated.

Tenfold serial dilutions of the broths were made in 0.9% (w/v) saline, the final dilution being 10^{-6}. Each dilution was plated in triplicate in 0.1 ml amounts onto BAB2 plates and the colonies were counted after 48 hr incubation at 35°C. The control cultures were treated in the same way.

ii) At varying concentrations of Acridine Orange (AO) (Sigma).

Acridine orange solution (2 mg/ml of distilled water) was prepared and autoclaved at 121°C for 15 min on the day of use. Appropriate amounts of acridine orange solution were added to sterile BHI broth to give final concentrations of 5 to 40 µg/ml of acridine orange before inoculation of the broth with 10^4 cells. The culture was then incubated at 35°C for 18 hr. A control broth containing no curing agent was similarly treated.

Tenfold serial dilutions of the broths were made in 0.9% (w/v) saline, the final dilution being 10^{-6}. Each dilution was plated in triplicate on BAB2 in 0.1 ml amounts. For the control broth only 10^{-4} to 10^{-6} dilutions were plated.

The plates were incubated for 48 hr at 35°C and colonies were counted.

iii) At varying concentrations of Acriflavine (AF) (BDH).

Acriflavine solution (1 mg/2 ml of distilled water) was prepared and autoclaved at 121°C for 15 min. The method used was the same as described above for acridine orange, except that the final dye concentration in BHI broth was 0 to 4 µg/ml.

iv) Growth in broth at different temperatures.

The method used was based on that of Jacob & Hobbs (1974).

Duplicate 10 ml amounts of BHI broth were inoculated with 10^4 cells and incubated with shaking at 37°C, 42°C and 45°C for 18 hr. Tenfold serial dilutions of each of the broths were made in 0.9% (w/v) saline, the final dilution being 10^{-6}. Each dilution was plated in triplicate on BAB2 in 0.1 ml amounts. The plates were incubated 48 hr at 35°C and colonies were counted.

v) At varying concentrations of Sodium Dodecyl Sulphate (SDS) (Fisons).

The method used was based on that of Tomoeda *et al*. (1968).

Sodium dodecyl sulphate solution (20%) was prepared and sterilized at 121°C for 15 min on the day of use. An appropriate amount of SDS solution was added to sterile BHI broth to give final concentration of SDS of 0.5%, 1%, 2% and 5% (w/v).

A 10 ml amount of each broth was placed in a test tube and incubated with 10^4 cells. The control culture was prepared without SDS. The tubes were incubated at 35°C in a shaker for 3 days. Samples were taken at 48 and 72 hr and tenfold serial dilutions to 10^{-6} were made in 0.9% (w/v) saline. Each dilution was plated in triplicate on BAB2 in 0.1 ml amounts. For the control broth only 10^{-4} to 10^{-6} dilutions were plated.

The plates were incubated for 48 hr at 35°C and colonies were counted.

B) Efficiency with which characters were cured.

For each curing agent (and at both sampling times for SDS), 1000 to 3000 isolated colonies were picked at random from the recovery plates used to determine survival rates, and patched onto a master plate. These were grown up at 35°C overnight, and then plated on to BAB2 containing the appropriate selective agent. The plates were incubated and examined for cured strains at 24 and 48 hr. In this way, the efficiency of different concentrations of curing agents to produce cured strains could be determined.

From these tests, it was found that a concentration of ethidium bromide (3 to 4 µg/ml), acridine orange (25 to 30 µg/ml), acriflavine (3 to 4 µg/ml) and growth at 42°C and 45°C were suitable for curing the plasmid studied, but SDS had no curing properties for the organisms studied.

Isolation of antibiotic-resistant mutants

Strains were grown in 100 ml BHI broth for 18 hr at 37°C. The culture was then diluted to 10^{-6} in fresh BHI broth and 0.1ml of the diluted culture was spread inoculated on BAB2 plates containing the appropriate antibiotic.

Fusidic acid resistant mutants were selected on BAB2 containing 25 and 50 µg of fusidic acid per ml. Mutant colonies resistant to 200 µg

of fusidic acid per ml arose at a frequency of 2×10^{-9} per viable cell.

Mutants resistant to rifampicin (Rif) were obtained by using the same technique as described above. The diluted cells were spread inoculated on BAB2 plates containing 150 and 200 µg of rifampicin per ml. Mutants resistant to 200 µg/ml Rif appeared at a frequency of 2×10^{-9}.

Determination of the minimal inhibitory concentration (MIC) of antibiotics.

Overnight cultures were diluted to 10^{-6} in sterile saline and 0.1 ml of the diluted suspension was plated on to medium containing a range of concentrations (0-4500 µg/ml) of antibiotics. The colonies were counted after incubation at 37°C for 48 hr.

The MIC was taken as the concentration of antibiotic that resulted in a decrease of the efficiency of plating by at least 50%.

Plasmid transfer experiment

Mating in liquid medium was performed as described by Jacob & Hobbs, (1974).

Donor and recipient strains were grown overnight in BHI broth, standing at 37°C. The following morning both the cultures were diluted to 10^{8} cells/ml with fresh BHI broth. Diluted donor and recipient cultures were mixed in the ratio 1:10 and incubated at 37°C with gentle agitation for 4 hr. Dilutions 10^{-3} to 10^{-8} of the mating mixture were plated on BAB2 plates containing selective markers. Control plates were incubated from the donor and recipient cultures alone at 35°C, with the selective plates seeded with the mating mixture. All the plates were checked for transconjugants after 24 hr and 48 hr of incubation.

Presumed transconjugant strains from doubly selective media were plated again for single colonies on the same media before further confirmatory tests were performed.

Mating on membrane filters was done according to Burdett (1980). Donor and recipient strains were grown in BHI broth standing at 37°C. Fresh overnight cultures of both donors and recipients were diluted 1:10 in

fresh BHI broth and allowed to grow to mid-exponential phase ($\sim 5 \times 10^8$ colony-forming units). Donor and recipient cultures were mixed at a ratio of 1 donor (0.05 ml) to 10 recipients (0.5 ml) in a final volume of 5 ml of BHI broth. One millilitre of this mixture was collected on a sterile membrane filter (type HA; pore size 0.45 µm, Millipore), which was then placed on the surface of a BAB2 plate and incubated at 35°C. After 18 hr incubation, the cells on the filter were suspended in 1 ml of BHI broth, aliquots (0.1 ml) of dilutions were plated on BAB2 plates selective for transconjugants. The mating mixtures were also plated on appropriate medium to determine the total number of donors and recipients present. Controls consisting of unmated donors and recipients were treated in the same way. Frequencies were expressed as the number of transconjugants per donor organism. The transfer frequency of plasmid at different time intervals from donor to recipients was calculated after 0, 15, 30 and 45 min, and 1, 2, 3, 4 and 18 hr of contact on membrane filter, followed by inoculating the mating mixture on selective plates. Colonies were counted after 48 hr..

Plasmid Screening Procedures

A) Routine plasmid screening was performed according to the method of Leblanc & Lee (1979). Cultures were grown overnight in 20 ml BHI broth containing 20 mM glucose and 10 mM L-threonine (BDH). Cells were harvested by centrifugation and washed with 20 ml of 10 mM sodium phosphate buffer, pH 7.0. Washed cells were suspended in 0.73 ml of 50 mM Tris-hydrochloride (Sigma, pH 8.0) followed by the addition of 0.72 ml of lysozyme (BDH, 40 mg/ml in Tris-hydrochloride). After 20 min of incubation at 37°C, 0.5 ml of 0.25 M EDTA (BDH, pH 8.0) was added. The cell suspension was chilled for 5 min in ice water and lysis was achieved by the addition of 0.5 ml of 20% sodium dodecyl sulphate (SDS) in TE buffer (50 mM Tris-hydrochloride-10 mM EDTA, pH 8.0). Cell lysis was enhanced by 15 sec. incubations at 55°C followed by five gentle inversions in 15 sec. to mix. This procedure was repeated eight times. DNA in the lysate was denatured by the addition of 0.25 ml of 3N NaOH

(made fresh daily from a 10N stock solution) and mixed by 20 gentle inversions per min for 3 min. Unless the NaOH solution is standardized on the day of use, the volume of alkali required may vary from day to day. Thus, it was critical to check at least one sample on any given day to be certain that after the gentle mixing the pH was between 12.25 and 12.40, as suggested by Currier & Nester (1976). After alkali denaturation, the volume of the lysate was raised to 2.95 ml by the addition of water. The lysate was rapidly neutralized by the addition of 0.5 ml of 2M Tris-hydrochloride (pH 7.0) and mixed by 10 gentle inversions in 30 sec. An additional 0.5 ml of 2M Tris-hydrochloride was added, and the lysate was mixed again as described above. High-molecular weight chromosomal DNA was removed by SDS-NaCl precipitation by adding 0.65 ml of 20% SDS in TE, followed rapidly by the addition of 1.25 ml of 5M NaCl and immediate mixing by 20 inversions in 1 min. The sample was stored overnight at 4°C, and the supernatant fraction was collected after centrifugation for 30 min at 18,000 rpm, 4°C, in an SS 34 rotor (Sorvall). The volume of the supernatant fraction was doubled by the addition of water, and RNAase (BDH, 2 mg/ml in water, heated at 100°C for 15 min) was added to a final concentration of 100 µg/ml. After a 60-min incubation at 37°C, single-stranded DNA was removed by extraction with an equal volume of phenol (BDH) which was previously saturated with NaCl. The aqueous phase was then extracted with an equal volume of chloroform-isoamyl alcohol (24:1). The DNA in the aqueous phase was separated from degraded RNA by ethanol precipitation. A $^{1}/_{20}$ volume of 3M sodium acetate and 2 volumes of 95% ethanol were added and, after mixing, the sample was stored at -20°C overnight. Precipitated DNA was recovered by centrifugation at 18,000 rpm for 30 min at 4°C, in an SS 34 rotor. The DNA was suspended in 100 µl of TES buffer (10 mM Tris-hydrochloride-10 mM EDTA-50 mM NaCl, ph.8.0), and 20 µl was examined by agarose gel electrophoresis.

B) Plasmid screening was also performed as outlined by Casse et al. (1979), with minor modifications. Cultures were grown overnight on BHI broth containing 20 mM glucose and 10 mM L-Threonine in a final volume of 50 ml. Cells were harvested by centrifugation and washed twice with 10 mM sodium phosphate buffer (pH 7.0). Washed cells were suspended in 2 ml of 25% glucose in 100 mM Tris-hydrochloride (pH 8.0), followed by the addition of 1.8 ml of lysozyme (40 mg/ml in 100 mM Tris-hydrochloride, pH 8.0). The mixture was incubated at 37°C for 1 hr. After incubation, 1.25 ml of 0.25M EDTA (pH 8.0) was added. The suspension was chilled for 5 min in iced water. The cells were lysed by adding 1.25 ml of 20% sodium dodecyl sulphate in TE buffer (100 mM tris-hydrochloride - 10 mM EDTA, pH 8.0). Cell lysis was enhanced by incubating the mixture at 55°C for 3 minutes followed by 15 gentle inversions to mix. DNA in the lysate was denatured by the addition of 0.625 ml of 3N NaOH and mixed by gentle inversions for 3 minutes. The lysate was rapidly neutralized by the addition of 1.25 ml of 2M Tris-hydrochloride (pH 7.0). The lysate was adjusted to 3% (w/v) NaCl and after 30 min 9 ml phenol (previously saturated with a solution of 3% (w/v) NaCl in water) was added. The two phases were mixed by stirring at 300 rev. per min for 10 sec, and further stirred for 2 min at 100 rev. per min. The mixture was then centrifuged at 12,000 r.p.m. for 10 min and the clear aqueous upper phase was transferred, using a Pasteur pipette, into a sterile tube. It was brought to 0.3M-sodium acetate and 2 volumes of cold (-20°C) 95% ethanol were added to precipitate the DNA. The tube was kept at -20°C overnight. The precipitated DNA was recovered by centrifuging at 18,000 r.p.m. for 30 min. The ethanol was removed from the tube and the DNA pellet was dissolved in 100 µl TES buffer (50 mM Tris-HcL-5 mM EDTA-50 mM NaCl, pH 8.0). The tubes were held under vacuum for 5 min to remove residual ethanol. The DNA sample was analyzed immediately by agarose gel electrophoresis or stored at -20°C until ready for use.

Detection and isolation of labelled plasmid DNA

Labelling and lysis of cells were performed according to Marder & Kayser (1977). Cells, grown overnight in BHI broth, were diluted to approximately $5x10^6$ cells/ml in fresh BHI broth containing 100 µci of [methyl-^{3}H] thymidine (24 ci/mmols; Radiochemical Centre Amershams) for 8 ml of medium. The cultures were grown to about 10^9 cells/ml with aeration. The cells were harvested and washed twice in TES buffer (50 mM NaCl-50 mM Tris-Hcl-5 mM Na_2 EDTA, pH 8.0) at 4°C. The cell pellet was suspended in 0.25 ml of TES buffer containing 100 mg of sucrose per ml. This was followed by the addition of 0.25 ml of TES buffer containing 2 mg of lysozyme per ml, 1 mg of RNAase (ribonuclease) per ml and 100 mg of sucrose per ml. The suspension was incubated for 30 min at 37°C and cooled for 5 min in ice. The cells were lysed by the addition of 0.25 ml Sarkosyl (Geigy; 2.4% in water) and gently mixed. After the addition of 0.5 ml of TES buffer at room temperature, the lysate was slowly drawn in and out of a 1 ml pipette 20 times. Ethidium bromide solution to a final concentration of 300 µg/ml was added to the sheared lysate followed by the addition of caesium chloride (Fisons) to a final concentration of 1.12 gm per gm of solution. The samples were centrifuged for 48 hr at 40,000 r.p.m. in a Ti 10x10 rotor at 22°C in an MSE65 ultracentrifuge. Gradiants were fractionated by puncturing the bottom of the centrifuge tube and 4-drop fractions were collected in each tube. Radioactivity in each fraction was determined by applying 20 µl samples of each fraction on to Whatman No. 1 filters which were then dried. The dried filters were washed twice in cold 5% TCA, rinsed in cold 96% ethanol, dried and placed in toluene-based scintillation fluid [PPO(2, 5-diphenyl oxazole), 5g per litre; dimethyl-POPOP (1, 4-bis-2-[4-methyl-5-phenyl oxazole]-benzene), 0.3 g per litre] and counted in a Packard 3255 liquid scintillation spectrophotometer. Fractions corresponding to the plasmid peak were pooled and the ethidium bromide was extracted by two successive treatments with CsCl-saturated isopropanol. After dialysis against four changes of TE buffer (10 mM

Tris-Hcl, 1 mM EDTA, pH 7.4), over 24 hr., the DNA samples were frozen and stored at -20°C.

When labelling of DNA was performed only to screen the presence of plasmid DNA in the bacteria, then radioactivity in each fraction was determined by adding scintillation fluid directly to the tubes containing different fractions and counts were performed in a Packard liquid scintillation spectrophotometer.

Preparation of plasmid DNA

The method of Courvalin et al. (1980) was used.

Cells were grown in 100 ml BHI broth containing 20 mM glucose and 10 mM L-Threonine at 37°C with shaking. After overnight incubation the cells were harvested by centrifugation and re-suspended in 10 ml of SET buffer (150 mM Nacl-100 mM EDTA-50 mM Tris-hydrochloride, pH 8.0) containing lysozyme (1 mg/ml) and RNase (500 µg/ml). This suspension was incubated at 37°C for 1 hr. To lyse the resulting protoplasts, Sarkosyl (2% in water) was added to a final concentration of 0.1% followed by incubation at 37°C for 15 min. The lysate was slowly drawn in and out of a 1 ml pipette 20 times. Ethidium bromide solution was then added to the sheared lysate to give a final concentration of 300 µg/ml. Then, 1.12 gm of caesium chloride (CsCl) was added per gram of solution. After mixing, the refractive index of the solution was adjusted to between 1.392 and 1.398. The sample was then centrifuged for 48 hr at 40,000 r.p.m. in a Ti 10x10 rotor at 22°C in a MSE65 ultracentrifuge. The plasmid DNA band was viewed by exposure to long-wave UV light and was removed by a syringe. Ethidium bromide was removed by two extractions with equal volumes of cesiumchloride saturated isopropanol.Caesium chloride was removed by dialysis against TE buffer (10 mM Tris-Hcl-1 mM EDTA, pH 7.4), allowing 2-3 hr for the first change of buffer and 4-6 hr between each change for the next three changes of buffer at 4°C. The dialysed plasmid DNA was collected in a sterile tube and the concentration of DNA in the preparation was determined by scanning 10 µl of DNA diluted to 1 ml at 220 nm - 300 nm with a Pye Unicom SP1800 spectro-

photometer. A concentration of 50 µg of DNA in the solution gives an optical density (O.D.) at 260 nm of 1.0. The purified plasmid DNA was stored at -20°C.

Restriction enzyme analysis

Plasmid DNA preparations (1 to 2 µg) maintained in 10 mM Tris-Hcl, 1 mM EDTA,(pH 7.4) were subjected to cleavage with restriction endonucleases Bam HI and Hind III according to the method of Davis et al. (1980). Bacteriophage λ (λcI 857) DNA was used as molecular weight markers and cleaved with restriction endonuclease Hind III alone or doubly digested with Hind III and Eco RI (Davis et al.,1980). Table 6 shows the molecular weights of the λ fragments generated by these restriction endonucleases(Daniels et al., 1980). All digestions were performed at 37°C for 3 hr followed by a 5 min heat pulse at 70°C to inactivate the enzyme. Restriction endonucleases Bam HI, Eco RI and Hind III were purchased from Bethesda Research Laboratories.

When double digestions were performed, the enzyme requiring the lower salt concentrations was used first, and appropriate adjustments were made to the reaction buffer before the addition of the second enzyme. The resulting DNA fragments were separated by electrophoresis in agarose gels of varying concentrations (see Figure legends for details).

The molecular weights of uncharacterized DNA molecules were estimated from their mobilities in agarose gels by comparison with the mobilities of λ DNA fragments of known size. Mobilities were measured from a photograph of the ethidium bromide stained agarose gel and molecular weights were calculated using the computer program of Schaffer & Sederoff (1981).

Agarose gel electrophoresis

Plasmid DNAs were analysed by electrophoresis in agarose (Miles Laboratories Ltd.) gels made in Tris-acetate buffer (40 mM Trisbase, 20 mM Acetic acid and 2 mM Na_2EDTA). The concentration of agarose and the type of gel used is detailed in the Figure legends. A dye solution consisting of bromophenol blue (0.025%), sodium dodecyl sulphate (5%) and glycerol (50%) in water was added to the DNA sample in the ratio of 1:4 prior to electro-

Table 6 λ DNA digested with different restriction enzymes, and the fragments generated are as follows:

λ Hind III	λ Hind III/Eco RI
Kb	Kb
23.4	21.57
9.6	5.37
6.8	5.055
4.42	4.222
2.29	3.60
1.95	1.945
0.585	1.8
	1.709
	1.33
	0.956
	0.820
	0.585

Kb = Kilobase

phoresis. The electrophoresis was carried out at 17 volts for 16 hr.

After electrophoresis was complete, the gel was stained in ethidium bromide (0.5 μg/ml) for about 30 min. The plasmid DNA bands were visualized and photographed under UV illumination.

Preparation of unlabelled 'total' cell Deoxyribonucleic acid (DNA) for DNA-DNA hybridization experiments.

Each strain was grown in 3 litres of BHI broth plus 20 mM glucose and 10 mM L-Threonine at 35°C. Cells were harvested in their late logarithmic or early stationary phase of growth. The wet packed cells were frozen at -20°C and stored at this temperature until used.

The method of Garvie (1976) was employed for the isolation and purification of total DNA.

(i) Lysing of cells

Cells (3-4 gm wet weight) were washed twice with distilled water and suspended in distilled water (10 ml of water per gm of cells). Proteinase K (Boehringer Mannheim; 5 mg/ml of cell suspension) was dissolved in a small amount of EDTA-saline (see Reagent section). Lysozyme (BDH; 10 mg per 2.3 gm cells) was dissolved in EDTA-saline. Both proteinase K and lysozyme were then added to the cell suspension and the whole mixture was incubated for 1 hr at 37°C. An appropriate volume of concentrated saline citrate (CSC, see Reagent section) was added to convert the cell suspension to standard saline citrate (SSC, see Reagent section). To this, 12% (w/v) 4-amino salicylic acid (Fisons) in SSC was added to give a final concentration of 3% (w/v). The whole suspension was then incubated overnight at 37°C.

(ii) Phenol Treatment

The cell suspension was centrifuged at 18,000 r.p.m. for 30 min. The supernatant was removed and chilled on ice while an aqueous solution of 6.25% (w/v) of the tri-isopropylnaphthalene sulphonic acid (IPNS; Eastman) was added to give a final concentration of 0.5% (w/v). The mixture was then incubated at 60°C for 10 min and chilled immediately in an ice bucket.

The viscous solution was deproteinized by shaking for 15 min with an equal volume of phenol water (see Reagent section) in a sinter round-bottomed flask at room temperature. An equal volume of chloroform iso-amyl alcohol (24:1, v/v) was added to help separate the layers on centrifuging. After centrifugation for 10 min at 15,000 r.p.m. the viscous aqueous upper layer containing DNA was removed by using blunt glass tubes. The DNA was then deproteinized by shaking for 15 min with an equal volume of chloroform iso-amyl alcohol (24:1, v/v). The layers in this mixture were separated by centrifugation at 15,000 r.p.m. for 10 min and the DNA in the aqueous upper layer was transferred into a graduated cylindrical beaker. The DNA was precipitated with twice its volume of cold ethanol. The ethanol was added by careful pouring down the side of the beaker, so that two layers were obtained. The DNA was then collected on a spooling rod and stored in dilute saline citrate (DSC, see Reagent section) at 4°C until the next deproteinization step.

(iii) Sodium deoxycholate treatment

The crude DNA obtained as described above was dissolved by gentle shaking for 15 to 30 min in a small volume of DSC in a water bath held at 37°C. The preparation was then transferred to a sintered glass cylinder and the total volume of dissolved DNA solution was recorded and adjusted to SSC with CSC.

Ribonuclease (BDH) 6 mg per 100 ml of DNA preparation was dissolved in a small volume of 0.15 M NaCl (see Reagent section) and incubated at 80°C for 10 min. The crude DNA solution was treated with the RNase solution for 1 hr at 37°C and then with proteinase K (5 mg/ml DNA solution) for 1 hr at 37°C. The volume was adjusted with SSC to make the total volume divisible by 9. An equal volume of 4M NaCl/citrate (see Reagent section) was added. The solution was then deproteinized with enough 2% (w/v) sodium deoxycholate in SSC to give a final concentration of 0.2% (w/v). After 1 hr incubation at 37°C, the solution was held overnight at 4°C. If a gel formed it was gently

dispersed and the DNA was separated from impurities by centrifugation at 15,000 r.p.m. for 10 min. The DNA was precipitated with a double volume of ethanol (AR) and collected on a spooling rod.

The procedure was then repeated, excluding the proteinase K treatment, and chloroform iso-amyl alcohol (24:1,v/v) was used for deproteinization in place of sodium deoxycholate.

(iv) Iso-propyl alcohol precipitation

The DNA was dissolved in a small volume of DSC and then the volume was adjusted with DSC so that the final volume was divisible by 9. EDTA-acetate solution was added to the DNA solution to a final concentration of 10% (v/v). A fine burette tilted at at angle of 30° was used to add isopropyl alcohol (equivalent to half the volume of the DNA solution) drop by drop with simultaneous spooling of DNA until the solution cleared. The spooled DNA on the rod was finally washed in ethanol/water mixtures, 80%, 85%, 90%, 95% and 100% (v/v) consecutively and then transferred to dram vials. To each vial 2 ml of DSC and a drop of chloroform were added and stored at 4°C.

Preparation of labelled total cell DNA for DNA-DNA hydridization experiments

Radioactively-labelled DNA were prepared by adding 100 µci amounts of [methyl-^{3}H] thymidine to each 1 litre of culture medium used. The labelled DNA was isolated and prepared as previously described for the unlabelled DNA.

Preparation of labelled plasmid DNA for DNA-DNA hybridization experiments

The details of this procedure are given on page 95 under "Detection and isolation of labelled plasmid DNA".

Hybridization of DNA

The method used was based on the membrane-filter method of Denhardt(1966). Filters were loaded with heat-denatured unlabelled DNA as described by De Ley & Titgat (1970) using 25 mm-diameter membrane filters (type HA; Millipore Corporation). The difference in optical density at 260 nm of the DNA solution before and after filtration was used to calculate the amount of DNA on each filter. The calculation assumed that 1 µg of

heated DNA per ml gave an optical density of 260 nm of 0.028.

The filters were washed with 10 ml of 6xSSC and allowed to dry at room temperature for 2 to 3 hr. They were then heated at 80°C for 2 to 3 hr and stored at 4°C in a vacuum jar with phosphorus pentoxide. Blank filters were also prepared, saturated with 6xSSC but without any DNA.

The DNA-loaded filters were cut into quarters and marked with a pencil for identification. Two quarters from each filter were used in each hybridization.

The labelled DNA was diluted in DSC, sheared by passing ten times through a hypodermic needle (diam. 25 G x $^{5}/_{8}$) and denatured by boiling in a boiling-water bath for 10 min, and then plunging into ice-water. The amount of labelled DNA was also estimated by measuring the optical density (260 nm) of the solution.

Two filter quarters containing the unlabelled DNA together with the blank filter were all put in one flask to which 2 ml of 30% dimethyl sulphoxide in 2xSSC was added. 0.50 ml of the labelled denatured DNA was then added and the flask was incubated in a shaking water bath at 60°C overnight. The filter quarters were then washed 3 times with 2xSSC. After the washing 200 µl of Nuclease S_1 (Sigma) in buffer (see Reagents, Sigma) were added and the whole incubated for 2 hr at 37°C. The filters were then washed 3 times with 2xSSC and dried under a UV light for 30 min. The dried filter quarters were put in scintillation vials and 3 ml of "Fiso Flour 1" scintillation cocktail (Fisons) were added. The count per minute was estimated for each vial using a Packard Scintillation Counter (Packard, Prias),

The hybridizations with radioactively labelled DNA were done twice, and the mean was taken as the figure quoted in Table 22 .

DNA-DNA duplex formation between labelled plasmid DNA and the whole cellular DNA of "Str. faecalis var. zymogenes" and "Str. durans" containing the appropriate plasmid species was assayed according to So et al.(1975) as previously described for hybridization of total labelled cell DNA with total unlabelled whole cell DNA ((Denhardt, 1966).

The labelled plasmid DNA was sheared by passing ten times through a hypodermic needle and denatured by boiling in a boiling-water bath for 10 min, and then plunging into ice-water.

Two filter quarters containing the unlabelled DNA from the 7 strains together with the blank filter were all put in one flask to which 4 ml of 30% dimethyl sulphoxide in 2xSSC was added. One ml of the labelled denatured plasmid DNA was then added and the flask was incubated in a shaking water bath at 60°C overnight. The filter quarters were then washed 3 times with 2xSSC. Washed filters were incubated with 200 µl Nuclease S_1 at 37°C for 2 hr. The filters were then washed 3 times with 2xSSC and dried under a UV light for 30 min. The dried filter quarters were put in scintillation vials and 3 ml of scintillation cocktail were added, and the count per minute was estimated.

Reagent Section

Concentrated Saline Citrate (CSC)

1.5 M Sodium Chloride	87.66 gm
0.15 M Trisodium Citrate	44.115 gm
Distilled water to	1 litre

The solution was made in a volumetric flask. The solid ingredients were dissolved in 800 ml distilled water and the pH was adjusted to 7.0 $\pm$ 0.2 with 2 or 3 drops of IN HCl. The volume was then made up to 1 litre.

Standard Saline Citrate (SSC)

This is a 10 x dilution of CSC, i.e. 10 ml CSC made up to 100 ml with distilled water.

Dilute Saline Citrate (DSC)

This is a 100 x dilution of CSC. It was best made by diluting CSC to SSC and SSC (10x) to DSC.

EDTA-Saline

0.15 M Sodium Chloride	8.8 gm
0.10 M Ethylene diamine tetra acetic acid (EDTA Sodium Salt)	37.2 gm
Distilled water to	1 litre

900 ml distilled water was added to solids. The solution was adjusted to pH 7.0 with 3 M NaOH (120 gm/litre) and the volume made up to 1 litre with distilled water.

Phenol water

Phenol (BDH, AR) 50 gm was dissolved in 5 ml distilled water by placing the flask in a water bath at 60°C for 30 min. The pH of the solution was adjusted to 6.8 by adding 1 ml of 1 M phosphate buffer. Approximately 20 ml of SSC was added to this solution and shaken for 10 min. The solution was transferred to a separating funnel and left at 4°C overnight. The lower layer of phenol was retained.

Sodium Chloride Citrate (4 M)

4 M Sodium Chloride	233.76 gm
0.1 M Sodium Citrate	29.41 gm
Distilled water to	1 litre

The solid ingredients were dissolved in 900 ml distilled water and the solution made up to 1 litre.

Acetate-ethylene diamine tetracetic acid

3 M Sodium Acetate (anhydrous)	246.09 gm
0.001 M EDTA	0.372 gm
Distilled water to	1 litre

The pH was adjusted to 7.0 with a few drops of 3 M acetic acid.

Nuclease S_1 buffer

2.5 M Sodium Chloride	13.6 gm
0.3 M Sodium Acetate	2.46 gm
45 mM Zinc Sulphate	0.612 gm
Distilled water	100 ml

The pH was adjusted to 4.5 with 3 N HCl. The buffer was diluted 10 times when used with Nuclease S_1 (Sigma).

Phosphate buffer (1 M)

A K_2HPO_4 8.709 gm dissolved in 50 ml distilled water

B KH_2PO_4 6.804 gm " " " " "

Solution A, 50 ml was added to 42 ml of solution B. The pH of the final solution was 6.8.

Detection of phenotypic traits

Haemolysis

Strains were streaked to give single colonies on BAB2 plates containing 7.5% (v/v) horse blood or BAB2 plates containing 5.0% (v/v) sheep blood. After overnight incubation at 35°C, the colonies were examined by the naked eye. The plates were inspected for areas of clearing (β haemolysis) or greening (α haemolysis) around the colonies. The results were then scored as β, α or non-haemolytic. "Str. faecalis var. zymogenes" JH2-27 was used as a positive control.

Gelatin hydrolysis

a) The medium was of the following composition:

BAB broth	1000 ml
Bacto-gelatin (Oxoid)	120 gm

The medium containing the gelatin was steamed for 30 min to dissolve the gelatin. The resulting solution was then dispensed in 10 ml amounts into universal bottles and autoclaved at 121°C for 15 min.

The medium in the universal bottles was stab inoculated and incubated at 35°C. After 5 and 14 days the bottles were refrigerated at 4°C for two hours and then examined for gelatin liquefaction.

Proteus vulgaris was used as a positive control. An uninoculated control was always included.

b) The method to be described is that of detection of a change in composition of the gelatin due to bacteria rather than the detection of liquefaction (Frazier, 1926). Gelatin agar medium used consists of 3 solutions and have the following ingredients:

Solution 1

	Grams per litre
NaCl	50.0
KH_2PO_4	5.0
K_2HPO_4	15.0

Solution 2

	Grams per litre
Bacto-gelatin (Oxoid)	10.0
Bacto-peptone	0.25
Dextrose	0.125
Beef infusion	12.5 ml

Solution 3

Agar No. 1 (Oxoid)	30.0

100 ml of solution 1 was mixed with 400 ml of solution 2, heated in a steamer and then mixed with 500 ml of solution 3. The pH of the medium was adjusted to 7.0 The gelatin-agar medium was then autoclaved at 121°C for 15 min, cooled to 60°C and dispensed into Petri dishes.

Two strains were inoculated, one on each side of the plate and incubated at 35°C for 48 to 72 hr.

After incubation the plates were flooded with an acid-solution of bichloride of mercury of the following composition:

$HgCl_2$	15 gm
HCl (conc.)	20 ml

Distilled water to 100 ml.

If the gelatin had been changed, a clear zone appeared around the colony. This clear zone was surrounded by the cloudy precipitate of unchanged gelatin. The reaction is slow and takes 15-30 min for its completion.

Motility

The hanging drop method was employed to examine motility of the culture at X400 magnification. Cultures of the organisms in BHI broth, incubated at 35°C for 12 hr., were used.

Pigment production

a) Basal medium agar (BAB2) plates were inoculated and incubated for 48 hr. The plates were then left in the light for 9 days and observed for development of pigment.

b) Pigment production by strains of streptococci of serological Group B was

investigated using Islam's medium (Islam, 1977). The composition of the medium was as follows:

	Grams per litre
Proteose peptone No. 3 (Difco)	23.0
Soluble starch	5.0
Sodium dihydrogen phosphate dihydrate	1.482
Disodium hydrogen phosphate	5.749
Agar No. 1 (Oxoid)	10.0

The pH of the medium was adjusted to 7.4 by dropwise addition of potassium hydroxide. The medium was steamed for 5 min, then autoclaved for 15 min at 121°C. After cooling to about 60°C, 50 ml of sterile horse serum (Difco) was added to the medium and the plates were poured.

Plates were then streaked with the strains to be tested and incubated overnight at 35°C under anaerobic conditions, using the "Gas Pak" system (Oxoid). Cultures were examined for the production of an orange coloured pigment.

Potassium tellurite reduction and sensitivity

A 10% (w/v) aqueous solution of potassium tellurite (Fisons) was sterilized by autoclaving at 121°C for 5 min. This was added to sterile BAB2 to give final concentrations of 0.1 and 0.5% (w/v) potassium tellurite. The medium was then dispensed into Petri dishes. Plates were streaked to give single colonies and incubated at 35°C and examined after 1 and 2 days for inhibition of growth and also for the reduction of potassium tellurite which is indicated by the blackening of the colonies and of the medium if profuse growth occurs. Basal medium (BAB2) control Petri dishes were also inoculated and compared with the test plates.

Tetrazolium reduction and sensitivity

A 10% (w/v) aqueous solution of 2, 3, 5-triphenyl tetrazolium chloride (Fisons) was filter sterilized.

The BAB2 plus 0.2% glucose were autoclaved together and cooled to about 50°C. Then the filter-sterilized solution of 2, 3, 5-triphenyl tetrazolium

chloride was added to give final concentrations of 0.01% (w/v) and 0.1% (w/v).

The medium was mixed and dispensed into Petri dishes. Plates were streaked to give single colonies, incubated at 35°C and examined after 1 and 2 days for inhibition of growth and reduction of the tetrazolium which was indicated by a reddish-pink colouration of the growth and medium.

Hydrogen sulphide production

This was tested for in a medium of the following composition:

BAB2 (Difco)	1 litre
Cysteine hydrochloride	0.5 gm

Lead acetate paper strips were prepared by moistening strips (1 x 8 cm) of Whatman No. 1 filter-paper with 10% (w/v) neutral lead acetate solution. After drying, the strips were placed in a glass dish and sterilized by autoclaving. The medium was dispensed in 5 ml amounts in 6x0.4" test tubes after sterilization by autoclaving, then sloped and cooled. The slopes were inoculated by streaking, and a strip of lead acetate paper was placed inside the top of the test tube so that it hung freely in the tube. The tubes were then incubated at 35°C and examined after 1, 5, 7, 11, 14 and 21 days for blackening of the lead acetate paper. An uninoculated control tube was included.

Antibiotic sensitivity

The antibiotic resistance phenotypes of the bacterial strains were determined using three different sets of Multodisks (Oxoid Nos. 4561E, 4562E and 4563E). Thus each bacterial strain was tested against a total of 24 antibiotics (see Table 7). BAB2 plates were spread inoculated with about 10^8 cells from a 24 hr BHI culture. A multodisk was transferred aseptically on to each plate, and the plates were left at room temperature for 30 min to allow diffusion of the antibiotics. The plates were then incubated at 35°C for 2 days and examined each day for zones of inhibition of growth around each disk. Total and partial inhibition was recorded.

TABLE 7 Antibiotics tested

Symbol	Antibiotic name	Amount in disk
P	Penicillin G	5 units
SF	Sulphafuraxole	500 mcg
PN	Ampicillin	25 mcg
OB	Cloxacillin	5 mcg
E	Erythromycin	10 mcg
CB	Methicillin	10 mcg
NV	Novobiocin	30 mcg
OL	Oleandomycin	10 mcg
FR	Furazolidine	100 mcg
PY	Carbenicillin	10 mcg
CT	Colistin sulphate	10 mcg
CN	Gentamycin	10 mcg
K	Kanamycin	30 mcg
NA	Nalidixic acid	30 mcg
F	Nitrofurantoin	200 mcg
PB	Polymixin B	300 mcg
TE	Tetracycline	50 mcg
CR	Cephaloridine	25 mcg
C	Chloramphenicol	50 mcg
CH	Chlortetracycline	50 mcg
N	Neomycin	10 mcg
OT	Oxytetracycline	50 mcg
S	Streptomycin	25 mcg
SXT	Sulphamethoxazole & Trimethoprim	25 mcg

Acid production from carbohydrates

The API 50CH system (API System, La Balme Les Grottes, 3890 Montalieu Vercien, France) was used to test the production of acids anaerobically from carbohydrates. The system was employed in accordance with the manufacturer's instructions.

The cells were scraped off from the BAB2 plates which were incubated overnight at 35°C, and suspended in a sterile medium of the following composition:

Peptone	1%
NaCl	0.5%
Bromocresol purple	0.002%

in distilled water, pH 7.8. Results were scored on 3 hr, 6 hr, 24 hr, 2 days, 3 days, 4 days, 5 days and 6 days respectively. Production of acid was noted by a change in the colour of the bromocresol purple indicator, from purple to yellow.

Lactose fermentation

Strains were streaked on MacConkey agar plates to give single colonies and incubated at 35°C for 24 to 48 hr. Fermentation of lactose was indicated by the development of pink colonies.

Starch hydrolysis

Medium:	BAB2 (Difco)	1 litre
	Soluble starch	2 gm

The medium was autoclaved and dispensed into Petri dishes. The plates were inoculated with the organism and incubated at 35°C for 14 days. After incubation the plates were flooded with Gram's iodine. Hydrolysis of starch was indicated by either a colourless or very pale blue area around the bacterial growth. Areas of unhydrolysed starch remained dark blue.

Slime production from sucrose

The medium consists of:	Tryptone (Oxoid)	10 gm
	Yeast Extract	5 gm
	Agar No. 3	12 gm
	Sucrose	50 or 100 gm
	Distilled water to	1000 ml

The medium was sterilized by autoclaving. Two concentrations of sucrose were used (5% (w/v) and 10% (w/v). Plates were poured, inoculated and incubated at 35°C for 48 and 72 hr. The plates were examined by eye for the production of slime as a visible change in colonial morphology.

RESULTS

Erysipelothrix rhusiopathiae

E. rhusiopathiae strains C50, C334, C335, C337, C338 and C524, were analyzed for the presence of plasmid DNA molecules. Plasmid screening was performed by using the procedures of LeBlanc & Lee (1979), Casse *et al.*, (1979), and by using dye-buoyant density gradient centrifugation (see Methods). No plasmid DNA was detected in any of the *E. rhusiopathiae* strains analyzed. Even when strain C50 was labelled with tritiated thymidine and the DNA was centrifuged to equilibrium in CsCl —ethidium bromide density gradient, the result was negative. "*Str. faecalis* var. *zymogenes*" strain JH2-27 was used as a positive control.

Listeria

L. monocytogenes strains which showed β-haemolysis on sheep blood agar plates and non-haemolytic *L. monocytogenes* strains were examined for the presence of plasmid DNA. In addition, strains of other species of the genus *Listeria* (see Table 3) that showed no phenotypic evidence for the presence of extrachromosomal DNA were also examined. The plasmid screening procedures of LeBlanc & Lee (1979), Casse *et al.*,(1979) and the CsCl-ethidium bromide gradient centrifugation method (see Methods) which routinely yielded plasmid from known strains, gave consistently negative results except for one haemolytic strain of *L. monocytogenes* (C659). This strain was found to contain plasmid DNA that banded separately from chromosomal DNA after CsCl-ethidium bromide gradient centrifugation. Figure 4 shows the presence of the plasmid in strain C659 as a discrete band in agarose gel electrophoretograms. To correlate the presence of the plasmid in strain C659 with β-haemolysis, several attempts were made to cure the haemolysis trait of the organism by using different curing agents (see Methods), but no cured strain was isolated. Therefore another approach was taken to determine whether haemolysin production was encoded by the plasmid. An attempt was made to transfer the plasmid from C659 to a non-haemolytic *L. monocytogenes* strain C201 and to *Str. faecalis* strain JH2-1. *Str. faecalis* strain JH2-1 is a

non-haemolytic plasmid free strain, resistant to fusidic acid (250 µg/ml). L. monocytogenes strain C201 was made resistant to fusidic acid (200 µg/ml). Selection was done on sheep blood agar plates containing Fus(200 µg/ml) in order to identify any recipients which had acquired a haemolytic character. However, no transconjugants were obtained after 18 hr contact on filter and also after 4 hr of mixed incubation in liquid culture.

Since the plasmid pC659 is present in the haemolytic L. monocytogenes strain C659, and no haemolytic negative variant was isolated after the curing experiment nor were any transconjugants obtained after mating experiments, it is not known whether the β-haemolysis trait of L. monocytogenes strain C659 is encoded by this plasmid or by the chromosome.

Streptococci

1) Streptococcus agalactiae or Group B Streptococcus

Streptococcus strains of serological Group B isolated from both human and bovine sources were screened (see Table 5) for the presence of plasmid DNA according to the method of LeBlanc & Lee (1979) and by CsCl-ethidium bromide density gradient centrifugation. Of the screened strains only two strains of Str. agalactiae B14 and B32 (Figure 1) were found to contain plasmid DNA molecules. There was no obvious correlation between the presence of plasmids in these strains and any phenotypic traits. No attempt was made to investigate whether these plasmids were determining any phenotypic traits or were merely cryptic.

2) Streptococcus bovis

Strains B16, D598, D599, PB78, PB80,were analyzed for the presence of plasmid DNA by the method of LeBlanc & Lee (1979) and CsCl-ethidium bromide density gradient centrifugation. Plasmid DNA was detected in Str. bovis strains B16 and D598, after CsCl-ethidium bromide density centrifugation method. Strain JH2-27 was used as a control (Figure 4).

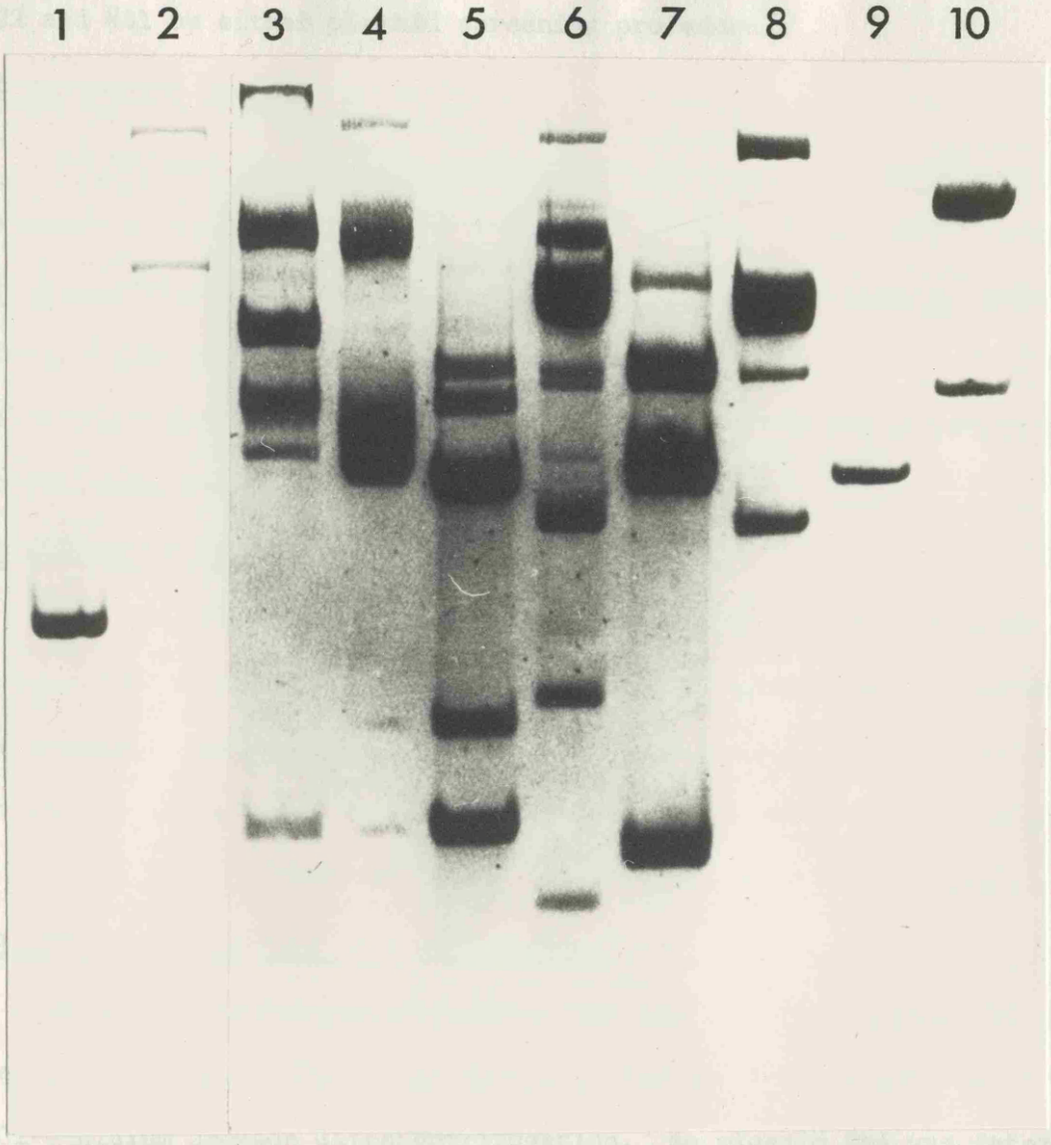
3)"Streptococcus durans"

Plasmid screening of haemolytic strains of "Str. durans" B17, W12

Figure 1 Detection of plasmid DNA by CsCl-ethidium bromide density gradient centrifugation method

Plasmid DNAs were isolated from CsCl-ethidium bromide density gradients and subjected to electrophoresis through a 0.7% agarose vertical gel for 16 hr at 17 volts. The tracks contain from left to right:

1) Str. faecalis strain JH2-27 containing the haemolytic plasmid pJH2
2) "Str. faecium var. casseliflavus" strain W107
3) "Str. faecium var. casseliflavus" strain W103
4) "Str. faecalis var. liquefaciens" strain PB85
5) "Str. faecalis var. liquefaciens" strain B20
6) Str. agalactiae strain B32
7) Str. agalactiae strain B14
8) "Str. faecium var. mobilis" strain W88
9) "Str. faecium var. casseliflavus" strain W99
10) "Str. faecium var. casseliflavus" strain W106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and non-haemolytic strains W22 and W31 were performed according to the method of LeBlanc & Lee (1979) and by using CsCl-ethidium bromide density centrifugation (see Methods). Plasmid DNA molecule was detected in both the haemolytic strains B17 and W12 (Figure 5), but no plasmid was detected in non-haemolytic strains of "Str. durans" W22 and W31 by either plasmid screening procedure.

4) Streptococcus equinus

Of the six Str. equinus strains (see Table 3) screened for the presence of plasmid DNA by the methods of LeBlanc & Lee (1979), Casse et al.(1979) and CsCl-ethidium bromide density centrifugation, only one strain D1090 was found to contain plasmid DNA (Figure 4). No obvious correlation between the presence of this plasmid and the phenotype of strain D1090 was noted.

5) Streptococcus faecalis

Strains B18, PB77, PB125 and W1 were examined for the presence of plasmid DNA, although their phenotype did not suggest such a presence. The method of LeBlanc & Lee was employed to screen the plasmid DNA. Strain JH2-27, a known plasmid containing strain was used as a control. No plasmid DNA was detected in any of the strains of Str. faecalis.

6)"Streptococcus faecalis var. liquefaciens"

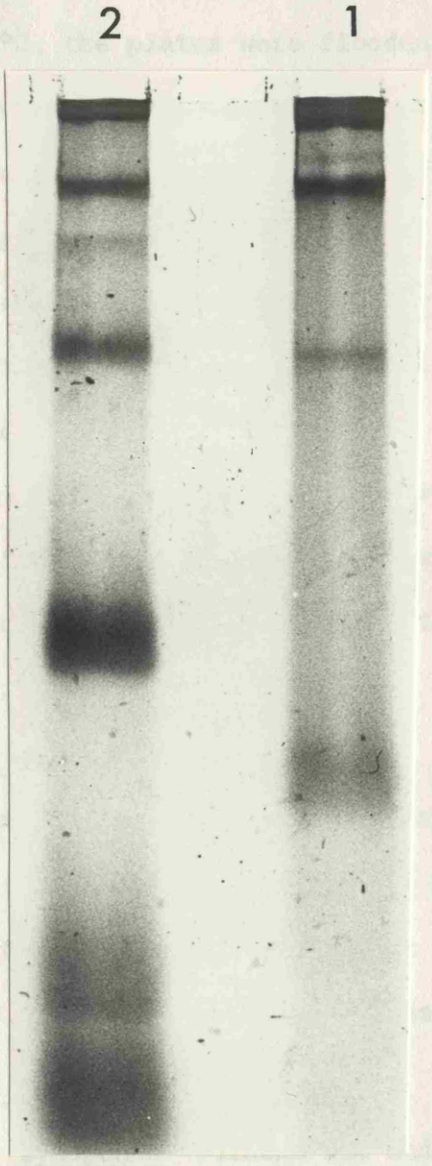
Gelatin liquefying strains B20, PB85 and W2 were analyzed for the presence of plasmid DNA by the method of LeBlanc & Lee (1979) and by CsCl-ethidium bromide ultracentrifugation. No plasmid DNA was detected in strain W2 by either method. In contrast, more than one plasmid molecule was found to be present in "Str. faecalis var. liquefaciens" strains B20 and PB85, after CsCl-ethidium bromide density centrifugation followed by agarose gel electrophoresis of purified DNA (see Figure 1).

Several attempts were made to cure the gelatin liquefying ability

Figure 2 Detection of plasmid DNA in "Str. durans" strain B17 and "Str. faecium var. casseliflavus" strain W98 using the method of LeBlanc & Lee (1979)

Plasmid DNAs were isolated from "Str. durans" strain B17 and "Str. faecium var. casseliflavus" strain W98 by the method of LeBlanc & Lee (1979). Ethanol precipitated DNA was subjected to electrophoresis through a 0.7% agarose vertical gel. The tracks on the gel are: from left to right:

1) "Str. durans" strain B17
2) "Str. faecium var. casseliflavus" strain W98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of the organism by growing cells in the presence of different curing agents. After growing cells for 18 hr in different culture conditions for curing (see Methods), 0.1 ml of different dilutions were plated on BAB2 plates. After 48 hr incubation at 35°C, colonies were transferred to the BAB2 master plate, and at the same time on gelatin plates. After 3 days incubation at 35°C, the plates were flooded with acid solution of bichloride of mercury, which highlights the clear zones of liquefaction and gelatin liquefying colonies. However, none of the strains had lost the ability to liquefy gelatin.

Since no plasmid was found in one of the "Str. faecalis var. liquefaciens" strain W2 and the gelatin-liquefying abilities of the plasmid carrying strains B20 and PB85 were not lost after growing cells in the presence of curing agents, it is not known for certain whether the gelatin-liquefying ability of the "Str. faecalis var. liquefaciens" strains examined is plasmid encoded, but the evidence of the experiments performed suggest that it is not.

7) "Streptococcus faecalis var. malodoratus"

Analysis of strains D846 and D847 were performed for the presence of plasmid DNA, according to the method of LeBlanc & Lee (1979), Casse et al., (1979), and by using dye-buoyant density gradient centrifugation methods. None of the strains was found to contain any plasmid DNA.

8) "Streptococcus faecalis var. zymogenes"

"Str. faecalis var. zymogenes" strains B21 and PB79 were screened for the presence of plasmid DNA by using CsCl-ethidium bromide density gradient centrifugation methods. Plasmid DNA was detected in both the strains (Figure 4).

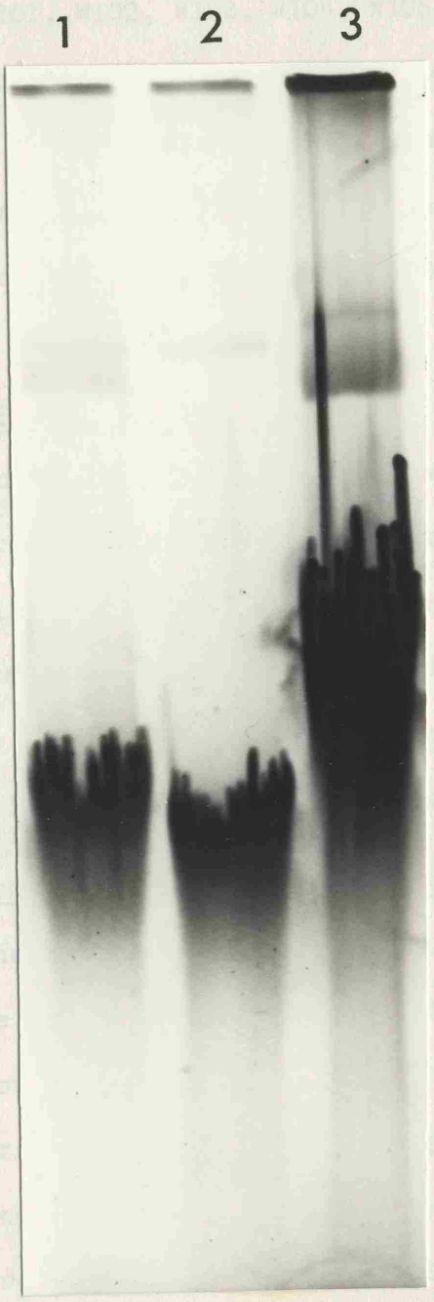
9) Streptococcus faecium

No plasmid DNA was detected in any of the Str. faecium (B19, W4 and W19) strains examined by the method of LeBlanc & Lee (1979) except in strain W100. This strain is resistant to high levels of the antibiotics

Figure 3 Screening of "Str. faecium var. casseliflavus" strains for detection of plasmids by the method of Casse et al. (1979).

Plasmid DNAs were isolated from "Str. faecium var. casseliflavus" strains W86 and W87 according to the method of Casse et al.(1979). Ethanol precipitated DNA (50 µl) was subjected to electrophoresis through a 0.7% agarose vertical gel for 16 hr at 17 volts. The tracks on the gel contain from left to right:

1) "Str. faecium var. casseliflavus" strain W86
2) Str. faecalis strain JH2-27, a known plasmid-containing strain
3) "Str. faecium var. casseliflavus" strain W87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kanamycin, neomycin, streptomycin and erythromycin. Strains B19, W4 and W19 did not show any phenotypic evidence for the presence of plasmid molecules.

10)"Streptococcus faecium var. casseliflavus"

Plasmid screening of "Str. faecium var. casseliflavus" strains W86, W87, W98, W99, W101, W102, W103, W104, W105, W106, W107, were performed according to the method of LeBlanc & Lee (1979), Casse et al., (1979), and by using CsCl —ethidium bromide density gradient centrifugation methods. Plasmid DNA was detected in "Str. faecium var. casseliflavus" strains W86, W87, W98, W99, W103, W106 and W107 (Figures 1, 2, 3).

11)"Streptococcus faecium var. mobilis"

"Str. faecium var. mobilis" strains, W88, which produce yellow pigment and a non-pigmented strain W92, were examined for the presence of plasmid DNA, by using CsCl —ethidium bromide density centrifugation methods. Plasmid DNA was found in the pigmented strain W88. No attempts were made to cure the plasmid from "Str. faecium var. mobilis" strain W88 to relate its presence with any phenotypic properties.

Genetic stability of haemolysin production traits of "Str. durans" strain B17 & W12

The stability of the haemolysin production traits of "Str. durans" strains B17 and W12 were tested by screening for the appearance of haemolysin-negative (Hly^-) clones on horse blood agar plates after growing cells in different culture conditions (Table 8). Curing experiments were done by the following agents: heating at 45°C, acriflavine, acridine orange and ethidium bromide. The results are summarized in Table 8 .No loss of haemolytic activity was observed in either strain after growth in broth at 37°C or growth in the presence of acridine orange. However, incubation of cultures at 45°C, growth of cells in the presence of acriflavine or ethidium bromide, resulted in loss of haemolytic activity from a low percentage of cells. The efficiency of curing was low. The haemolysin production trait of "Str. durans" strains B17 and W12 is clearly a relatively unstable property.

Table 8 Effect of curing agents on the stability of the haemolytic trait of "Str. durans" strains B17 and W12.

Host	Treatment	No.of colonies screened	No. of non-haemolytic colonies	Non-haemolytic colonies (%)
	Incubation in broth:			
	At 37°C	2004	0	
	At 45°C	2044	9	0.44
	Incubation with chemical			
B17	curing agents:			
	Acriflavine (3.5 µg/ml)	1782	2	0.11
	Control [a]	1500	0	
	Acridine Orange (25 µg/ml)	2083	0	
	Control [a]	2100	0	
	Ethidium bromide (3 µg/ml)	2425	3	0.12
	Control [a]	2000	0	
	Incubation in broth:			
	At 37°C	1800	0	
	At 45°C	2204	12	0.54
	Incubation with chemical			
W12	curing agents:			
	Acriflavine(3.5 µg/ml)	1775	2	0.11
	Control [a]	1700	0	
	Acridine Orange (25 µg/ml)	2000	0	
	Control [a]	1908	0	
	Ethidium bromide (3 µg/ml)	2045	2	0.09
	Control [a]	1895	0	

[a] Control cultures were treated in the same way as the test cultures, but without the curing agent

One haemolysin-negative variant of each strain was tested for spontaneous reversion to haemolysin producing activity; no reversion was observed when blood agar plates were seeded with 10^9 cells.

Transferability and frequency of haemolysis trait transfer from "Str. durans"

Filter matings were performed in an attempt to transfer the haemolysin determining plasmid conjugally from "Str. durans" strains B17R and W12R to Str. faecalis JH2-1. "Str. durans" strains B17 and W12 were made resistant to rifampicin (200 µg/ml) and designated B17R and W12R respectively. Strain JH2-1 which is resistant to Fus(250 µg/ml), lacks haemolytic activity and is free of plasmid DNA, was used as a recipient. Str. faecium strain B19-1, and haemolytic negative variants B17-4F and W12-1F(derived from "Str. durans" strains B17 and W12 after curing) were made resistant to fusidic acid (200 µg/ml) and were used as recipients.

After 18 hr contact on a membrane filter, the growth was suspended in sterile saline and 0.1 ml of various dilutions of the mating mixture were spread inoculated on selective media. Selection was done on horse blood agar plates containing Fus(200 µg/ml) to select for the recipient and to detect recipient clones producing haemolysin. Diluted mating mixtures were also plated on BAB2 containing rifampicin (200 µg/ml) to select for the donors. After 24 and 48 hr incubation at 35°C, the blood agar plates were checked to detect whether the recipients had acquired haemolytic activity. Haemolytic recipients were purified by re-streaking several times on BAB2 plates containing horse blood. Purified haemolytic transconjugants obtained from the crosses between strain B17R and JH2-1 and between strain W12R and JH2-1 were tested by the API 50CH system together with both donors, B17R and W12R and the recipients JH2-1.This was carried out to assess whether the transconjugants differed from the parent and recipient strains in any other characters.

The haemolysin trait was found to transfer from "Str. durans" strains B17R and W12R to the recipient strains JH2-1 at a high frequency in the range of 10^{-6}. In contrast, when mating was performed with the recipient

Table 9 Transfer frequency of haemolysin trait from "Str. durans" to non-haemolytic streptococci of serological group D

Donor strain	Recipient strain	Frequency of haemolytic recipients per donor
B17R	JH2-1	1.5×10^{-6}
	B19-1	-
	B17-4F	-
	W12-1F	-
W12R	JH2-1	5×10^{-6}
	B19-1	-
	B17-4F	-
	W12-1F	-

- = No transconjugant obtained

strains B19-1,B17-4F and W12-1F,no transconjugants were isolated (Table 9).

Since no transfer of plasmid occurred in liquid culture, when both the donor strains B17R and W12R were mated with the recipient JH2-1, it can be concluded that the transfer requires that both donor and recipient be held in close contact on filter.

Stability of the haemolysin trait of "Str. faecalis var. zymogenes" strains (B21, PB4, PB79).

The stability of the haemolysin production trait in "Str. faecalis var. zymogenes" strains B21, PB4 and PB79, was tested by screening for the appearance of haemolysin-negative (Hly^-) clones on blood agar plates containing 7.5% (v/v) horse blood after growing the cells in different culture conditions, and in the presence of different curing agents. After growing cells for 18 hr in different culture conditions for curing (see Table 10), the cultures were diluted and 0.1 ml portions of diluted culture were spread inoculated on BAB2 plates containing 7.5% (v/v) horse blood and incubated at 35°C (see Methods). After 48 hr incubation, colonies which were growing on blood agar plates but which had lost the ability to haemolyse the blood were isolated. Presumed-cured strains were re-streaked several times on horse blood agar plates. For further identification, haemolysin-negative variants were grown on tellurite and tetrazolium plates and also tested by the API 50CH system together with the parental strains.

No loss of haemolytic activity was detected after growth in broth at 37°C, but incubation at 45°C resulted in loss of the character from a high percentage of cells. The results are summarized in Table 10 . Incubation of culture at 45°C was found to be the most effective curing agent and was able to produce a higher percentage of haemolysin-negative variants of"Str. faecalis var. zymogenes". None of the Hly^- variants had lost the capacity to ferment sucrose or glycerol (anaerobically). The latter character is particularly diagnostic for Str. faecalis.

Three Hly^- variant clones (B21-1, PB4-1, PB79-2) were also examined for

Table 10 Curing of haemolytic trait of "Str. faecalis var. zymogenes" strains by different curing agents

Host	Treatment	No. of colonies screened	No. of non-haemolytic colonies	Efficiency of curing (%)
	Incubation in broth:			
	At 37°C	2275	0	
	At 45°C	2543	30	1.1
B21	Incubation with chemical curing agents:			
	Acriflavine (4 µg/ml)	2403	2	0.9
	Control [a]	1409	0	
	Acridine Orange (30 µg/ml)	2705	2	0.07
	Control [a]	2000	0	
	Ethidium Bromide (4 µg/ml)	2300	8	0.34
	Control [a]	2123	0	
	Incubation in broth:			
	At 37°C	2409	0	
	At 45°C	2445	14	0.57
PB4	Incubation with chemical curing agents:			
	Acriflavine (4 µg/ml)	2113	1	0.04
	Control [a]	2100	0	
	Acridine Orange (30 µg/ml)	2323	2	0.08
	Control [a]	2005	0	
	Ethidium bromide (4 µg/ml)	2231	5	0.22
	Control [a]	2000	0	
	Incubation in broth:			
	At 37°C	2213	0	
	At 45°C	2415	15	0.61
PB79	Incubation with chemical curing agents:			
	Acriflavine (4 µg/ml)	2521	2	0.07
	Control [a]	2043	0	
	Acridine Orange (30 µg/ml)	2223	1	0.04
	Control [a]	2101	0	
	Ethidium bromide (4 µg/ml)	2380	5	0.21
	Control [a]	2000	0	

[a] Control cultures were treated in the same way as the test cultures, but without the curing agent.

spontaneous reversion to haemolysin activity; no reversion was observed.

Although the frequency of curing was low, it was clear that the haemolysin producing trait is an unstable property in the "Str. faecalis var. zymogenes" strains tested.

Transfer of haemolysin plasmid from "Str. faecalis var. zymogenes" strains (B21R,PB4R and PB79R) to plasmid-free Str. faecalis strain JH2-1

Haemolysis traits were transferred from "Str. faecalis var. zymogenes" strains B21R, PB4R and PB79R to Str. faecalis strain JH2-1 during mixed incubation. Strain JH2-1 is a plasmid free haemolysin-negative (Hly^-) variety of Str. faecalis and is resistant to fusidic acid (250 µg/ml). "Str. faecalis var. zymogenes" strains B21, PB4 and PB79 were made resistant to rifampicin (200 µg/ml) and called B21R, PB4R and PB79R respectively.

The filter mating technique (see Methods) was used for the detection of the conjugal transfer of the haemolysin plasmids from "Str. faecalis var. zymogenes" strains to the recipient strain JH2-1. Strain JH2-27 which contains the haemolysin plasmid was used as a control. After 18 hr contact on membrane filter, 0.1 ml of various dilutions of the mating mixture were spread inoculated on to horse blood agar plates containing fusidic acid (200 µg/ml) to select for the recipient and to indicate recipient clones producing haemolysin. The mating mixtures were also plated on BAB2 containing rifampicin (200 µg/ml) in order to select for the donors. After 24 and 48 hr of incubation at 35°C, the blood agar plates were checked to detect any recipients which had acquired haemolysin plasmids. Haemolytic recipients were purified by patching several times on horse blood agar plates containing fusidic acid (250 µg/ml). To prove that transfer had occurred, purified haemolytic transconjugants obtained from B21R, PB4R and PB79R in the crosses with JH2-1 shown in Table 11 , were analyzed for the non-selected chromosomal antibiotic resistances. All the clones tested were sensitive to rifampicin (200 µg/ml) and resistant to fusidic acid (250 µg/ml).

Table 11 Transfer frequency of haemolysin trait from "*Str. faecalis* var. *zymogenes*" strains to Hly^- variant of *Str. faecalis*

Donor strains	Recipient strains	Frequency of haemolytic recipients per donor
B21R	JH2-1	4×10^{-4}
PB4R	JH2-1	1×10^{-5}
PB79R	JH2-1	5×10^{-4}
JH2-27*	JH2-1	2.5×10^{-5}

* *Str. faecalis* strain JH2-27 is a known plasmid-containing strain and was used as a control.

Although the frequency of transfer of the haemolysin trait by filter mating was always in the range of 10^{-4} to 10^{-5} per donor, the frequency of transfer in liquid medium was considerably lower (10^{-7} to 10^{-8} per donor organism), when all donors including the control strains were crossed with Str. faecalis strain JH2-1 (Table 11).

Efficiency of Hly$^-$ variants as recipients of the plasmid-borne haemolysin trait

One haemolytic-negative variant of each strain of "Str. faecalis var. zymogenes" was tested to determine whether they would act as recipients for the transfer of haemolytic plasmids pB21, pPB4 and pPB79 from strains B21R, PB4R and PB79R. All the haemolytic-negative variants were made resistant to fusidic acid (200 µg/ml). The frequency of haemolysin trait transfer was determined after 18 hr of contact on membrane filter and the results are presented in Table 12 . Transconjugants were selected on horse blood agar plates containing fusidic acid (200 µg/ml). All the Hly$^-$ variants from strains B21, PB4 and PB79 received plasmids pB21, pB4 and pPB79 at a frequency similar to that of the efficient recipient strain JH2-1.

Demonstration of the plasmid which codes for haemolysin production in "Str.. faecalis var. zymogenes", "Str. durans" and their cured derivatives and transconjugants

The loss of haemolysin production by "Str. faecalis var. zymogenes" strains B21, PB4 and PB79, and "Str. durans" strains B17 and W12 during their growth in presence of curing agent, led to the suggestion that the β-haemolysis of these strains are also mediated by plasmids. To test this possibility and to prove that the haemolysin plasmid was transferred to the plasmid-free Str. faecalis strain JH2-1, the haemolysin-producing strains B21R, PB79R, B17R, W12R, their respective cured derivatives B21-1, PB79-2, B17-4, and transconjugant haemolytic strains JH2-21 and JH2-17, were all analyzed for the presence of extrachromosomal DNA molecules. Strain JH2-27 was also included as a marker strain. By using CsCl-ethidium bromide density gradient centrifugation

Table 12 Transfer frequency of haemolysin trait to Hly^- variants

Donor strain	Recipient strain	Frequency of haemolytic recipients per donor
B21R	B21-1	4×10^{-4}
	PB4-1	1×10^{-5}
	PB79-1	2.5×10^{-4}
PB4R	B21-1	1×10^{-5}
	PB4-1	5×10^{-4}
	PB79-1	2.5×10^{-4}
PB79R	B21-1	5.7×10^{-5}
	PB4-1	2×10^{-5}
	PB79-1	5×10^{-4}

Figure 4 Detection of plasmid DNA by CsCl-ethidium bromide density gradient centrifugation method

Agarose gel electrophoresis of DNAs isolated from streptococci of serological group D and L. monocytogenes. Plasmid DNAs were isolated from CsCl-ethidium bromide density gradients and subjected to electrophoresis through a 0.7% agarose vertical gel for 16 hr at 17 volts. The tracks contain from left to right:

1) Str. faecalis strain JH2-27 containing the haemolytic plasmid pJH2
2) "Str. faecalis var. zymogenes" strain PB79
3) "Str. faecalis var. zymogenes" strain B21
4) Str. faecalis strain JH2-21 (transconjugant)
5) Str. bovis strain B16
6) Str. bovis strain D598
7) L. monocytogenes strain C659
8) Str. equinus strain D1090

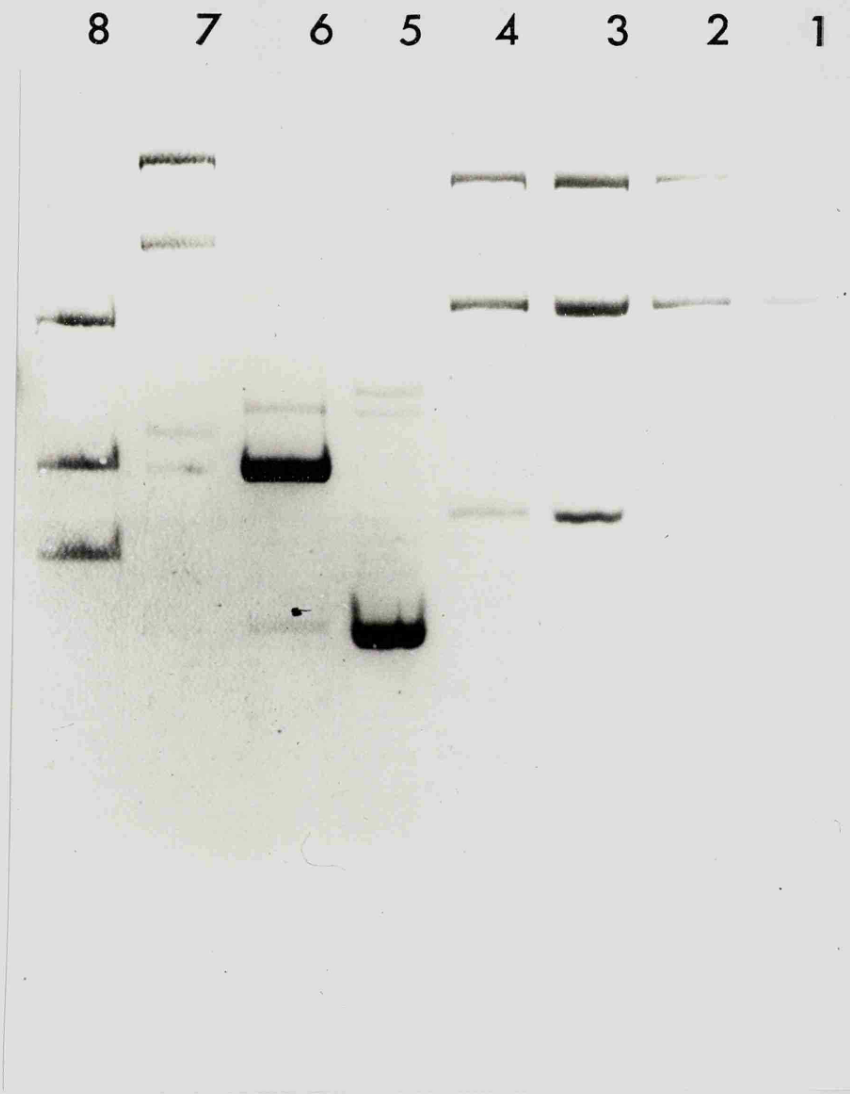
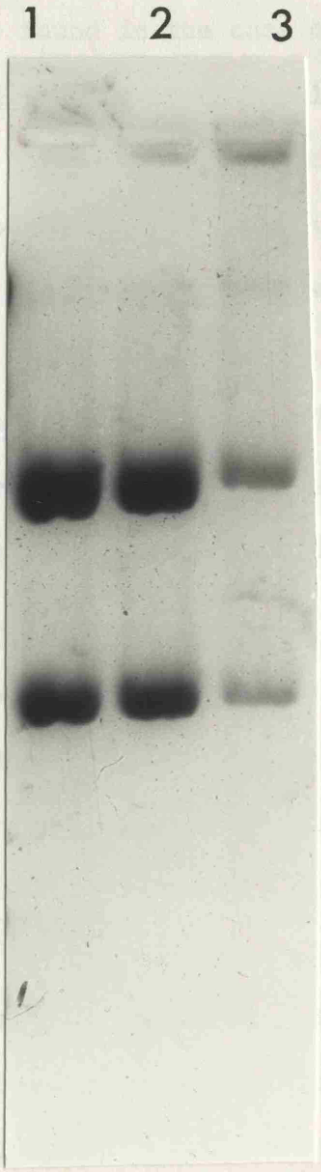

8
7
6
5
4
3
2
1

Figure 5 Detection of plasmid DNA in "Str. durans" strain B17, its transconjugant Str. faecalis strain JH2-17 and "Str. durans" strain W12 by using CsCl-ethidium bromode density gradient centrifugation methods

Agarose gel electrophoresis of plasmid DNA isolated from "Str. durans" strains B17, W12 and Str. faecalis strain JH2-17. Plasmid DNAs were isolated from dye-buoyant density gradients, and were subjected to electrophoresis through a 0.7% agarose horizontal slab gel for 16 hr at 17 volts. The tracks on the gel from left to right contain lysates of the following:

1) "Str. durans" strain W12
2) "Str. durans" strain B17
3) Str. faecalis strain JH2-17


1
2
3

followed by agarose gel electrophoresis, it was found that the parent haemolytic strains B21R, PB79R, B17R and their transconjugants JH2-21 and JH2-17 all contained plasmid DNA (Figures 4 & 5). Plasmids from the transconjugant (JH2-21) appeared identical in mobility to the plasmid DNA molecule isolated from the parental strain B21R, PB79R and the control strain JH2-27 (Figure 4). It was also found in the case of "Str. durans" strain that the position of the plasmid DNA in the gel isolated from transconjugant strain (JH2-17)was the same as the plasmid DNA obtained from the parent strain B17R and W12R (Figure 5). No plasmid DNA was detected in any of the haemolysin-negative variants (strains B21-1, PB79-2, B17-4) isolated after curing.

It was concluded that the genes conferring haemolysin production in "Str. faecalis var. zymogenes" strains B21, PB79, are borne on self-transferable plasmid called pB21, pPB79, and in "Str. durans" strain B17 are linked to the transferable plasmid pB17, respectively.

Genetic stability of drug-resistance traits in Streptococcus faecium

Str. faecium, W100, codes for resistances to kanamycin (Km), neomycin (Nm), erythromycin (Em) and streptomycin (Sm) (Table 3). The stability of the antibiotic resistance traits of the strain was tested by screening for the appearance of antibiotic-sensitive segregant clones by replica plating techniques. After growing cells in several culture conditions and in the presence of chemical agents known to promote plasmid curing in other systems, aliquots of the diluted culture were spread inoculated on BAB2 plates (see Methods). After 48 hr incubation at 35°C, colonies were picked at random and plated on BAB2 master plates and at the same time on selective plates containing kanamycon (1000 μg/ml). Presumed-cured clones picked from the BAB2 master plate were re-streaked on to BAB2 plates containing kanamycin (4000 μg/ml) or neomycin (4000 μg/ml) or streptomycin (2000 μg/ml) or erythromycin (2500 μg/ml) to confirm whether the cured strains had lost all the resistances simultaneously. For further identification antibiotic-sensitive segregants and the parent strain were tested on tellurite and tetrazolium plates and by the API 50CH system.

No loss of resistance was observed after growth in broth at 37°C. However, incubation of cultures at 45°C resulted in simultaneous loss of resistances to Km, Nm, Sm and Em. Similar antibiotic-sensitive clones were isolated after growth in the presence of acridine orange, acriflavine and ethidium bromide. The results are summarized in Table 13 . Thus, although the efficiency of curing was low, resistances to the antibiotics is clearly an unstable property. The MICs of the antibiotics for all antibiotic-sensitive clones, and for the parental strain, are shown in Table 14 .

Ethidium bromide was found to be the most effective curing agent to eliminate the drug resistances.

One of the antibiotic-sensitive segregant strains W100-1 was further examined for spontaneous reversion to high-level resistance. No reversion to Km, Nm, Sm and Em resistances was observed.

Transfer of multiple antobiotic resistances from Str. faecium W100

The genes conferring resistances to kanamycin (Km), neomycin (Nm), streptomycin (Sm) and erythromycin (Em) were transferred from the donor strain Str. faecium W100 to the recipients during mixed incubation. Str. faecalis strain JH2-1 is a plasmid free strain resistant to Fus(250 µg/ml) and was used as a recipient. Strain B19-1 is a fusidic acid (200 µg/ml) resistant derivative of the antibiotic-sensitive strain Str. faecium B19, and was not found to contain plasmids after lysing the cells followed by CsCl-ethidium bromide centrifugation. In addition to strain JH2-1, strain B19-1 was also used as a recipient in plasmid transfer experiments. The donor strain W100 was made resistant to rifampicin (200 µg/ml) and designated W100R. As a control, strain JH2-25 was used in the plasmid transfer experiments. Strain JH2-25 contains the drug-resistance plasmid pJH1.

After 18 hr contact on a membrane filter, the growth was suspended in sterile saline and 0.1 ml of various dilutions of the mating mixture was spread inoculated on to selective plates. Selection was done on BAB2 plates containing either Km (1000 µg/ml) and Fus(200 µg/ml), or

Table 13 Effect of curing agents on the stability of antibiotic resistance of Str. faecium, strain W100

Treatment	No. of colonies screened	No. of antibiotic-sensitive colonies [a]	Antibiotic-sensitive colonies (%)
Incubation in broth:			
At 37°C	2200	0	
At 45°C	2779	4	0.14
Incubation with chemical curing agents:			
Acriflavine (3.25 µg/ml)	2007	2	0.09
Control [b]	1535	0	
Acridine orange (25 µg/ml)	2251	1	0.04
Control [b]	2123	0	
Ethidium bromide (3 µg/ml)	1963	5	0.25
Control [b]	1645	0	

[a] All colonies had lost resistances to Km, Nm, Sm and Em, simultaneously

[b] Control cultures were treated in the same way as the test cultures, but without the curing agent.

Table 14 MICs* of antibiotics for inhibition of Str. faecium W100 and some antibiotic-sensitive variants

Strain	Source	MICs of antibiotics (µg/ml)							
		Km	Nm	Sm	Em	TC	Gen	Fus	Rif
W100	Parent strain	4000	>4000	2000	2500	2	20	4	2
W100-1	Incubation at 45°C	40	20	60	10	2	20	4	2
W100-2	"	40	20	60	10	2	20	4	2
W100-3	"	35	20	60	10	2	20	4	2
W100-4	"	40	40	60	20	1.5	20	4	2
W100-5	Incubation in AF	40	20	80	10	2	20	4	2
W100-6	"	40	20	60	10	2	20	4	2
W100-7	Incubation in AO	40	20	60	150	2	20	4	2
W100-8	Incubation in EB	40	20	60	10	2	20	4	2
W100-9	"	35	20	60	10	2	20	4	2

* MICs = minimal inhibitory concentrations

Em(1000 µg/ml) and Fus(200 µg/ml) to select for the recipients which had acquired resistance markers. It should be noted that both types of antibiotic containing plates were used. Fusidic acid was used to counter select the plasmid donor, and kanamycin was used to select those recipients which had acquired resistance markers. Diluted mating mixtures were also plated on BAB2 containing rifampicin (200 µg/ml) to select for the donors. After 48 hr incubation at 35°C, the selective plates were checked, to determine the number of transconjugants and the number of donor bacteria per ml.

It was found that the number of transconjugants obtained was very high in the matings between Str. faecium strain W100R with the recipient strain JH2-1. A very low number of transconjugants was obtained when strain W100R was crossed with strain B19-1. The results are shown in Table 15 . The control strain JH2-25 transferred the drug resistances to strain JH2-1 at a very high frequency whereas no transconjugant was detected when strain B19-1 was used as a recipient. Presumed transconjugants isolated from the selective plates were re-streaked on BAB2 plates containing Km (1000 µg/ml) or Nm (1000 µg/ml) or Sm (1000 µg/ml) or Em(1000 µg/ml) to find out whether the transoonjugants had acquired all the resistance markers simultaneously. In addition, transconjugants were grown on tellurite and tetrazolium plates (see Methods), and also tested by the API 50CH system with both donors and recipients as controls. The MICs of antibiotics of transconjugants, donors and recipients are shown in Table 16).

In all cases, the selection for transfer of Km, Nm, Sm and Em resistance revealed co-transfer of all four resistances simultaneously, except that in one case Em resistance alone was transferred.

The Em resistance clone was isolated from the selective plate containing both Em(1000 µg/ml) and Fus(200 µg/ml). The Em resistant clone was sensitive to Km, Nm and Sm.

However, the above results suggest that the genes determining resistance to Km, Nm, Sm and Em in Str. faecium strain W100 are carried by a conjugative plasmid.

Table 15 Frequency of transfer of resistance determinants from *Str. faecium* strain W100 to recipient strains JH2-1 and B19-1

Donor strain	Recipient strain	R plasmid transfer frequency per donor cell	Resistance markers transferred
	JH2-1	5×10^{-4}	Km, Nm, Sm, Em
W100	JH2-1	7×10^{-8}*	Em
	B19-1	7×10^{-8}	Km, Nm, Sm, Em
JH2-25	JH2-1	2.5×10^{-5}	Km, Nm, Sm, Em, Tc

* Selection of transconjugants was done on Fus and Em

Table 16 MICs of donor strain Str. faecium W100, recipient strains JH2-1, B19-1 and recipient strains having acquired resistances

Strain	MICs of antibiotics (µg/ml)						
	Km	Nm	Sm	Em	Fus*	Rif*	Gen
W100R	4000	4000	2000	2500	4	200	20
JH2-1	20	40	40	0.125	256	2	20
B19-1	20	40	40	1.0	200	1.5	20
JH2-100	4000	4000	2000	2000	250	2	20
JH2-101	20	40	40	1500	250	2	20
JH2-104	4000	4000	2000	2500	250	2	20
B19-100	3500	4000	1500	2000	200	1.5	20

* Resistances to fusidic acid in strain B19-1 and to rifampicin in strain W100R were introduced by mutagenesis and by selection of spontaneous mutants.

Resistance plasmid transfer frequency from Str. faecium W100

The transfer frequency of the R (resistance) plasmid from the donor strain W100 to the recipient strains JH2-1 and B19-1 was calculated after 0, 15, 30 and 45 min and 1, 2, 3, 4 and 18 hr of contact on membrane filter. Selection for transconjugants was done on plates containing both Km (1000/µg/ml) and Fus(200 µg/ml). The results are shown on Table 17 . The findings were, no transconjugants were obtained after 0, 15, 30, 45 min and 1 hr of mating; a high number of detectable transconjugants appeared after 2 hr of mating with strain JH2-1 and a low number with B19-1 after 4 hr of mating. The transfer frequencies of the plasmid to both the recipient strains JH2-1 and B19-1 was maximum after 3 hr and 4 hr of mating and did not change even after 18 hr of contact.

Plasmid content of Str. faecium strain W100 and its derivatives and transconjugant strains

Strains W100R, W100-1, W100-2, JH2-100 and JH2-104 were analyzed for the presence of plasmid DNA molecules. By using the plasmid screening procedure of LeBlanc and Lee (1979) followed by agarose gel electrophoresis, both strains W100R and JH2-100 were shown to contain a single plasmid DNA molecule. Plasmid from transconjugant JH2-100 appeared identical in mobility to the plasmid DNA molecule isolated from the parental strain W100-R (Figure 6). No plasmid DNA could be found in either of the two antibiotic-susceptible variants (strains W100-1 and W100-2) of strain W100 and the Em resistant transconjugant JH2-104 by using dye-buoyant density gradient centrifugation or by labelling the DNA with tritiated thymidine.

Tritiated thymidine labelled DNAs from the antibiotic resistant parent strain W100 and the antibiotic sensitive strain W100-1 were subjected to CsCl-ethidium bromide density gradient centrifugation. The banding profile of the DNA isolated from strain W100 and W100-1 (a cured derivative) are depicted in Figures 7a & b).Antibiotic resistant strain W100 contained a satellite peak corresponding to plasmid DNA in addition to the main peak

Table 17 Resistance plasmid transfer frequency of strain W100 to JH2-1 and B19-1 recipients

No. of donor bacteria/ml (viable counts)	Time of mating contact (hr)	Recipient strain	Plasmid transfer frequency / donor cell
3×10^{7}	0	JH2-1	-
		B19-1	-
4.2×10^{7}	0.25	JH2-1	-
		B19-1	-
5.5×10^{7}	0.50	JH2-1	-
		B19-1	-
1×10^{8}	0.75	JH2-1	-
		B19-1	-
1.8×10^{8}	1	JH2-1	-
		B19-1	-
3×10^{8}	2	JH2-1	5×10^{-6}
		B19-1	-
7.5×10^{8}	3	JH2-1	2.5×10^{-4}
		B19-1	-
1.1×10^{9}	4	JH2-1	4.6×10^{-4}
		B19-1	7.0×10^{-8}
8×10^{8}	18	JH2-1	4.0×10^{-4}
		B19-1	1.5×10^{-8}

- = no transconjugant obtained

Figure 6 Screening of Str. faecium strain W100 and its transconjugant strain JH2-100 for detection of plasmid DNA by the method of LeBlanc & Lee (1979)

Plasmid DNAs were isolated from Str. faecium strain W100 and Str. faecalis strain JH2-100 using the method of LeBlanc & Lee (1979). Ethanol precipitated DNA (40 µl) was subjected to electrophoresis through a 0.7% agarose vertical gel for 16 hr at 17 volts. The tracks on the gel from left to right contain lysates of the following:

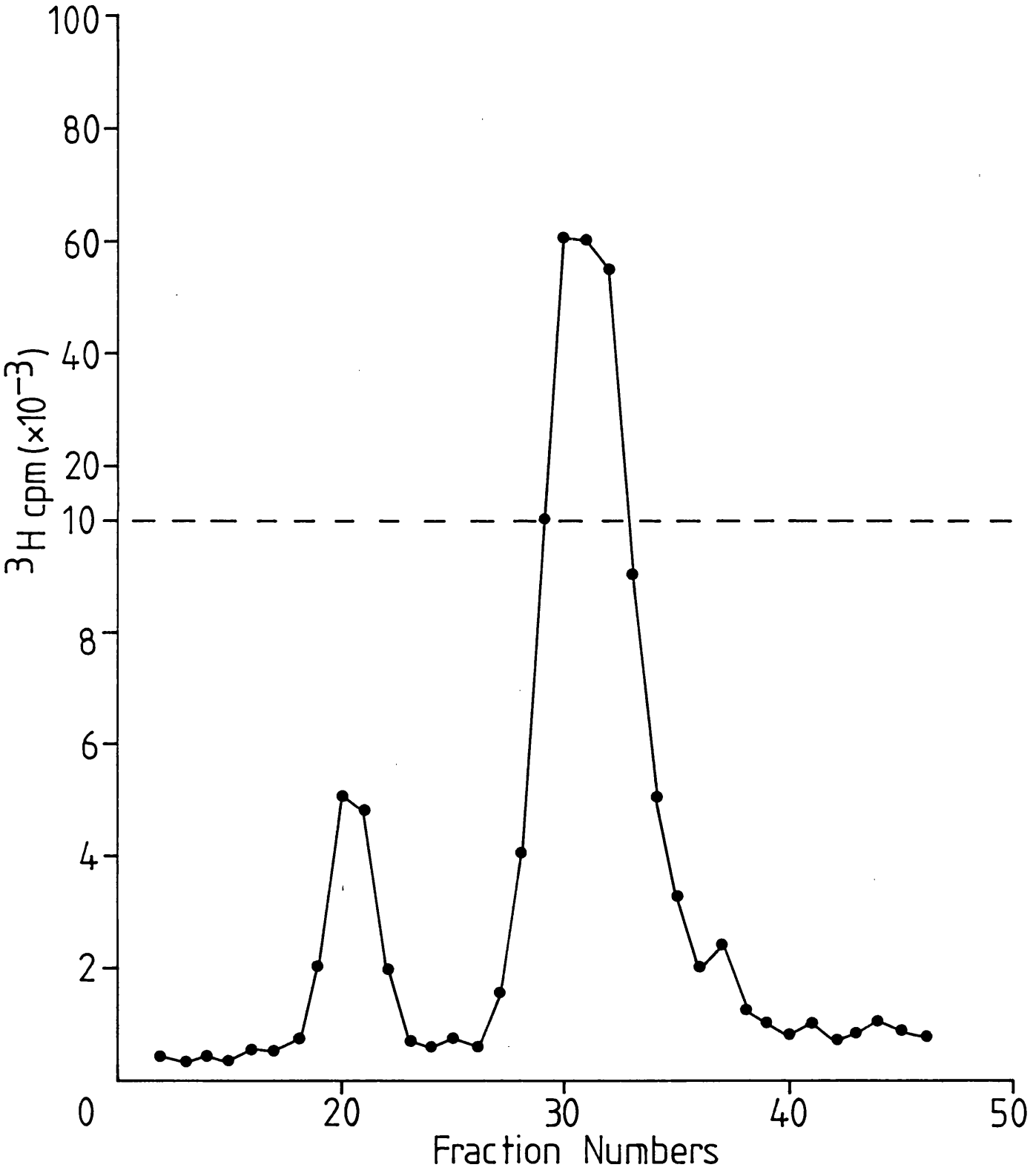
1) Str. faecium strain W100
2) Str. faecalis strain JH2-100
3) Str. faecalis strain JH2-27, a known plasmid-containing strain


1
2
3

Figure 7 a & b — Fractionation of CsCl-ethidium bromide density gradients containing ^{3}H-labelled DNA

Fractionation of CsCl-ethidium bromide density gradients containing ^{3}H-labelled DNA from a) Str. faecium strain W100 and b) its cured derivative W100-1. Fractions were collected and 20 µl of each were spotted and counted. Note the change in scale at 10^{4} counts/min.

a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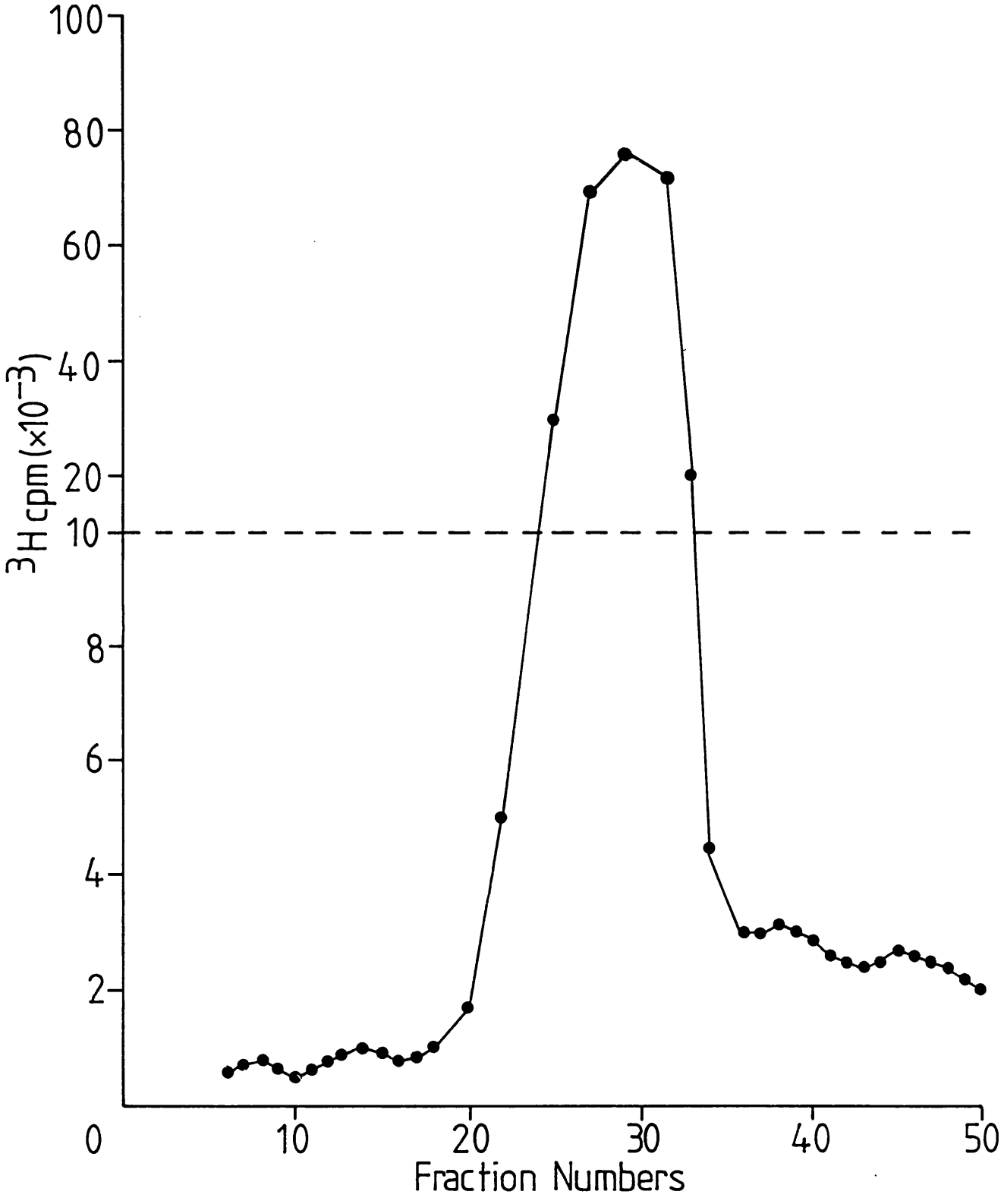
80
60
40
20
10
8
6
4
2
0
20
30
40
50
^{3}H cpm (x10^{-3})
Fraction Numbers

b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50
^{3}H cpm (x10^{-3})
Fraction Numbers

which corresponds to chromosomal DNA, whereas the satellite peak which corresponds to plasmid DNA was absent in the cured strain W100-1.

The loss of the plasmid DNA in the cured strain W100-1 associated with the loss of resistance provides evidence that determinants of resistance in strain W100 are linked to the transferable plasmid pW100.

Stability of pigment production in "Str. faecium var. casseliflavus" strains

The stability of the pigmentation trait in "Str. faecium var. casseliflavus" strains W99 and W106 was tested by screening for the appearance of non-pigmented (white rather than yellow) strains on BAB2 plates after performing curing experiments. Curing experiments were done using the following agents: 45°C, acridine orange, acriflavine and ethidium bromide. After growing cells under different culture conditions, 0.1 ml of diluted culture was spread inoculated on to BAB2 plates (see Methods). After 48 hr incubation at 35°C, the plates were inspected by eye to detect any non-pigmented colonies. The results are summarized in Tables 18 & 19. No loss of pigment production was detected after growing strains W99 and W106 in the presence of acridine orange, but a very low percentage of cells were found to lose pigment production after treatment with ethidium bromide, acriflavine and growth at 45°C.

Presumed-cured strains were tested by the API 50CH system together with the parental strains for confirmation of identity of the strains. The motility of the cured strains and the parent strains was also examined. It was inferred from the findings that the pigmentation trait is not a stable property in "Str. faecium var. casseliflavus" strain W99 and W106 and may well be plasmid encoded.

Detection of plasmid DNA in "Str. faecium var. casseliflavus" strains W99 and W106 and their derivatives

"Str. faecium var. casseliflavus" strains W99, W106 and their cured non-pigmented derivatives W99-1, W106-1 and W106-2 were analyzed for the presence of extrachromosomal DNA by CsCl-ethidium bromide ultracentrifugation followed by agarose gel electrophoresis of DNA. Both the parent strains

Table 18 The Effect of curing agents on the stability of pigment production in "Str. faecium var. casseliflavus" strains W99 and W106

Host	Treatment	No. of colonies screened	No. of non-pigmented colonies	Non-pigmented colonies (%)
	Incubation in broth:			
	At 37°C	1938	0	
	At 45°C	2278	1	0.04
	Incubation with chemical curing agents:			
W99	Acriflavine (3.5 µg/ml)	2432	2	0.08
	Control	2072	0	
	Acridine Orange (30 µg/ml)	2241	0	
	Control	2019	0	
	Ethidium Bromide (2.5 µg/ml)	2742	4	0.14
	Control	2035	0	
	Incubation in broth:			
	At 37°C	2045	0	
	At 45°C	2403	3	0.12
	Incubation with chemical curing agents:			
W106	Acriflavine (3.5 µg/ml)	2118	2	0.09
	Control	2000	0	
	Acridine Orange (30 µg/ml)	1980	0	
	Control	1845	0	
	Ethidium Bromide (2.5 µg/ml)	2109	1	0.04
	Control	2234	0	

Table 19 Characters differing between the pigmented parent strains and the cured strains

Strain	Source	Pigmentation	Motility
W99	Parent strain	+	+
W99-1	Incubation at 45°C	-	+
W99-2	Incubation with AF	-	+
W99-3	" "	-	+
W99-4	Incubation with EB	-	+
W99-5	" "	-	+
W99-6	" "	-	+
W99-7	" "	-	+
W106	Parent strain	+	+
W106-1	Incubation at 45°C	-	+
W106-2	" "	-	-
W106-3	" "	-	+
W106-4	Incubation with AF	-	+
W106-5	" "	-	-
W106-6	Incubation with EB	-	+

W99 and W106 were found to contain a plasmid DNA molecule; but no plasmid DNA was detected in the cured strains. It is concluded from the result that the wild type strains W99 and W106 harbour a plasmid which determines pigment production in "Str. faecium var. casseliflavus", and the loss of plasmid in the cured strain was accompanied by the loss of pigmentation.

Stability of lactose fermentation in Str. bovis strain B16

Variants unable to ferment lactose were obtained from Str. bovis strain B16 by growing cells at 42°C and in the presence of chemical agents (see Table 20).

After growing strain B16 for 18 hr in the different culture conditions the cultures were diluted and 0.1 ml portions of the diluted cultures were spread inoculated on to MacConkey agar plates. After 48 hr incubation at 35°C, isolated colonies which were unable to produce acid from lactose on the indicator plate were recovered and purified on MacConkey agar plate. For further confirmation, lactose negative colonies were tested by the API 50CH system together with the parent strain. The results are shown in Table 21 . Growth of the culture at 42°C resulted in the complete loss of lactose-fermenting ability from a low percentage of cells. No loss of lactose utilizing ability was observed after growth at 37°C or growth in the presence of acriflavine. It was found that acridine orange concentrations of 20 to 25 µg/ml yielded a high percentage of lactose negative variants. Treatment of Str. bovis strain B16 with all curing agents tested resulted in only partial loss of lactose-fermenting ability in a high percentage of colonies, (1.0%) and such colonies were designated as Lac^d derivative or lactose defective mutant. These latter mutants produced acid slowly from lactose on the indicator agar. These results indicate that fermentation of lactose is not a stable property in Str. bovis strain B16.

Three lactose-negative (Lac^-) colonies of Str. bovis strain B16 independently isolated were tested for spontaneous reversion to lactose utilizing ability, but no full-reversion was observed; only they were able to revert to a partial lactose-fermenting phenotype, whereas all Lac^d

Table 20 Effect of curing agents on the stability of lactose metabolism by Str. bovis, strain B16

Treatment	No. of colonies screened	No. of lactose-negative colonies	Lactose-negative colonies (%)
Incubation in broth:			
At 37°C	2114	0	
At 42°C	2703	12	0.48
Incubation with chemical curing agents:			
Acriflavine (4 µg/ml)	2531	0	
Control	2232	0	
Acridine Orange (25 µg/ml)	2042	20	0.97
Control	2000	0	

N.B. Those colonies which appeared to have partially lost the ability to ferment lactose (Lac^d mutant) are not included in this Table.

Table 21 Characters differing between the lactose utilizing Str. bovis strain B16 and the cured strains

Strain	Source	Lactose utilization	Galactose utilization
B16	Parent strain	+	+
B16-1	After incubation at 42 °C	-	-
B16-2	" " " 42 °C	-	±
B16-3	" " with AO	-	±
B16-4	" " "	-	+
B16-5	" " "	-	+

AO = Acridine Orange

derivatives tested were able to revert to full lactose-fermenting ability.

Screening of Str. bovis strain B16 and its cured derivatives for detection of plasmid DNA molecule

The results presented here confirm the presence of a plasmid in Str. bovis strain B16 and its absence in the lactose-negative derivatives, strains B16-1 and B16-2, after curing experiments.

Since it was found that the lactose utilizing ability of Str. bovis strain B16 can be lost easily, it was of interest to determine if the plasmid molecule is missing in the cured strains. The parent strain B16 contained an extra band of DNA corresponding to plasmid DNA in addition to chromosomal DNA, after CsCl-ethidium bromode density gradient centrifugation. The plasmid band from the parent strain was pooled and then analyzed on agarose gel electrophoresis (Figure 4); whereas no plasmid was detected in the cured strains B16-1 and B16-2. Even when strain B16 and B16-1 (a cured derivative) was labelled with tritiated thymidine and the DNA was centrifuged to equilibrium in CsCl —ethidium bromide density gradient, no satellite peak characteristic of plasmid DNA was found in cured strain B16-1; whereas a satellite peak characteristic of plasmid DNA was observed together with the main peak of chromosomal DNA in the parent strain B16.

Four lactose defective mutants of Str. bovis strain B16 were also analyzed by CsCl-ethidium bromide ultracentrifugation methods after labelling with tritiated thymidine to detect the presence of plasmid DNA. All the Lac^d derivatives examined were found to contain the plasmid DNA molecule.

The loss of plasmid DNA in the cured (Lac^-) strains associated with the loss of lactose utilizing ability in these strains provides evidence that lactose fermentation in Str. bovis strain B16 is mediated by a plasmid.

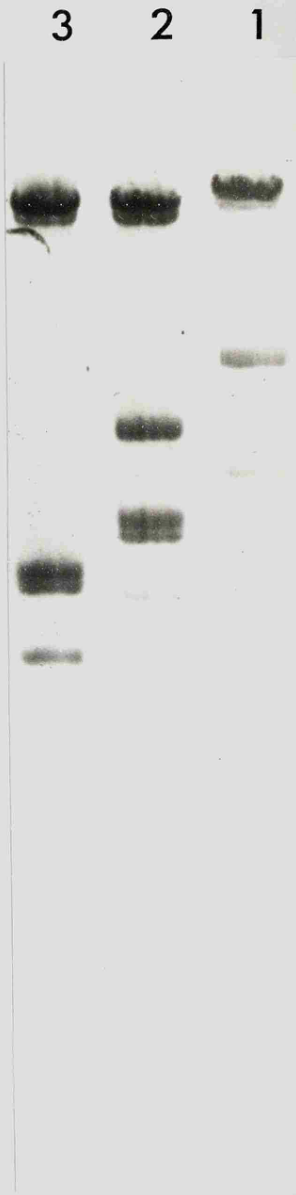
DNA-DNA hybridization studies

DNA-DNA reassociation was performed to determine the degree of polynucleotide sequence homology among strains of "Str. faecium var. casseliflavus" ,"Str. faecium var. mobilis" and Str. faecium.

Figure 8 Restriction enzyme analysis of plasmids obtained from Str. bovis strains B16 and D598 and from Str. equinus strains D1090

Agarose gel electrophoresis of plasmid DNA isolated from Str. bovis strains B16, D598 and Str. equinus strain D1090. Plasmid DNAs were obtained from dye-buoyant density gradients and cleaved by restriction endonuclease Bam HI. Bam HI-cleaved DNA was subjected to electrophoresis on 0.7% horizontal slab gel and run for 16 hr at 17 volts. The tracks on the gel from left to right contain:

1) Bam HI digest of the cryptic plasmid from Str. equinus strain D1090
2) Bam HI digest of the plasmid from Str. bovis strain D598
3) Bam HI digest of the plasmid from Str. bovis strain B16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Labelled whole cell DNA was prepared from Str. faecium strain W100, "Str. faecium var. casseliflavus" strain W99 and "Str. faecium var. mobilis" strain W88. As a source of unlabelled whole cell DNA Str. faecium strain W100, "Str. faecium var. casseliflavus" strains W98 and W99, and "Str. faecium var. mobilis" strains W88 and W92 were used.

The results of hybridization experiments carried out at 60°C are presented in Table 22 . The degree of DNA duplex formation between "Str. faecium var. mobilis" strains W88 and W92, and "Str. faecium var. casseliflavus" strains W98 and W99 was always at a level of 90% or greater: but, DNA from all of the "Str. faecium var. casseliflavus" and "Str. faecium var. mobilis" strains examined showed low (less than 25%) homology with DNA from strains of Str. faecium, W100.

DNA homology among plasmids encoding for haemolysis in streptococci of serological group D

Hybridization studies were performed to determine the degree of relatedness among haemolytic plasmids derived from "Str. faecalis var. zymogenes" strains (B21, PB4, PB79, JH2-27). Similar studies were conducted on the haemolytic plasmids derived from "Str. faecalis var. zymogenes" and "Str. durans" strains.

Labelled plasmid DNA was isolated from Str. faecalis strains JH2-17 and JH2-21 carrying haemolytic genes were chosen as probes for the study. Unlabelled whole cell DNA was extracted from strains JH2-4, JH2-12, JH2-17, JH2-21, JH2-27 and PB 79 (see Table 23). Str. faecalis strain JH2-1 is a plasmid free, non-haemolytic strain and served as a control.

The sheared labelled plasmid DNA isolated from strains JH2-17 and JH2-21 was denatured and added to the solutions containing filter quarters loaded with unlabelled whole cell DNA from all the strains (see Methods). Two separate flasks were used: one for the DNA from JH2-17 and one for the DNA from JH2-21. The DNA of Str. faecalis strain JH2-1 on the filter served as a control. The mixtures were incubated at 60°C for 18 hr. Non-specific binding of labelled plasmid DNA to the filters was tested for each

Table 22 Reassociation of DNA from strains of *Str. faecium*, "*Str. faecium* var. *casseliflavus*" and "*Str. faecium* var. *mobilis*"

Source of unlabelled DNA from:		Percentage of hybridization of unlabelled DNA with labelled DNA from[a]:		
Species	Strain	*Str. faecium* W100	"*Str. faecium* var. *casseliflavus*" W99	"*Str. faecium* var. *mobilis*" W88
Str. faecium	W100	100	21	18
"*Str. faecium* var. *casseliflavus*"	W98	13	105	96
	W99	17	100	98
"*Str. faecium* var. *mobilis*"	W88	10	95	100
	W92	15	90	92

[a] Each value shown is the average of four reactions.

Table 23 DNA homology among haemolytic plasmids derived from "Str. faecalis var. zymogenes" and 'Str. durans"

Total unlabelled DNA from: Species	Origin	Plasmid no.	Phenotype	Percentage of reassociation with labelled plasmid DNA from [a] pB21	pB17
Str. faecalis	JH2-21	pB21	Haemolysin	100	12
" "	JH2-4	pB4	"	92	14
"Str. faecalis var. zymogenes"	PB79	pPB79	"	97	13
" "	JH2-27	pJH2	"	95	10
Str. faecalis	JH2-17	pB17	"	17	100
" "	JH2-12	pW12	"	14	87

[a] The results indicate the percentage reassociation of ^{3}H-labelled plasmid DNA with unlabelled plasmid DNA relative to the reassociation with whole cell DNA extracted from a plasmid-free Str. faecalis strain JH2-1. Each value shown is the average of three reactions.

hybridization by using blank filters. Table 23 summarizes the results of these experiments. Haemolytic plasmid pB21 isolated from strain JH2-21 and plasmid pB17 from strain JH2-17 were found to share roughly 7% of their DNA sequences with the DNA of Str. faecalis strain JH2-1 (plasmid free). Taking the reassociation with whole cell DNA or chromosomal DNA extracted from Str. faecalis strain JH2-1 into account, it can be seen that reassociation among haemolytic plasmids originating in "Str. faecalis var. zymogenes" is high (>90% see Table 23). In contrast, plasmid pB21 from "Str. faecalis var. zymogenes" shared only 17% and 14% of its DNA sequences with the haemolytic plasmids derived from "Str. durans" strains B17 (JH2-17) and W12 (JH2-12).

DNA-DNA duplex studies performed with ^{3}H-labelled haemolytic plasmid pB17 originating from "Str. durans" showed that the plasmid did not share a significant proportion of its nucleotide sequences (10-17%) with any haemolytic plasmid originating from "Str. faecalis var. zymogenes" strains that have been examined; whereas plasmid pB17 shared 87% of its sequences with the DNA of "Str. durans" strain W12. This is in keeping with the results noted above when both plasmids pB17 and pB21 were used.

Restriction enzyme analysis of haemolysin determining plasmid

Plasmid DNAs were isolated from "Str. durans" strain B17 and "Str. faecalis var. zymogenes" strain B21 by the CsCl-ethidium bromide density gradient centrifugation method and cleaved by restriction endonuclease Hind III. Fragments obtained by digestion of λ DNA with restriction endonucleases Hind III + EcoRI were used as molecular weight size markers (Figure 9 ; lane 2).

Cleavage of plasmid pB17 obtained from "Str. durans" strain B17 with Hind III generated nine fragments (in addition to two faint bands of partial digests) (Figure 9 ; lane 1). Cleavage of the λ plasmid pB21 isolated from "Str. faecalis var. zymogenes" strain B21 with Hind III generated twelve fragments (Figure 9 ; lane 3). The sizes of the fragments generated after digestion of plasmids with restriction endonucleases was calculated according to Schaffer & Sederoff (1981) (Table 24, i and ii).

Table 24 i) Fragments generated after digestion of pB17 with restriction endonuclease Hind III

Hind III fragments	Molecular size in Kb
A	10.572
B	9.072
C	5.251
D	4.351
E	3.488
F	2.981
G	2.749
H	2.448
I	2.163

ii) Fragments generated after digestion of pB21 with restriction endonuclease Hind III

Hind III fragments	Molecular size in Kb
A	27.892
B	5.378
C	4.306
D	4.134
E	3.083
F	2.942
G	2.412
H	2.225
I	2.030
J	1.883
K	1.608
L	1.311

Figure 9 Restriction enzyme analysis of haemolysin determining plasmid of "Str. faecalis var. zymogenes" and "Str. durans"

Agarose gel electrophoresis of plasmid DNA isolated from "Str. durans" strain B17 and "Str. faecalis var. zymogenes" strain B21. Plasmid DNAs were isolated from dye-buoyant density gradients and cleaved by restriction endonuclease Hind III. Fragments obtained by digestion of λ DNA with restriction endonucleases Hind III and EcoRI were used as molecular weight size markers. The molecular weights of the Hind III + EcoRI fragments of λ DNA were taken from Daniels et al. (1980). The fragments generated after digestion of DNA with restriction endonucleases were subjected to electrophoresis. Electrophoresis was carried out in a 1% agarose vertical gel for 16 hr at 17 volts. The tracks on the gel from left to right contain:

1) Hind III digest of the haemolytic plasmid from "Str. durans" strain B17
2) Hind III + EcoRI digest of λ DNA
3) Hind III digest of the haemolytic plasmid from "Str. faecalis var. zymogenes" strain B21

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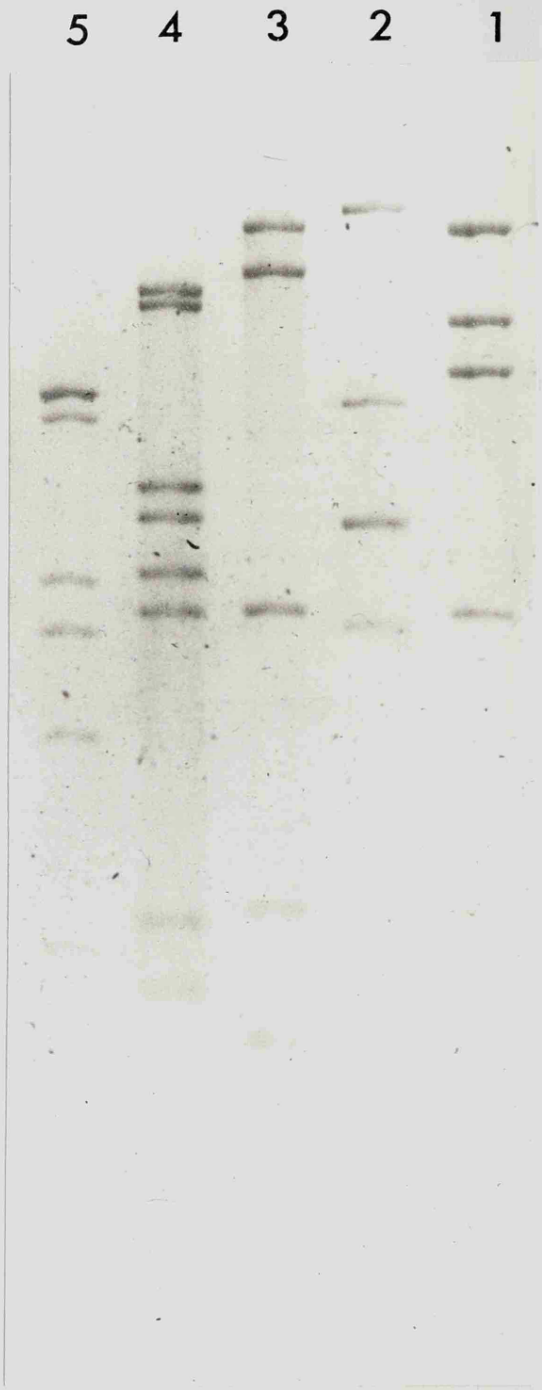
Figure 10 Restriction enzyme analysis of plasmids from streptococci of serological group D and the cryptic plasmids from L. monocytogenes serovar 5

Restriction endonuclease Bam HI cleavage pattern of haemolytic plasmids isolated from "Str. faecalis var. zymogenes" strain B21, "Str. durans" strain B17, and the cryptic plasmid from L. monocytogenes strain C659. The plasmid which codes for the production of pigments in "Str. faecium var. casseliflavus" strains W99 and W106 was also cleaved by restriction endonuclease Bam HI. Plasmid DNAs were isolated from dye-buoyant density gradients. Bam HI—cleaved DNA was then analyzed by electrophoresis on 0.7% agarose horizontal slab gel for 16 hr at 17 volts. The tracks on the gel contain from left to right:

1) plasmid pW106 isolated from "Str. faecium var. casseliflavus" strain W106
2) cryptic plasmid pC659 from L. monocytogenes strain C659
3) plasmid pW99 isolated from "Str. faecium var. casseliflavus" strain W99
4) haemolytic plasmid from "Str. faecalis var. zymogenes" strain B21 and
5) haemolytic plasmid from "Str. durans" strain B17.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Molecular weights of the plasmids were determined by summing the size of the fragments. It was found that the sizes of plasmids are 43.07 kilobases for plasmid pB17 isolated from "Str. durans" strain B17, and 59.204 kilobases for plasmid pB21 isolated from "Str. faecalis var. zymogenes" strain B21.

Restriction enzyme analysis of the drug resistance plasmid pW100

Plasmid DNA (pW100) isolated from Str. faecium strain W100 by the CsCl-ethidium bromide density gradient centrifugation method was cleaved with restriction endonuclease Hind III. Hind III-cleaved DNA was subjected to electrophoresis through a 1% agarose gel (Figure 11).

Cleavage of plasmid pW100 with the enzyme Hind III generated eight fragments (Figure 11 ; lane 2). Molecular weight size markers were obtained by digestion of λ DNA with Hind III. The molecular weights of the Hind III fragments of λ DNA were taken from Daniels et al.(1980). The size of the first four fragments generated after digestion of plasmid pW100 with Hind III was calculated according to Schaffer & Sederoff (1981) (Table 25). Since the last four fragments generated after digestion of pW100 with Hind III was much smaller that the smallest Hind III fragment generated after digestion of λ DNA, the sizes of these fragments are unknown.

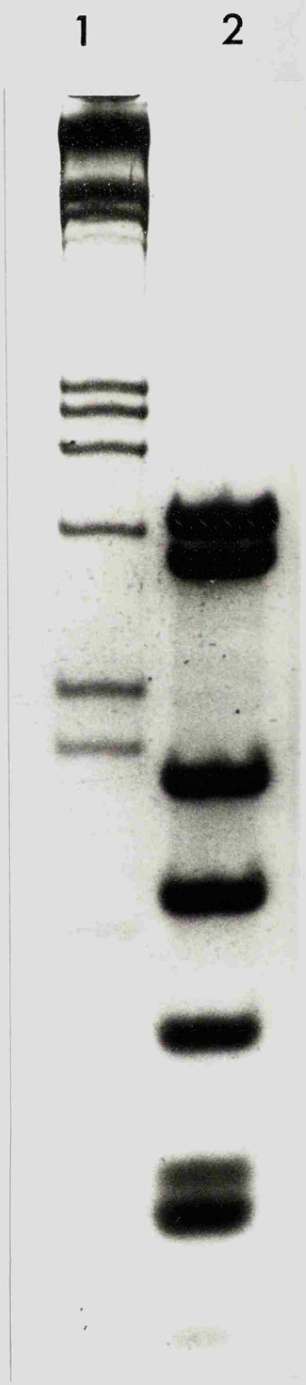
Table 25 Fragments generated after digestion of plasmid pW100 with restriction endonuclease Hind III

Hind III fragments	Molecular size in Kb
A	4.860
B	4.140
C	1.483
D	0.638
E	-
F	-
G	-
H	-

- = Size of the fragments unknown.

Figure 11 Restriction enzyme analysis of drug resistance plasmid pW100

Agarose gel electrophoresis of plasmid DNA isolated from Str. faecium strain W100. Plasmid DNA isolated by dye-buoyant density gradient centrifugation, and cleaved by restriction endonuclease Hind III. Hind III-cleaved DNA was analyzed by electrophoresis on 1% agarose vertical gel for 16 hr at 17 volts. Track 1 on the gel contains a Hind III digest of λ DNA used as a molecular weight marker; track 2 contains the drug resistant plasmid pW100 cleaved by Hind III.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DISCUSSION

The results of the work presented here indicate that of the bacterial species investigated, evidence for plasmid encoded characters was obtained only amongst certain strains of the genus Streptococcus.

No plasmid DNA was detected in the strains of Erysipelothrix rhusiopathiae, Streptococcus faecalis, and "Streptococcus faecalis var. malodoratus" examined.

Plasmid DNA detected in Listeria monocytogenes serovar 5, Streptococcus agalactiae, Streptococcus equinus, "Streptococcus faecalis var. liquefaciens" and "Streptococcus faecium var. mobilis" was not found to be correlated with any phenotypic property of the respective strains. Only in Streptococcus bovis, "Streptococcus durans","Streptococcus faecalis var. zymogenes", Streptococcus faecium and "Streptococcus faecium var. casseliflavus" strains could the presence of plasmid DNA be correlated with phenotypic properties. These particular results will be discussed in detail later.

Taxa where plasmid DNA not detected

Of these taxa, only "Str. faecalis var. malodoratus" will be discussed because there was no obvious correlation of phenotype with the presence of extrachromosomal DNA in E. rhusiopathiae and Str. faecalis.

"Str. faecalis var. malodoratus"

"Str. faecalis var. malodoratus" strains (D846 and D847) were studied because they produce significant amounts of H_2S whereas most Str. faecalis produce slight amounts of H_2S from cysteine after 2 and 3 days. A possible explanation of this observation is that the H_2S production by "Str. faecalis var. malodoratus" strains may be encoded by a plasmid. Since there is circumstantial evidence that H_2S production is a plasmid-mediated property in E. coli (Magalhães & Véras, 1977; Ørskov & Ørskov, 1973), it was of interest to know whether "Str. faecalis var. malodoratus" contained any plasmids. Possible explanations for not detecting a plasmid are: (1) absence of plasmid DNA in those strains; (2) strains D846 and 847 may have contained a plasmid but the plasmid screening methods employed to detect its presence

might not have worked properly for these strains; (3) yield of plasmid DNA was very low due to poor lysis of the cells or due to significant loss of plasmid DNA during the steps of the isolation procedure.

Although no plasmid was detected in "Str. faecalis var. malodoratus" strains D846 and D847, it would have been interesting to determine whether H_2S production trait could be transferred to other strains of Str. faecalis which do not produce H_2S and also whether the trait could be lost by using chemical curing agents.

Taxa where plasmid DNA detected

In this section will be discussed the taxa where plasmid DNA was detected but no correlation could be found between its presence and any phenotypic character.

Listeria monocytogenes serovar 5

Plasmid DNA was detected in one strain of Listeria monocytogenes serovar 5 (C659) but no correlation was noted between the presence of plasmids and any phenotypic character (Figure 4). This result is in partial keeping with the findings of Perez-Diaz et al. (1981). The lack of correlation between the presence of plasmid DNA and phenotypic characters in the strain C659 was disappointing because haemolytic activity amongst listeriae is rather easily lost and it was hoped that it could be shown that the property was plasmid encoded.

As another approach to determine whether the plasmid pC659 isolated from a strain of L. monocytogenes serovar 5 (C659) shares a common evolutionary origin with the plasmid pB17 of the haemolytic "Str. durans" strain B17 or with the pB21 of "Str. faecalis var. zymogenes" strain B21, restriction endonuclease digestion of the plasmids pC659, pB17 and pB21 were performed and the patterns of fragments generated were compared on agarose gel. The results show (Figure 10) that the fragments generated after digestion of all the plasmids are completely different from each other suggesting that the origin of the plasmids is different.

No haemolytic transconjugant was isolated when several attempts were made to transfer the haemolytic trait from L. monocytogenes serovar 5 strain C659 to other bacteria. Possible explanations for these results are: 1) haemolysin production in the genus Listeria may not be encoded by plasmids; 2) plasmids may indeed encode for the production of haemolysin but are non-conjugative; 3) since no plasmid was found to be present in other L. monocytogenes serovar 5 strains, it is possible that the various degrees of haemolysis by L. monocytogenes may be a chromosome borne trait.

Streptococcus agalactiae

Str. agalactiae strains (Table 5) were chosen for the study on the basis of various phenotypic properties of the strains studued by Nakkash (1981). Of the 39 strains screened, only two Str. agalactiae strains, B14 (type strain) and B32 of bovine origin were found to contain plasmids. The plasmid patterns obtained after electrophoresis on agarose gels (Figure 1) indicate that the two strains B14 and B32 contain two different sets of plasmids. None of the plasmids was found to correlate with any phenotypic trait. All 39 strains of serological Group B including those which contained plasmids, gave almost the same pattern of sensitivity to antibiotics.

The presence of plasmid DNAs in the type strain of the species is interesting because the strain B14 was isolated in the late 1940's at a time when there was intensive use of penicillin in the treatment of cases of bovine mastitis. Strain B32 was also isolated from a cow before 1954. It is possible that during excessive use of penicillin, the bovine strains acquired drug-resistance plasmids from other strains of the same species or from different species of the same genera. However, both strains are penicillin-sensitive. Perhaps the plasmid containing drug resistance genes may be present, but the lack of resistance to penicillin may be due to the formation of a defective β-lactamase enzyme. This could be the result of spontaneous mutation in the gene encoding for β-lactamase activity.

Streptococcus equinus

As can be seen from the Results section plasmid DNA was detected in only one of the Str. equinus strains studied,(Figure 4). There did not appear to be any phenotypic character possessed by the plasmid containing strain and any of the other strains of Str. equinus. The taxonomic relationship already noted between Str. equinus and Str. bovis would have been a good reason for further investigation of the transfer of plasmids between these strains and the study of homology between the plasmids from the two taxa, but time did not allow for this investigation.

"Streptococcus faecalis var. liquefaciens"

The results presented here show that, with one exception, all the "Str. faecalis var. liquefaciens" strains examined contained plasmid DNA molecules (Figure 1). However, no correlation was found between the presence of plasmid DNA in these strains and their gelatin liquefying ability which is the only distinguishing feature separating Str. faecalis and "Str. faecalis var. zymogenes" from "Str. faecalis var. liquefaciens". Nor did any other phenotypic property of these strains appear to be plasmid coded. This result is consistent with the findings of Oliver et al.(1977). These workers also reported both the presence and absence of plasmid DNA in "Str. faecalis var. liquefaciens" strains but found no relationship between the presence of plasmid DNA and gelatin liquefying ability.

Over many years several workers have questioned the validity of the subspecies "Str. faecalis var. liquefaciens". Since gelatin liquefaction has not been reported to be lost spontaneously or by using chemical agents, and no transconjugants have been isolated which have acquired the ability to liquefy gelatin, it is most probable that the gelatin liquefying ability of "Str. faecalis var. liquefaciens" is a stable character and encoded by chromosomes.

In this context it would be interesting to determine whether the cryptic plasmids of "Str. faecalis var. liquefaciens" could be mobilized to plasmid-free strains by using other plasmids which can transfer non-conjugative plasmids. Gelatin liquefying ability of the transconjugants could then be tested.

"Streptococcus faecium var. mobilis"

The presence of plasmid DNA in a yellow-pigmented strain of "Str. faecium var. mobilis" (W88) and its absence in the non-pigmented strain (W92) indicated a strong possibility that this trait was plasmid encoded. The detection of a high degree of DNA-DNA homology between the strains of "Str. faecium var. mobilis" and "Str. faecium var. casseliflavus" studied (Table 22) suggests that "Str. faecium var. mobilis" and "Str. faecium var. casseliflavus" are very closely related species, or members of the same species, and constitute a distinct taxon. This result is in accord with the views expressed by Amstein & Hartman (1973), Collins & Jones (1979), Roop et al.,(1974), and Vaughan et al.(1979).

It would have been interesting to study the degree of homology between the plasmids isolated from "Str. faecium var. mobilis" and the pigment encoding plasmid of"Str. faecium var. casseliflavus" by digesting the plasmids with different restriction endonucleases and comparing the patterns of the fragments generated. Further, hybridization of the plasmid DNA isolated from "Str. faecium var. mobilis" and "Str. faecium var. casseliflavus" could have shown the extent of homology of deoxyribonucleotide sequences of the two plasmids or the source of origin of the plasmids.

Taxa where plasmid DNA was detected and could be correlated with phenotype

In this section the strains studied will be discussed under that phenotypic trait for which this is good evidence or some evidence that the trait is plasmid encoded. The traits are lactose fermentation,haemolysis, drug resistance and pigmentation.

Lactose fermentation

The metabolic properties of Str. bovis strain B16 appear to be governed in part by DNA associated with extrachromosomal elements. At least a distinct plasmid species has been observed in both Str. bovis strains B16 and D598 (Figure 4). The results presented here provide evidence that lactose activity may well be associated with specific plasmids.

Evidence that lactose-fermenting ability in Str. bovis strain B16 is

plasmid-linked comes from several observations: 1) the ability to ferment lactose was readily cured when cells were exposed to acridine orange; 2) the plasmid was found to be missing in the Lac^- mutants.

Of further interest regarding Str. bovis strain B16 is the fact that lactose defective (Lac^d) strains of the organism were able to utilize lactose slowly. However, these Lac^d variants appeared after incubation with different curing agents. It was found that although lactose-fermenting ability is associated with a plasmid in strain B16, Lac^d derivatives still contain the plasmid DNA. These Lac^d derivatives containing the lactose plasmid were able to revert to full lactose-fermenting ability, whereas Lac^- mutants which had lost the plasmid were able to revert only to a partial lactose-fermenting phenotype. The ability of Lac^d derivatives to revert to a fully lactose-metabolizing phenotype suggests a point mutation on the lactose plasmid. This suggests that the organisms may have an alternative mechanism for lactose utilization, which could be carried on the chromosome in addition to the plasmid locus. This chromosomal location of the β-galactosidase or lactose gene would be required for Lac^- mutants without a plasmid to acquire a partial Lac^+ phenotype and coincident β-galactosidase activity. Although several enzymes essential to lactose metabolism may be linked to the plasmid, the evidence provided in this work indicates that the loss of this plasmid results in loss of β-galactosidase activity. The observation that all three independently isolated Lac^- mutants reverted to the partial Lac^+ phenotype is additional evidence that the genes enabling slow lactose metabolism are located on the only common genetic determinant, the bacterial chromosome. Similar observations have been reported by Anderson & McKay (1977) with regard to lactose metabolism in Str. cremoris. They showed that lactose-fermenting ability is associated with a 36-Mdal plasmid in Str. cremoris strain B1, and spontaneous Lac^- derivatives were isolated which maintained this plasmid. Those spontaneous Lac^- derivatives containing the lactose plasmid were able to revert to full lactose-fermenting ability, whereas Lac^- mutants

which had lost the 36-Mdal plasmid were able to revert only to a partial lactose-fermenting phenotype. The reversion of Lac^- mutants to a partial Lac^+ phenotype was also reported in Str. lactis C_2 (McKay et al., 1972). The electrophoretic patterns of the DNA fragments obtained after restriction endonuclease treatment of the plasmids from Str. bovis strains B16 and D598, Str. equinus strain D1090(Figure 8) indicated that the plasmids are completely different.

In any event, the results presented here strongly imply the involvement of a plasmid with the ability of Str. bovis strain B16 to utilize lactose. These data also provide evidence that cells which have lost the plasmid and subsequently become Lac^- still possess lactose activity and thus retain their distinction from Str. equinus.

Further research is needed in this area. The development of genetic transfer systems could lead to further clarification of the role of plasmid DNA in Str. bovis strains and its relationship to lactose fermentation by this bacterial species.

Lactose fermentation as noted earlier is an important criterion for differentiating Str. bovis from Str. equinus. Str. bovis ferments lactose, Str. equinus does not. While taxonomic studies show that the two species are distinct, and indeed the pattern of phenotypic traits is different for each, lactose fermentation is the one single constant characteristic which differentiates Str. bovis from Str. equinus (see Jones, 1978). Thus it is taxonomically important to establish whether lactose fermentation is a plasmid-mediated property.

Haemolytic activity

It was concluded from the results that the genes determining haemolysin production in "Str. durans" strains B17 and W12 and in "Str. faecalis var. zymogenes" strains B21, PB4 and PB79 are borne on self-transmissible plasmids. The instability of this trait and the correlation between their loss and acquisition and the absence and presence of plasmids, are the evidence for this conclusion.

Plasmids from all "Str. durans" strains (B17 and W12) and "Str. faecalis var. zymogenes" strains (B21, PB4 and PB79) tested, transferred to other Str. faecalis strains at a high frequency (generally in the range of 10^{-4} to 10^{-6} per donor organism) (Tables 9 & 11).Although these matings were quite reproducible by the membrane filter method described, attempts to effect transfer of the haemolytic plasmid from"Str. durans" strains in broth cultures were unsuccessful. However, in similar broth cultures, "Str. faecalis var. zymogenes" strains transferred the haemolysin determining plasmid to other Str. faecalis strains at a low frequency (10^{-7} to 10^{-8} per donor organism). This result is poor compared to previous studies by other workers (Jacob et al., 1975) who found high transfer frequency of the haemolytic plasmid of "Str. faecalis var. zymogenes" when mating was carried out in liquid medium. The mechanism of transfer of the haemolysin determining plasmids by "Str. durans" and "Str. faecalis var. zymogenes" strains might be a conjugation like phenomenon as has been inferred by several workers (Jacob & Hobbs, 1974; Jacob et al., 1975; Horodniceanu et al., 1979). This mechanism is suggested by the fact that a very close cell-to-cell contact between a very high number of donor and recipient cells is essential for conjugation.

"Str. faecalis var. zymogenes" strains B21, PB4 and PB79 could readily transfer the plasmid determining haemolysin to Str. faecalis strain JH2-1 when the mating was performed on membrane filters. This is in accord with the views of Clewell (1981) based on his interpretation of the findings of several workers. Clewell (1981) placed the conjugative plasmids of Str. faecalis into two general categories: one group which transfers at a relatively high frequency (10^{-1} to 10^{-3} per donor) in broth (Clewell et al., 1980; Dunny et al., 1978, 1979; Jacob et al., 1975), and the others which transfer poorly in broth (usually less than 10^{-6} per donor) but rather efficiently (10^{-2} to 10^{-4} per donor) when the matings are carried out on membrane filters (Hershfield,1979; Malke, 1979). The findings reported here

are consistent with the latter category of conjugative plasmids. Clewell (1981) speculated that these differences in the efficiency of plasmid transfer could be explained on the basis of sex pheromones which generate cell-to-cell contact in these strains where transfer is high.

The haemolysin determining plasmid pJH2 of Str. faecalis strain JH2-27 has been reported to be transferred to a plasmid free Str. faecalis strain in broth at a very high frequency (Jacob et al., 1975). However, efforts to confirm this observation were unsuccessful. It is likely that either the plasmid pJH2 in strain JH2-27 did not respond to the pheromone produced by plasmid-free Str. faecalis strain JH2-1, or Str. faecalis strain JH2-1 did not produce enough pheromone to facilitate the formation of donor-recipient mating aggregates under the conditions of the experiment. Another possibility is that, although I followed the standard mating conditions in liquid culture as described by Jacob et al. (1975), it may have happened that the mating conditions were not appropriate for the donors and recipients tested. However, it is difficult to explain why the plasmid transfer frequency was poor for haemolysin determining plasmid pJH2 in broth. It would have been interesting to know whether the plasmid transfer frequency of pJH2 could be changed, by changing some of the mating conditions, e.g. size of the inoculum, duration of contact, or by allowing the strains to mate in standing culture or by agitation at different speeds during mating.

The observation that β-haemolysis in "Str. faecalis var. zymogenes" strain B21 and in "Str. durans" strain B17 is plasmid mediated suggested the possibility that the haemolysin plasmid of "Str. durans" might be an immediate ancestor,or alternatively, a derivative of the haemolytic plasmid or, indeed, the same haemolytic plasmid as that found in "Str. faecalis var. zymogenes" strains. Clearly, the observations reported here are not consistent with this view. The haemolytic plasmids isolated from "Str. durans" strain B17 and from "Str. faecalis var. zymogenes" strain B21 were of 43.07 and 59.204 kilobases (Kb) respectively: whereas the molecular

weight of haemolytic plasmids isolated from two "Str. faecalis var. zymogenes" strains by Jacob et al. (1975) was 38-Mdal. Further, the haemolytic plasmid pB17 isolated from "Str. durans" strain B17 and pB21 isolated from "Str. faecalis var. zymogenes" strain B21, showed completely different restriction endonuclease digestion patterns (Figures 9 & 10).Consistent with the completely different patterns of haemolytic plasmid DNA from pB17 and pB21, it was also found that the percentage of hybridization of DNA between haemolytic plasmids pB17 and pB21 was very low. In contrast, the reassociation of DNA among haemolytic plasmids isolated from "Str. faecalis var. zymogenes" strains was significantly higher (Table 23). These results once again indicate the probable diverse source of origin of haemolytic plasmids obtained from "Str. durans" strains and "Str. faecalis var. zymogenes" strains. Of course, the structural genes determining haemolysin biosynthesis are expected to occupy but a small portion of the plasmid genome. It is possible, therefore, that the haemolytic activity specified by the haemolytic plasmids of "Str. faecalis var. zymogenes" strains is closely related to the haemolysin synthesized by "Str. durans" strains containing haemolytic plasmids. Our data simply indicate that the major nucleotide sequences of the molecular vehicles carrying the haemolysin genes are not closely related.

During hybridization experiments, a plasmid-free Str. faecalis strain JH2-1 was used as a control. The haemolytic plasmids pB17 and pB21 were reassociated with the DNA extracted from Str. faecalis strain JH2-1 (plasmid-free) to determine the degree of nucleotide similarity between these plasmids and the host chromosome. It was found that haemolytic plasmids pB17 and pB21 shared roughly 7% of their DNA sequences with the total or chromosomal DNA of Str. faecalis strain JH2-1. This level of relatedness was also observed between plasmids and host chromosomal DNA (So et al., 1975). The nature of the sequences held in common between the haemolytic plasmids and chromosomal DNA is unknown, although the reassociation experiments were carried out at 60°C, which sets stringent criteria for the formation of DNA-DNA duplexes and discriminates against imperfectly matched base pair

sequences (Crossa et al., 1973).

The beta-haemolytic activity of Str. faecalis strain B21, PB4 and PB79 characterizes them as being the "zymogenes" variety (Sherman, 1938). It has been found that these properties are linked to a transferable plasmid and and are lost easily from a host cell at high frequency, which is in complete agreement with the results of other workers (Jacob et al.,1975). Spontaneous loss of haemolytic activity is a property commonly observed for "zymogenes" strains (Deibel, 1964; Sherman, 1938). Tomura et al.(1973) have shown that another Str. faecalis strain carried transferable haemolysin and bacteriocin traits. These data lead us to suggest that the beta-haemolysin trait of "Str. faecalis var. zymogenes" strains will prove to be commonly, if not invariably, plasmid borne. Since the haemolytic activity is the property that distinguishes the "zymogenes" variety from Str. faecalis (Sherman, 1938), the results presented here endorse the view of Deibel (1964) that the varietal status of "zymogenes" should be dropped.

"Str. durans" is now considered to be a variety of Str. faecium (see Jones, 1978). It should be noted that although the strain of "Str. durans" which was considered to be the type strain is haemolytic, very few "Str. durans" strains possess this property.

Drug resistance

The data presented here show that the genes determining resistance to Km, Nm, Sm and Em in Str. faecium strain W100 are carried by a conjugative R plasmid. The criteria used to assign these resistances to the plasmid, are several. The loss of resistances by Str. faecium strain W100 at a high frequency during certain growth conditions (Table 13) and the correlation between their loss and acquisition and the absence and presence of plasmid DNA, are the evidence for this interpretation.

Multiple antibiotic resistances were transferred from Str. faecium strain W100 into recipient strain JH2-1 (Str. faecalis) and B19-1 (Str. faecium) (Table 15). Plasmid transfer from Str. faecium strain W100 to either of the recipients (JH2-1 or B19-1) could not be carried out by using

the classical method in liquid medium. The use of the membrane technique which allows a close contact between a very high number of cells is necessary for the transfer of antibiotic resistances from Str. faecium strain W100 to Str. faecalis strain JH2-1 and Str. faecium strain B19-1. This is not consistent with the findings of Courvalin et al.(1978), who showed transfer of Km, Sm and Em resistance from a strain of Str. faecium to a strain of Str. faecalis during mixed incubation in broth. The mechanism of transfer of resistance markers carried by Str. faecium strain W100 might be a conjugation-like phenomenon. This mechanism is suggested by the fact that a very close cell-to-cell contact between donor and recipient cells appears to be essential for conjugation. By analogy with the conjugal transfer of R factors between the Enterobacteriaceae, it would be expected that cell-to-cell contact between a donor and a recipient cell would be essential for conjugation (Watanabe, 1963).

The kinetics of transfer of the conjugative plasmid pW100 from Str. faecium strain W100 to a plasmid-free recipient strain JH2-1 show that a considerable number of transconjugants were detected after 2 hr, and that the highest frequency of transfer was observed after 3 hr. In contrast, in matings with strain B19-1, a low number of transconjugants were detected after 4 hr of contact, and the frequency did not change after 18 hr (Table 17). It was found that the plasmids harboured by Str. faecium strain W100 and by the control strain JH2-25, are transferred at a frequency of 1000 to 10000 times higher when crossed with strain JH2-1 than with B19-1. It was concluded that the increase of plasmid transfer frequency was related to the mating time rather than to the increase of the donor cell number, when Str. faecium strain B19-1 was used as recipient. In contrast, transfer frequency appears to depend on donor cell number rather than time when Str. faecalis strain JH2-1 was used as a recipient. The different kinetics of acquisition of transferred resistances (Table 17) by different recipient strains JH2-1 and B19-1 are difficult to interpret since it was not understood why the rate of transfer should markedly decrease when Str. faecium

strain B19-1 was used as a recipient.

Str. faecium strain W100 always transferred resistances to Km, Nm, Sm and Em simultaneously to the recipient strains JH2-1 and B19-1, except in one case, where an Em-resistant transconjugant was isolated when strain W100 was crossed with JH2-1. Em-resistance was never transferred alone except in the one case. Analysis of the Em-resistant transconjugant by the CsCl-ethidium bromide density gradient centrifugation method showed that the transconjugant does not contain plasmid DNA. These results suggest that: (i) the Em resistance marker might be located on a transposable element and inserted in the host chromosome as a part of a transposable element after transfer. This would be consistent with the findings that the tetracycline resistance transposon Tn916 in Str. faecalis is capable of conjugal transfer when mated with plasmid-free recipients on membrane filters (Franke & Clewell, 1981). The interpretation was based on the fact that no plasmid DNA was detected in Em-resistant transconjugant strain JH2-104, after analysis of the strain by CsCl-ethidium bromide density gradient centrifugation. Previous workers have demonstrated the presence of an erythromycin resistance transposon Tn917 on the non-conjugative plasmid, which has been shown to transpose to a co-resident plasmid at a frequency of 10^{-6} (Tomich et al., 1980). (ii) Since the selection of transconjugants was done on a plate containing a high concentration of Em (1000 µg/ml) and the frequency of transconjugants obtained was very low (10^{-8}), the probability of a mutation event occurring cannot be ruled out. However, the insertion of Em resistance coding genes in the host chromosome as part of a transposable element seems the more likely. It is difficult, however, to decide at this point whether an erythromycon-resistant transconjugant is just an Em-resistant mutant of Str. faecalis strain JH2-1, or whether an erythromycin resistance determinant located on a transposable element was transferred to Str. faecalis strain JH2-1 and integrated into the host chromosome. Since it has been postulated here that the Em resistance determinant might reside on a transposon, it was of interest to determine whether the erythromycin resistant plasmid-free

transconjugant strain(JH2-104) could act as a donor in the transfer of Em-resistance; because of lack of time, no attempts were made in the present study to transfer erythromycin resistance from transconjugant strain JH2-104 to other recipient strains.

Attempts were made to determine the molecular weight of the drug resistance plasmid pW100 of Str. faecium strain W100 by digesting the plasmid DNA with restriction endonuclease Hind III, and summing the size of the fragments. As a molecular weight marker, λ DNA digested with Hind III was used; but some of the fragments generated after digestion of the plasmid DNA (pW100) with restriction endonuclease Hind III were smaller than the smallest fragment generated after digestion of λ DNA (Figure 11). Consequently, the size of the fragments generated after digestion of pW100 plasmid DNA with Hind III, could not be determined. The sizes of the first four fragments generated after digestion of pW100 plasmid DNA with Hind III are 4.860, 4.140, 1.483 and 0.638 Kb respectively. In order to determine the molecular weight of the smaller fragments, a restriction endonuclease digest of smaller plasmid DNA of known size should have been used, in addition to λ DNA as a molecular weight size marker. But again, due to shortage of time it was not possible to perform this experiment.

While drug resistance can be important taxonomically because such characters are used to define a phenotype, no bacteria would be classified on the sole basis of drug resistance or sensitivity. However, plasmids encoding for drug resistance may carry other genes which are capable of phenotypic expression. Therefore, the presence of plasmid DNA encoding for drug resistance can mean that that organism may be phenotypically different in other respects.

Drug resistance in streptococci is interesting because it has not spread through the genus to the same extent as in Staphylococcus.

Pigment production

The data presented here show that the genes determining the production of pigment in "Str. faecium var. casseliflavus" strain W99 and W106 are

almost certainly borne on a plasmid. The instability of the trait and the correlation between its loss and acquisition and the absence and presence of plasmids, are the evidence for this conclusion.

Of the 11 strains of "Str. faecium var. casseliflavus" screened for the presence of plasmids, only seven strains were found to contain plasmid DNA. It would be of interest to know whether the plasmids in these strains could be correlated with any of the phenotypic properties of the respective strains, such as the plasmid encoded pigmentation trait in "Str. faecium var. casseliflavus" strains W99 and W106.

Further work is required in this area to determine whether the plasmid which codes for yellow pigmentation in "Str. faecium var. casseliflavus" strains can be transferred to other plasmid-free strains of Str. faecalis or any other streptococci; and, if so, whether the plasmid can be stably maintained in the new host.

The electrophoretic patterns of the DNA fragments obtained after restriction endonuclease treatment of the plasmids from strain W99 and W106 indicated that the plasmids were very similar to each other (Figure 10). This suggests that the plasmid may have evolved from a common ancestor. It would have been interesting to know the extent of polynucleotide sequence homology between the two plasmids by DNA-DNA hybridization experiments. Unfortunately, time did not allow us to do such work.

It was suggested by Liaaen-Jensen (1965) that carotenoid pigments may protect membrane-bound functions from photodynamic damage. It is reasonable to speculate that the pigment in "Str. faecium var. casseliflavus" has survival value for those enterococci which are most often found in soil and plant material but this is pure speculation.

The loss of motility noted in the two of the cured strains of "Str. faecium var. casseliflavus" (W99 and W106) may perhaps have been due to altered flagella synthesis or a change in the cell surface. Since other cured strains of "Str. faecium var. casseliflavus" retained their motility it is suggested that motility in "Str. faecium var. casseliflavus" strains

W99 and W106 is not a plasmid encoded property.

The study was mainly concerned with the detection of plasmid encoded characters in certain lactic acid bacteria. Of the bacterial taxa investigated only the results obtained with representatives of the genus Streptococcus proved to be of interest. Haemolytic activity in "Str. durans" and "Str. faecalis var. zymogenes" was found to be determined by transferable plasmids of two different types. The results of the investigation of "Str. faecalis var. zymogenes" are in agreement with the work of previous investigators but the detection of a plasmid encoding for haemolysis in "Str. durans" is, to my knowledge, the first report.

It would be interesting to know whether the plasmids encoding for haemolysin production in the different strains of "Str. faecalis var. zymogenes", including the control strain obtained from Dr. A. Jacob, are the same. It would also be interesting to know whether plasmids encoding for haemolytic activity in the different strains of "Str. durans" are identical or very similar to each other and to the plasmid of "Str. faecalis var. zymogenes". The one example investigated did not reveal a close base pair homology between the plasmids from the two different taxa.

It is reasonable to speculate that strains of Str. faecalis and "Str. durans" which possess the haemolysis trait may have certain advantages over strains of the same taxa which do not contain the plasmid. It may be that the presence of the plasmid enables the strains to adapt and survive in a particular environment. Since it has been reported by other workers, as noted earlier, that in the cases investigated the same protein determines both haemolysin and bacteriocin activities in streptococci, it is

possible that the toxin helps to establish these bacteria which produce it as the predominant bacterial flora in a particular environment.

The evidence obtained indicated that lactose utilization by the Str. bovis strain studied was probably governed by genes located on both chromosome and plasmid. In the absence of the plasmid, the cured derivatives of the Str. bovis strain had lost their full lactose utilizing ability and became partially lactose positive. Further work is required to determine whether lactose utilization by all Str. bovis strains is a plasmid-mediated property; if so, whether all lactose utilizing plasmids are similar.

Str. faecium strain W100 was shown to contain a conjugative plasmid pW100, carrying the genes for resistances to Km, Nm, Sm and Em. The presence of a drug-resistance encoding plasmid in this Str. faecium strain was not surprising because the strain was isolated from clinical sources. Thus, the acquisition of the plasmid by the organism enabled it to adapt and survive in an "antibiotic-rich" environment.

In two strains of"Str. faecium var. casseliflavus", the evidence indicated that pigment production was probably a plasmid-mediated property. Moreover, "Str. faecium var. casseliflavus" and "Str. faecium var. mobilis" were found to be closely related species on the basis of DNA-DNA hybridization studies. Since pigmentation has been reported to protect the cell from photodynamic damage, it is possible that these yellow strains which were isolated from soil and plant material may have better survival chances than other bacteria lacking pigment.

No relationship was found between the presence of plasmid DNA and any phenotypic trait of the strains of L. monocytogenes serovar 5, Str. agalactiae, Str. equinus, "Str. faecalis var. liquefaciens" and "Str. faecium var. mobilis".

What effect plasmid-borne characters have on classification is not yet known, although preliminary work with the genus Proteus and Shigella suggests not very much when the classification is based on many features (McKell, 1977; Dodd, 1979), but they can have a marked effect on identification if the diagnostic scheme used relies heavily on such characters.

The effect of plasmid DNA on the ability of bacteria to adapt and survive in a changing environment is only just beginning to be studied. Evidence from the study of R plasmids indicates that the presence of these extrachromosomal entities is very important in "antibiotic-rich" environments and the same is probably true for other environments.

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A B S T R A C T

A survey was conducted of a number of lactic acid bacteria of the genera Erysipelothrix, Listeria and Streptococcus to investigate the presence of detectable plasmids. When plasmids were detected an attempt was made to correlate their presence with a phenotypic trait. In addition some plasmids were investigated to determine certain physical characteristics, e.g. molecular weight and also the degree of base pair homology between plasmids isolated from different bacteria.

The results indicate that of the strains of Erysipelothrix investigated none appeared to contain extrachromosomal DNA. Of the strains of the genus Listeria, plasmids were detected only in one representative of Listeria monocytogenes serovar 5. This particular serovar is characterized by enhanced haemolytic activity when compared with strains of the various other species in the genus Listeria. However, no evidence was obtained to indicate that the haemolysis was plasmid encoded. Plasmid deoxyribonucleic acid was detected in a number of strains representing a variety of species in the genus Streptococcus.

The results obtained confirmed earlier reports that the haemolytic activity of "Streptococcus faecalis var. zymogenes" is plasmid encoded. The results also indicate that the haemolytic activity of haemolytic strains of bacteria previously named "Streptococcus durans" was also plasmid encoded. Investigation of the properties of the plasmid DNA from both these sources however did not show a high degree of homology, thus indicating that the plasmids had not been derived from the same source. Plasmids conferring drug resistance were isolated from representatives of the species Streptococcus faecium. Pigmentation in some strains of "Streptococcus faecium var. casseliflavus" appeared to correlate with the presence of plasmid DNA. Strong evidence was also obtained which indicated that lactose fermentation in Streptococcus bovis was at least, in part, due to the presence of plasmids.

Although plasmids were detected in representatives of the species

Streptococcus agalactiae, Streptococcus equinus, "Streptococcus faecalis var. liquefaciens", "Streptococcus faecium var. mobilis", their presence could not be correlated with any particular phenotypic trait.

No plasmids were detected in those strains of Str. faecalis and "Str. faecalis var. malodoratus" examined.