ANTIBIOTIC RESISTANCE IN VIRIDANS STREPTOCOCCI

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A thesis submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements of Doctor of Philosophy.

Johannesburg 1994

ABSTRACT

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POTGIETER, ELSA, Ph.D. University of the Witwatersrand, Johannesburg, 1994.

Viridans streptococci are aetiological agents of endocarditis, bacteraemia, dental caries and abscesses of the liver, brain and joints. Viridans streptococci had until recently generally been considered to be uniformly susceptible to antimicrobial agents active against Gram-positive cocci. The susceptibility of 211 viridans streptococci isolated from blood in South Africa between 1988-1991 to eight antimicrobial agents was determined. Thirty-eight percent of the isolates were found to be resistant to penicillin (MICs $\geq 0.25\ \mu$g/ml). Five *Streptococcus mitis* isolates exhibited increased (MIC 64 and 128 μg/ml) or high-level (MIC $\geq 500\ \mu$g/ml) resistance to gentamicin. In vitro synergy between penicillin and gentamicin was not demonstrated with these isolates.

Inter- and intra-specific transfer of penicillin resistance genes between *S. mitis, Streptococcus oralis* and *Streptococcus pneumoniae* was also investigated. Of the 18 *S. mitis* / *S. oralis* isolates selected, 16 strains were shown to transform the laboratory recipient *S. pneumoniae* R6 to increased levels of penicillin resistance. All 13 *S. pneumoniae* donors tested transformed the *S. mitis* NCTC 10712 recipient. The high frequency with which penicillin resistance determinants were shown to transfer between the oropharyngeal commensals *S. mitis* and *S. oralis*, and *S. pneumoniae*

has significant implications for the in-vivo transfer of penicillin resistance and the search for the origins of penicillin-resistant genes in *S. pneumoniae*. Penicillin resistance development in *S. mitis* was investigated using penicillin-binding protein (PBP) affinity studies. *S. mitis* NCTC 10712 was used as a recipient to investigate PBP alterations which could occur as a result of spontaneous mutation and intra- and inter-specific transfer of penicillin resistance genes. *S. mitis* NCTC 10712 possesses seven major PBPs ranging in molecular mass from 49-82 kDa. One *S. mitis*, one *S. oralis* and two *S. pneumoniae* penicillin-resistant clinical isolates were used as donors in transformation experiments. The most consistant PBP alteration accociated with increasing resistance in *S. mitis* NCTC 10712 was seen with PBP 3 (74 kDa).

The findings from the reciprocal transfer and PBP studies were extended to investigate PBP 2b similarities between *S. mitis, S. oralis* and *S. pneumoniae* using DNA fingerprinting and sequencing. Polymerase chain reaction (PCR) was performed on 41 penicillin-susceptible and -resistant clinical isolates of *S. mitis* and *S. oralis* using the susceptible *S. pneumoniae* R6 PBP 2b primers. PCR products were then analysed using *Hin*fI and *Sty*I restriction enzymes. Of the 41 *S. mitis* / *S. oralis* isolates 15 strains produced a PCR product of a similar size to that of *S. pneumoniae* R6. On fingerprinting these 15 strains, 11 different patterns were seen using *Sty*I restriction enzyme and 12 different patterns with *Hin*fI. The PBP 2b genes of the *S. mitis* and *S. oralis* isolates studied were found to be very heterogeneous.

The PBP 2b genes of two *S. mitis* isolates MICs (0.5 and 2 μg/ml) were sequenced. These PBP 2b genes were found to possess a mosaic structure when compared to those of other *S. pneumoniae* and viridans streptococcal

species. Analysis of these mosaic blocks indicates that both *S. mitis* strains contain areas which originated from *S. pneumoniae* as well as regions of unknown origin.

PBP 2b sequence comparisons of a susceptible *S. oralis* with reported sequences of *S. pneumoniae* R6 and *S. mitis* NCTC 10712 revealed what appears at this stage to be nucleotide regions unique to *S. oralis*. A penicillin-resistant *S. oralis* strain contained a pneumococcal region of 272 bp which was flanked by *S. oralis* sequences. It is still necessary to continue studies in this area to determine amino acid alterations which are directly associated with penicillin resistance development and to locate recombination sites which with time have been responsible for the mosaic structures seen in PBP 2b genes of streptococci.

Results of this study indicate the importance of monitoring antibiotic resistance trends in viridans streptococci particularly with respect to penicillin and aminoglycoside resistance. The study also highlights how the presence of penicillin-resistant *S. mitis* and *S. oralis* commensals can continue to influence penicillin resistance development in *S. pneumoniae*.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or any examination in any other University.

Elsa Potgieter

30th day of January, 1994.

To Matthys and Francois and to my Dad, with much love.

ACKNOWLEDGEMENTS

I wish to thank the following people and institutions for their help and support during this study:

My supervisor, Dr L. J. Chalkley whose expertise, advice and active interest have encouraged me throughout this study.

Marion Carmichael, Jean Saunders and the staff of the Library, Illustration and Photographic Units at the South African Instituite for Medical research for their efficient and friendly assistance.

Professor H. J. Koornhof for his support, advice and editorial assistance.

The Medical Research Council of South Africa, the South African Institute for Medical Research and the University of the Witwatersrand for financial support.

PUBLICATIONS

POTGIETER, E. & CHALKLEY, L.J. (1991) Reciprocal transfer of penicillin resistance genes between *Streptococcus pneumoniae, Streptococcus mitior* and *Streptococcus sanguis. Journal of Antimicrobial Chemotherapy* **28**, 463-465.

POTGIETER, E., KOORNHOF, H.J. & CHALKLEY, L.J. (1992) Penicillin-binding proteins in *Streptococcus mitis*. *Current Microbiology* **24**, 289-294.

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CHALKLEY, L., SCHUSTER, C., POTGIETER, E. & HAKENBECK, R. (1991) Relatedness between *Streptococcus pneumoniae* and viridans streptococci: transfer of penicillin resistance determinants and immunological similarities of penicillin-binding proteins. *FEMS Microbiology Letters* **90**, 35-42.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Infections caused by viridans streptococci.

The viridans or oral streptococci are normal flora of the upper respiratory tract as well as the gastrointestinal tract (Isenberg & D'Amato, 1991). In the oral cavity and pharynx where these streptococci are normally present in large numbers, each species is associated with specific oral surfaces (Frandsen, Pedrazzoli & Kilian, 1991). Viridans streptococci play an important role in preventing colonization of potential pathogens (Bernstein, 1992). It has been shown that people with disturbed normal flora are more prone to recurrent infections, eg. tonsillitis/pharyngitis and that replacement of the viridans streptococci after initial antibiotic treatment of the infection will prevent recurrent infections (Roos et al., 1993).

The viridans streptococci, particularly *Streptococcus mutans*, are responsible for dental caries, and although the condition is not life threatening, it is amongst one of the most expensive infections that many individuals have to contend with during a lifetime (Loesche, 1986).

Transient bacteraemia may result from trauma and other procedures including dental manipulation, surgery to the upper respiratory tract such as tonsillectomy, or surgery and instrumentation of the genitourinary or gastrointestinal tract. Detectable bacteraemia may also result from eating and toothbrushing (Working Party of the British Society for Antimicrobial Chemotherapy, 1982). Septicaemia caused by viridans streptococci in cancer patients has been reported as a complication of bone marrow transplantation (Classen et al., 1990) and as a result of mucositis, following chemotherapy (Awada et al.,1992). When the oral streptococci gain access to the blood stream, individuals with damaged heart valves as well as

cancer patients run a high risk of serious infection and even death (Bisno et al., 1989; Awada et al., 1992; Steiner et al.,1993).

The viridans streptococci are the most important aetiological agents in subacute infective endocarditis superimposed on native heart valves, damaged by rheumatic fever or congenital cardiac abnormalities (Bisno et al., 1989; Manford, Matharu & Farrington, 1992; Mansur, et al., 1992). Such infections are life threatening, and while they are no longer universally fatal especially when appropriate antibiotics are prescribed (Scheld & Sande, 1985), they may require long periods of expensive treatment and are still capable of producing a mortality rate of approximately 10% (Bisno et al., 1989).

In cancer patients significant mortality related to bacteraemia due to viridans streptococci has been extensively reported (Cohen et al., 1983; Bostrom & Weisdorf, 1984; Henslee et al., 1984; Venditti et al., 1989; Classen et al., 1990; Awada et al.,1992; Donelly et al., 1993). At particular risk are patients with severe mucositis, secondary to antineoplastic chemotherapy and/or possibly some viral infections (Cohen et al., 1983; Bostrom & Weisdorf, 1984; Brown, 1984; Ringden et al., 1984). Lately an apparently new manifestation of this infection, "alpha strep shock syndrome", with a substantial mortality rate has been reported for patients undergoing autologous bone marrow transplantation (Elting, Bodey & Keefe, 1992; Steiner et al., 1993).

Serious infections caused by the *Streptococcus anginosus* group [which includes *S. milleri, S. intermedius, S. constellatus*] are well documented (Miller, Mauff & Koornhof, 1983; Gossling, 1988; Rouff, 1988; Singh et al., 1988; Piscitelli et al., 1992; Whiley et al., 1992). The *S. anginosus* group cause various localised suppurative infections affecting sites such as liver,

brain, lung and intramural heart muscle. They cause in addition dental abscesses, pleural empyema, primary pneumonia, septic arthritis, wound sepsis, appendicitis, peritonitis, maxillary sinusitis and less frequently, endocarditis, neonatal sepsis and meningitis (Gossling, 1988; Ruoff, 1988). In one series of cases mortality due to liver abscesses caused by this group has been reported to be 13% (Chua, Reinhart & Sobel, 1989).

Other reports on the pathogenic role of viridans streptococci include various infections in neonates (Broughton, Krafka & Baker, 1981; Haffar, Fuselier & Baker, 1983), sinusitis (Edelstein et al., 1993), septicaemia (Geerdes et al., 1992), meningitis (Goldfarb, Wormser & Glaser, 1984; Quinn et al., 1988), infections of the cornea (Hunts et al., 1993), pneumonia (Marrie, 1993) and thoracic empyema (Shinazo et al., 1993).

Failure to identify the specific aetiology in most of these viridans streptococcal infections makes empiric antibiotic choices difficult and results in increased mortality in the patient.

1.2 Identification of the viridans streptococci.

1.2.1 *Classification systems*

The viridans streptococci are a heterologous group of streptococci which even today are very difficult to identify to species level (Facklam & Washington, 1991). Until the early 1970s few laboratories speciated these streptococci for two reasons: a) most strains were susceptible to penicillin, therefore a patient could be treated effectively, regardless of whether the viridans streptococcus causing the infection had been specifically identified or not (Facklam, 1977) and b) there were no satisfactory differentiation

schemes available (Facklam, 1977). The most authoritative taxonomy textbook, Bergy's Manual of Determinative Bacteriology (Diebel & Seeley, 1974) did not recognise all the viridans species and the schemes recommended for the differentiation of the accepted species were not practical for routine purposes (Facklam, 1977).

Between 1968 and 1977 three identification schemes were published (Carlsson, 1968; Colman & Williams, 1972; Facklam, 1977) of which the schemes of Colman & Williams (Colman & Williams, 1972) and of Facklam (Facklam, 1977) were subsequently widely adopted, while Carlsson never claimed to have established a differentiation scheme. Colman & Williams used transformation studies and cell wall analyses to complement their studies (Colman & Williams, 1965; Colman, 1969). The Colman & Williams scheme was mainly used in British and other European laboratories and recognised five species (*Streptococcus mutans, Streptococcus sanguis, Streptococcus mitior, Streptococcus salivarius* and *Streptococcus milleri*) while the Facklam scheme was used in the USA, and classified Colman & Williams's *S. sanguis* and *S. mitior* strains as *S. sanguis I* and *S. sanguis II* or *S. mitis* and divided strains of *S. milleri* into *Streptococcus anginosus-constellatus* and *Streptococcus MG-intermedius*. The differences in these two systems in both classification and nomenclature resulted in considerable confusion (Beighton, Hardie & Whiley, 1991) and in most studies some strains remained unidentified no matter which identification scheme was used (French, 1989).

The Approved Lists of Bacterial names published in 1980 (Skerman, McGowan & Sneath, 1980) included the following species: *S. mutans, S. sanguis, S. mitis, S. salivarius, S. anginosus, S. constellatus* and *S. intermedius*. The name *S. mitior* was not included on the Approved Lists of Bacterial

Names (Skerman, McGowan & Sneath, 1980) although it was still extensively used until about 1989. The species *S. mitis* formally existed, but it was not clearly defined (Kilian, Mikkelsen & Henrichsen, 1989a) and the type strain did not fit the description of the species (Coykendall, 1989a; Kilian, Mikkelsen & Henrichsen, 1989b). In the meantime a new species *S. oralis* had been described (Bridge & Sneath, 1982) but the type strain chosen was in fact a typical strain of *S. mitior* and the species appeared to be heterogenous (Kilpper-Bälz, Wenzig & Schleifer, 1985; Kilian, Mikkelsen & Henrichsen, 1989a).

Today, although several comprehensive taxonomic studies have been performed (Carlsson, 1968; Colman & Williams, 1972; Bridge & Sneath, 1983; Coykendall, 1989b; Facklam, 1977; Kilian, Mikkelsen & Henrichsen, 1989a; Beighton, Hardie & Whiley, 1991) international consensus on classification and nomenclature has still not been obtained. As a consequence several synonyms have been applied to the same species (Facklam, 1984; Facklam & Washington, 1991) while several species remain heterologous (Coykendall & Specht, 1975; Coykendall, 1977; Coykendall & Munzenmaier, 1978; Whiley & Beighton, 1991).

Lack of clearly defined and consistent distinguishing phenotypic traits has, however, left these problems unresolved (Kilian, Mikkelsen & Henrichsen, 1989a). Biochemical and physiological tests have either failed to detect species diversity or have yielded conflicting concepts of the phylogenetic relationships of the species (Gilmour et al., 1987). Serological classifications were not satisfactory because antigenic determinants were found not to be species specific (Gilmour et al, 1987). Even recent genetic studies have not resolved these problems, in fact they may have added to the confusion (Coykendall, 1989b; Facklam & Washington, 1991).

Table 1.1. Identification of viridans streptococci. (Facklam & Washington, 1991).

Group	Test[a] MAN	SORB	VP	ARG	ESC	URE
S. mutans (includes *S. cricetus, S. downei, S. ferus, S. macace, S. rattus* [ARG+], *S. sobrinus*)	+	+	+	–	+	–
S. salivarius (includes *S. intestinalis, S. vestibularis*)	–	–	+	–	+	±
S. sanguis (includes *S. gordonii*, group H and W streptococci, *S. parasanguis*,[b] *S. crista*[c])	–	–[d]	–	+	+	–
S. mitis (includes *S. mitior, S. oralis, S. sanguis* biotype II)	–	–	–	–	–	–
S. anginosus (includes *S. intermedius, S. constellatus, S. milleri,* DNA groups I and III)	–[d]	–[d]	+[d]	+	+	–

[a]MAN, mannitol; SORB, sorbitol; VP, Voges-Proskauer; ARG, arginine; ESC, aesculin; URE, urease; +, positive reaction; -, negative reaction; ±, some strains positive, other strains negative.

[b]Whiley et al, 1990.

[c]Handley et al, 1991.

[d]Occasional exception.

Despite all this confusion, a great deal of information has been obtained and strains can without much trouble be placed in one of five groups (Facklam & Washington, 1991): [1] *S. mutans* group, [2] *S. salivarius* group, [3] *S. sanguis* group, [4] *S. mitis* group and [5] *S. anginosus* group (Table 1.1).

1.2.2 *S. mutans group:*

This group was first isolated from carious human teeth by Clarke, (1924). The primary habitat of *S. mutans* is the tooth surface where the numbers are usually greater if the person has dental caries (Loesche, 1986). *S. mutans* is occasionally isolated from blood of patients with infective endocarditis (Hardie, 1986). Identification of this group is very easy. They ferment mannitiol and most of the other sugars used to identify the viridans streptococci, produce acetoin and hydrolyse aesculin (Coykendall, 1989b). Like most of the other groups of viridans streptococci this group is heterogeneous and was initially divided into four species (Coykendall, 1977) based on DNA base composition and DNA hybridization studies (Coykendall, 1971; Coykendall, 1974). Later three more species were added (Coykendall, 1983; Beighton et al, 1984; Whiley et al, 1988). Whiley et al. (1988) provided a useful summary of the properties of all seven species. Only *S. mutans* senso stricto and occasionally *S. sobrinus* are isolated from humans, the other five species occur in animals.

1.2.3 *S. salivarius group*:

S. salivarius was first described by Andrewes & Horder, (1906). *S. salivarius* is found on the dorsum of the tongue and on the oropharyngeal mucosa (Frandsen, Pedrazzoli & Kilian, 1991) and is on rare occasions isolated

from blood in cases of infective endocarditis (Hardie, 1986). It is Voges Proskauer positive, hydrolyses aesculin but not arginine and forms fructan from sucrose (Coykendall, 1989b). Genetic studies indicated that *S. salivarius, S. thermophilus* and *S. vestibularis* are related, but they are also sufficiently different to warrant three different species (Farrow & Collins, 1984; Whiley & Hardie, 1988). The fact that some *S. salivarius* strains will grow on aesculin-bile medium, can cause confusion with some *S. bovis* strains (Rouff et al, 1984; Coykendall, 1989b). *S. intestinalis* has not been isolated from humans. *S. vestibularis* resembles *S. salivarius* phenotypically but can be distinguised from *S. salivarius* by its ability to produce hydrogen peroxide and by not producing glucan or fructan from sucrose (Whiley & Hardie, 1988).

1.2.4 *S. sanguis group*:

S. sanguis was first described by White & Niven, 1946 as α-haemolytic streptococci which hydrolysed arginine and aesculin and produced glucan from sucrose (Niven, Kiziuta & White, 1946). Many authors have since broadened their species definition to include strains that are dextran negative (Colman & Williams, 1972; Facklam, 1977), arginine negative Facklam, 1977) or aesculin negative (Colman & Williams, 1972). The cell walls of *S. sanguis* strains contain glycerol teichoic acid and rhamnose (Kilian, Mikkelsen & Henrichsen, 1989a) and the peptidoglycan type is Lys-Ala$_{1-3}$ (Kilpper-Bälz, Wenzig & Schleifer, 1985). Price et al. (1986) showed that typical *S. sanguis* strains which contained significant amounts of rhamnose in their cell walls were arginine positive but they could be either aesculin positive or negative. *S. sanguis* strains can therefore be differentiated from the *S. mitis* group by their ability to hydrolyse arginine

and from other viridans strains which also hydrolyse arginine by the fact that *S. sanguis* strains do not produce acetoin (Coykendall, 1989b). Coykendall & Specht (1975) divided *S. sanguis* strains that hydrolysed both arginine and aesculin into two genomic groups on the basis of DNA-DNA hybridization studies. They called the two types *S. sanguis* subsp. *carlsonii* and *S. sanguis* subsp. *sanguis*, but because of a lack of correlating phenotypic differences this subdivision has never been practically employed (Kilian, Mikkelsen & Henrichsen, 1989a).

Kilian, Mikkelsen & Henrichsen (1989a) redefined *S. sanguis* senso stricto with NCTC 7863 as the type strain (Coykendall & Specht's *S. sanguis* subsp. *carlsonii*) as strains which do not produce alkaline phosphatase or α-fucosidase. The *S. sanguis* group was separated into four biovars by biochemical and serological characteristics (Kilian, Mikkelsen & Henrichsen, 1989a), but Beighton, Hardie & Whiley, (1991) using another type of synthetic fluorogenic glycosidase substrate and different strains could only suggest three biotypes for *S. sanguis*. In the Kilian classification scheme it is not clear why *S. mitis* biovar 2 is not included with the *S. sanguis* group (Colman, Efstratiou & Morrison, 1991). *S. mitis* biovar 2 strains hydrolyse arginine which usually correlates with the cell wall composition expected in *S. sanguis* strains (Price et al., 1986). Furthermore *S. mitis* strains have the ability to bind α-amylase, while *S. sanguis* strains do not, but in a study by Kilian & Nyvad, (1990) only 30% of *S. mitis* biovar 2 strains bound α-amylase.

S. sanguis is associated with initial plaque formation and is prominent on the buccal mucosa (Frandsen, Pedrazzoli & Kilian, 1991). *S. sanguis* account for a high percentage of streptococcal endocarditis cases (Coykendall, 1989b).

S. gordonii, typestrain NCTC 7865, originally described as *S. sanguis* subsp. *sanguis* by Coykendall & Specht (1975), has been proposed by Kilian, Mikkelsen & Henrichsen, (1989a). *S. gordonii* is found in small numbers on the oropharyngeal mucosa and in mature subgingival plaque (Frandsen, Pedrazzoli & Kilian, 1991), and is also an important cause of bacterial endocarditis (Colman, Efstratiou & Morrison, 1991). Genetic evidence to support separation of *S. gordonii* from *S. sanguis* was obtained by DNA-DNA hybridization (Coykendall & Specht, 1975) and multilocus enzyme electrophoresis analyses (Gilmour et al., 1987). Key characteristics are: Voges Proskauer negative, hydrolysis of arginine and aesculin, production of alkaline phosphatase, α-fucosidase and β-glucosaminidase; fermentation of amygdalin and lack of IgA1 protease activity (Kilian, Mikkelsen & Henrichsen, 1989a). Kilian, Mikkelsen & Henrichsen, (1989a) divided *S. gordonii* into 3 biovars by biochemical and serological characteristics, but again Beighton, Hardie & Whiley, (1991) could only find justification for one biovar.

Coykendall (1989b) noted the presence of two further genomic groups within the *S. sanguis* group. One has now been named *S. parasanguis* (Whiley et al., 1990) and the other *S. crista* (Handley et al., 1991).

S. parasanguis type strain ATCC 15912 was first described by Whiley et al. (1990) and were originally isolated from human throats, blood and urine (Whiley et al., 1990). DNA hybridization studies revealed a group distinct from all other species of viridans streptococci (Whiley et al., 1990). Key phenotypic characteristics are the inability to produce acetoin, production of H_2O_2, α-glucosidase and ammonia from arginine and the ability to bind salivary α-amylase (Whiley et al., 1990; Beighton, Hardie & Whiley, 1991).

S. crista strains have lateral fibrils organised in a very tense tuft on only one side of the cell (Handley et al., 1985). These tufted *S. sanguis* strains have been referred to as "*S. sanguis* I" (with tufts of fibrils) (Handley et al., 1985), the "CR group" (Douglas, Pease & Whiley, 1990) and the "tufted-fibril" group (Beighton, Hardie & Whiley, 1991). They are known to coaggregate with *Corynebacterium matruchotii,* forming characteristic corn cob configurations in mature plaque (Mouton et al., 1979; Mouton, Reynolds & Genco, 1980), a property not found in other *S. sanguis* strains (Handley et al., 1985). *S. crista* strains hydrolyse arginine but not aesculin; do not produce alkaline phosphatase or β-glucosidase, but produce α-fucosidase; produce hydrogen peroxide but not acetylmethylcarbinol (Voges Proskauer) (Handley et al., 1991) and bind salivary α-amylase (Douglas, Pease & Whiley, 1990).

1.2.5 *S. mitis group*:

S. mitis was described by Andrewes & Horder (1906) as streptococci which were rarely pathogenic, grew in short chains and failed to ferment inulin or raffinose. Because of the lack of detail in the description from these workers, the definition of *S. mitis* is usually based on the description provided by Sherman, Niven & Smiley, (1943) who noted that this was a heterogeneous group which did not ferment inulin or synthesized polysaccharide from sucrose. Carlsson (1968) and Facklam (1977) supported *S. mitis* as a defined group, but many microbiologists tended to abuse the term, naming any viridans streptococci which could not be placed directly into another described species, as *S. mitis* (Coykendall & Munzenmaier, 1978). Colman & Williams (1972) supplied a clearer definition and recommended the use of the name *S. mitior*, which was first

described in 1903 (Schottmuller, 1903), for isolates which were arginine and aesculin negative, formed hydrogen peroxide, fermented sucrose and lactose but rarely mannitol, sorbitol or inulin. Parker & Ball (1976) recognised dextran-positive and dextran-negative varieties of *S. mitior*, but dextran production was found not to be a genetically significant characteristic (Coykendall & Munzenmaier, 1978). Facklam (1977) divided *S. mitior* strains into *S. mitis* and *S. sanguis* II on the basis of raffinose fermentation but Coykendall & Munzenmaier (1978) could find no genetic justification for this. DNA hybridization studies indicated that *S. sanguis* I and *S. sanguis* II did not belong in the same species (Welborn et al., 1983).

Although *S. mitior* was not included on the Approved List of Bacterial Names (Skerman, McGowan & Sneath, 1980), the name *S. mitior* was still widely used until about 1989. The name *S. mitis,* type strain NCTC 3165 was included in the Approved List of Bacterial Names (Skerman, McGowan & Sneath, 1980), with an official description based on that proposed by Sherman, Niven & Smiley, (1943) in Bergy's Manual of determinative bacteriology (Diebel & Seeley, 1974). *S. mitis* as described at the time consisted of two genetic groups with no phenotypic tests to differentiate them (Coykendall & Munzenmaier,1978). Unfortunately the type strain chosen for *S. mitis* (NCTC 3165) was actually a strain of *S. sanguis* (Welborn et al, 1983; Coykendall, 1989a; Kilian, Mikkelsen & Henrichsen, 1989b). As it was found to hydrolyze arginine and aesculin and ferment raffinose and inulin it therefore fitted the description of *S. sanguis* and not *S. mitis* (Coykendall, 1989a). In addition its DNA hybridized with that of *S. sanguis* strains in the genetic group that is represented by *S. sanguis* NCTC 7865 and not with that of any *S. mitis* strains (Coykendall, 1989b).

Bridge & Sneath (1982) described a new species *S. oralis*, and in 1985 Kilpper-Bälz, Wenzig & Schleifer proposed an amended description of the species. Some of the strains of *S. oralis* had previously been described as *S. mitior*, *S. sanguis* II or *S. viridans* (Coykendall & Munzenmaier, 1978; Kilpper-Bälz, Wenzig & Schleifer, 1985). Coykendall (1989b) reported that hybridization experiments showed *S. mitis* and *S. oralis* to be genetically identical and described the history of *S. mitis* as a history of confusion. The strain Coykendall described as a typical *S. mitis* strain, strain NCTC 7864, had in fact been described as *S. oralis* (Welborn et al, 1983; Kilian, Mikkelsen & Henrichsen, 1989a).

Kilian, Mikkelsen & Henrichsen, (1989a) used chromogenic substrates included in the API ZYM and API ZYM osidase kits to detect glycosidase activities, in order to differentiate *S. oralis* from *S. mitis*. These reactions together with tests for the detection of IgA protease, neuraminidase activity as well as conventional carbohydrate/fermentation, hydrolysis and serological tests provided amended descriptions for both *S. oralis* and *S. mitis*. These authors (Kilian, Mikkelsen & Henrichsen, 1989a) disagreed with the phenotypic description of Bridge & Sneath, (1982) and Kilpper-Bälz, Wenzig & Schleifer (1985) and reported that *S. oralis* as described by them was phenotypically and genetically homogenous and well separated from other species including *S. mitis*. They proposed strain NCTC 11427 as the type strain and confirmed that the species contained strains previously described as *S. mitior* and *S. sanguis* II (Kilian, Mikkelsen & Henrichsen, 1989a). They did, however, recognise that *S. oralis* and *S. mitis* were related by having a cell wall composition that is distinct from that of *S. sanguis*. The cell walls of *S. oralis* and *S. mitis* strains contain ribitol teichoic acid but lack significant amounts of rhamnose, the

peptidoglycan type in both these species is Lys-direct (Kilian, Mikkelsen & Henrichsen, 1989a). Colman, Efstratiou & Morrison, (1991) have also warned that *S. oralis* and the pneumococci have a common cell wall antigen which may pose problems in serological identification (Holmberg et al., 1985).

The second genotype in the *S. mitis* group described by Coykendall & Munzenmaier, (1978) was proposed with *S. mitis* NCTC 12261 as the type strain by Kilian, Mikkelsen & Henrichsen, (1989a). They described two biovars for this species. Even though *S. oralis* appears more likely to form dextran from sucrose, be tolerant of 4% NaCl (w/v) in broth, produce neuraminidase, IgA1 protease, β-glucosaminidase and α-maltosidase than *S. mitis* (Kilian, Mikkelsen & Henrichsen, 1989a) the phenotypic differentiation between these two species will remain a problem until more cultures are studied and more tests become available (Colman, Efstratiou & Morrison, 1991).

Later, Beighton, Hardie & Whiley (1991) used different fluorogenic glycosidase substrates to differentiate between *S. oralis* and *S. mitis*, improving differentiation, however they only described one biovar for *S. mitis*. *S. oralis* is associated with initial dental plaque formation, *S. mitis* biovar 1 is found on buccal mucosa , while *S. mitis* biovar 2 can be found on the dorsum of the tongue (Frandsen, Pedrazzoli & Kilian, 1991). Both species cause bacterial endocarditis (Colman, Efstratiou & Morrison, 1991).

1.2.6 *S. anginosus group*:

This group was first described by Andrewes & Horder, (1906). The *S. anginosus* group is by far the most prominent in subgingival plaque

(Frandsen, Pedrazzoli & Kilian, 1991). *S. intermedius* is associated with abscesses of the brain and liver; *S. anginosus* and *S. constellatus* are isolated from a wider range of infections, while *S. anginosus* predominates in urogenital infections (Whiley et al., 1992). Despite its diversity the *S. anginosus* group has characteristics which seldom vary (Coykendall, 1989b) ie. production of acetoin and alkaline phosphatase, hydrolysis of arginine, fermentation of trehalose and failure to split hippurate or ferment ribose. Whiley et al. (1990) used 4-methylumbelliferyl-linked fluorogenic substrates for the detection of glycosidase activity, together with the production of hyaluronidase to distinguish between the three species within this group namely, *S. constellatus, S. intermedius* and *S. anginosus*.

In all there seem to be five genotypes within this group, but how they will be identified using conventional tests is not yet clear (Facklam & Washington, 1991). There also seems to be much disagreement still as to whether the group should remain one species (Welborn et al., 1983; Coykendall, Wesbecher & Gustafson, 1987), or whether further subdivisions should be made (Kilpper-Bälz et al., 1984; Knight & Shlaes, 1988; Whiley & Hardie, 1989; Whiley & Beighton, 1991).

1.2.7 *Remaining problems in identification of viridans streptococci.*

Despite all the efforts by many microbiologist over the last five years (Coykendall, 1989b; Kilian, Mikkelsen & Henrichsen, 1989a; Beighton, Hardie & Whiley, 1991) identification of the viridans streptococci to species level is still extremely difficult (Facklam & Washington, 1991). The reasons for this are many and varied.

Although there seems to be a general agreement on five main groups within the viridans streptococci no international concensus has yet been

reached on dividing strains within the groups (Coykendall, 1989b; Kilian, Mikkelsen & Henrichsen, 1989a; Colman, Efstratiou & Morrison, 1991; Facklam & Washington, 1991; Beighton, Hardie & Whiley, 1991). Kilian, Mikkelsen & Henrichsen, (1989a) describe four biovars in the *S. sanguis* group, three biovars in the *S. gordonii* group but only one species in the *S. anginosus* group and one in the *S. mutans* group; while Beighton, Hardie & Whiley (1991) describe three biovars in the *S. sanguis* group, one in the *S. gordonii* group, three species in the *S. anginosus* group and two in the *S. mutans* group.

Lack of distinguishing phenotypic characteristics remain a problem (Kilian, Mikkelsen & Henrichsen, 1989a). Genetic studies, although essential can only be applied if the genotypes also have clear phenotypic characteristics by which they can be separated (Coykendall, 1989b). For instance, Coykendall & Specht (1975) had already in 1975 shown two genotypes within the *S. sanguis* group but it was not until the end of 1989 that Kilian, Mikkelsen and Henrichsen found enough phenotypic evidence to separate this group into *S. sanguis* and *S. gordonii*. Likewise, Coykendall & Munzenmaier (1978) described two groups within the *S. mitis* group, however, due to lack of distinguishing phenotypic characteristics, it is still difficult to distinguish between the two species *S. mitis* and *S. oralis*.

Genetic studies can and have caused confusion, when studies by different workers yielded conflicting data (Coykendall, 1989b; Facklam & Washington, 1991). Different conclusions have been reached in DNA-DNA hybridization studies as a result of comparing different clinical isolates with the inclusion of only a small number of reference strains (Gilmour et al., 1987) or by inherent variations in the experimental hybridization stringencies used (Whiley & Beighton, 1991).

Biochemical tests used to differentiate species also need to be standardised, as small variations in a test may give different results (French et al., 1989; Kilian, Mikkelsen & Henrichsen, 1989a).

Kilian, Mikkelsen & Henrichsen, (1989a) reported that different results were obtained in the test for aesculin hydrolysis depending on whether Tryptic soy broth or Trypticase peptone was used in the medium, claiming that Trypticase peptone gave better results. Similarly French et al. (1989) reported that the tests for arginine and aesculin hydrolysis as found in the API 20-STREP system gave more reliable results than conventional tests.

Kilian, Mikkelsen & Henrichsen, (1989a) also reported that carbohydrate fermentation test discrepancies were most probably due to the use of different pH indicators and suggested pH measurements should be made both before and after incubation.

Also the new chromogenic substrates employed to distinguish between some species tend to give different results, depending on the substrate used. Kilian, Mikkelsen & Henrichsen, (1989a) showed that results obtained testing for identical glycoside hydrolyses but using two different commercial kits (API ZYM & API ZYM osidase) were quite different. They also reported that the substrate used for propagation of the inoculum as well as the cell resuspension seemed to be very important when testing for enzyme activity. The pH of the suspending buffer should be as close as possible to the optimum pH of the relevant enzymes in order to obtain correct results. Beighton, Hardie and Whiley, (1991) used yet another type of synthetic fluorogenic substrate to demonstrate glycosidase activity and distinguish between the viridans species. Where finite differentiation of two species relies on one or two specific of tests as found with glycosidase activity, in the case of *S. mitis* and *S. oralis* (Beighton, Hardie & Whiley,

1991; Kilian, Mikkelsen & Henrichsen, 1989a) such tests must be standardised.

Two of the tests used in the study of Kilian, Mikkelsen & Henrichsen, (1989a), the IgA1 protease and neuraminidase tests, although useful for distinguishing between some of the species, require specialised equipment which preclude their use in routine laboratories.

Another factor which undoubtedly has caused many problems is that the viridans streptococci are notoriously difficult to obtain in pure culture and that up to five subcultures are sometimes required to ensure culture conformity (Kilian, Mikkelsen & Henrichsen, 1989a).

Atypical strains have always been and still are a problem. Coykendall, (1989b) has warned that atypical *S. bovis* strains would be difficult to differentiate from *S. salivarius* strains. Another major problem lies with atypical *Streptococcus pneumoniae* strains. *S. oralis* and *S. pneumoniae* are genetically related and both species contain ribitol and choline in their cell walls (Kilpper-Bälz, Wenzig & Schleifer, 1985). *S. oralis* and *S. pneumoniae* also have a common cell wall antigen, the C polysaccharide and cross-reactions may cause problems in serological identification, resulting in *S. oralis* being identified as *S. pneumoniae* (Wasilauskas & Hampton, 1984; Sjörge & Holme, 1987). In one study as many as 68% of viridans streptococci showed antigenic cross reactions with pneumococcal omniserum (Holmberg et al, 1985). Atypical *S. pneumoniae* strains may be optochin resistant, bile insoluble and some cannot be typed serologically (Fennol et al., 1990). Fennol et al., (1990) therefore developed a new DNA probe to separate atypical *S. pneumoniae* strains from *S. oralis* strains and suggest that this is the only reliable way to distinguish between these strains.

Susceptibility patterns of the viridans streptococci have changed in recent years and these streptococci can no longer be assumed to be susceptible to most antibiotics. Recognition of the role of viridans streptococci in specific diseases has placed more emphasis on identification to species level as a basis for rational antibiotic therapy.

1.2 Antibiotic resistance in viridans streptococci.

1.2.1 *Treatment of viridans infections.*

For the treatment of viridans streptococcal endocarditis the American Heart Foundation has suggested various regimens depending on the penicillin and aminoglycoside MICs of the isolates and the age and condition of the patient involved (Bisno et al., 1989).

This ranges from a two-week penicillin/streptomycin course of treatment for younger patients with uncomplicated penicillin sensitive (MIC ≤ 0.1 μg/ml) viridans streptococcal endocarditis, to four weeks of penicillin alone for elderly patients or patients with impaired renal function, while in complicated cases an initial two weeks of streptomycin treatment followed by four weeks of penicillin is recommended. In cases where there is a history of complications or if the disease has lasted for longer than 3 months, a 4-week period of combined penicillin/aminoglycoside treatment is suggested. Where patients have prosthetic valves or other prosthetic implants, the treatment is a 6 weeks course of penicillin plus an aminoglycoside for the first two weeks. If high-level streptomycin resistance is present or if the strains have a penicillin MIC of ≥ 0.2 and ≤ 0.5 μg/ml, penicillin/gentamicin combination treatment is proposed by

the American Heart Foundation (Bisno et al., 1989). Netilmicin is claimed to be less toxic than gentamicin and has been recommended as an alternative to gentamicin by the Working Party of the British Society for Antimicrobial Chemotherapy (1985). Penicillin alone has been suggested as adequate and sometimes preferable for treatment of endocarditis caused by relatively resistant (MIC ≥ 0.2 and $\leq 0.5\ \mu$g/ml) (DiNubile, 1990) viridans streptococci. For viridans streptococci with penicillin MICs $> 0.5\ \mu$g/ml the treatment should be the same as for enterococcal endocarditis, ie. a cell wall-active antibiotic such as vancomycin, used in combination with an aminoglycoside. Cephalothin, cefazolin or vancomycin is recommended for patients allergic to penicillin (Bisno et al., 1989).

Although the treatment regimes as suggested by the American Heart Foundation (Bisno et al., 1989) are safe and effective, there are some disadvantages. Firstly prolonged hospitalization periods are necessary as constant or frequent intervenous infusions or six intramuscular injections per day may be required (Wilson, 1992). Interest in the treatment of viridans streptococcus infections therefore focused on the development of protocols which permit once-a day dosing and/or outpatient treatment. Very good results have been reported for the once daily treatment of penicillin-susceptible viridans streptococcal endocarditis with netilmicin plus ceftriaxone (Ter Braak, 1990), ceftriaxone alone for four weeks (Stamboulin et al., 1991; Francioli et al., 1992) or ceftriaxone for two weeks followed by amoxicillin for another two weeks (Stamboulin et al., 1991). Apart from the obvious financial and convenience advantages of a once daily outpatient treatment regimen, it has also been suggested that higher doses of aminoglycosides given at less frequent intervals improved their efficacy and reduced their toxicity (Kapusnik & Sande, 1986).

Optimal antimicrobial therapy in cases of multiply aminoglycoside resistant enterococci or in cases of penicillin and/or aminoglycoside resistant viridans streptococci, has not been established although imipenem alone or in combination with gentamicin has been reported to be highly effective against penicilillin-resistant viridans streptococci in rabbits (Garcia, Luengo & Valdès, 1993).

Antibiotic prophylaxis of infective endocarditis is recommended for patients at risk from endocarditis before selected dental, surgical and genitourinary tract manipulations that might cause bacteremia (Durack, 1990). Depending on the procedure and the medical history of the patient involved, the following antibiotics are recommended: Amoxicillin with or without gentamicin, ampicillin with gentamicin or cefazolin and for the penicillin allergic patients, erythromycin, clindamycin or vancomycin with or without gentamicin (Durack, 1990; Endocarditis working party of the British Society for Antimicrobial Chemotherapy, 1990). Despite the fact that many prophylactic regimens have been suggested in the literature, the recommendations made are not well understood and are therefore often ignored in practice (Durack, 1990).

Vancomycin is recommended for treatment or prophylaxis of viridans streptococcal septicemia in cancer patients (Cohen et al., 1983; Brown, 1984; Venditti et al., 1989; Classen et al., 1990) and has been used with great success as prophylaxis in bone marrow transplant patients (Henslee et al., 1984). Imipenem, teichoplanin and vancomycin have been suggested in cases of septicemia caused by penicillin resistant viridans streptococci (Venditti et al., 1989).

For infections caused by the *S. anginosus* group (*S. anginosus, S. intermedius* and *S. constellatus*) penicillin is still the antibiotic of choice (Gossling, 1988). Effective alternatives are penicillin plus an aminoglycoside or cephalosporins, alone or in combination with other antibiotics, vancomycin, erythromycin, clindamycin or trimethoprim-sulfamethoxazole (Gossling, 1988).

1.2.2 *Antibiotic resistance trends.*

Viridans streptococci had for many years been considered to be universally susceptible to penicillin (Faber et al., 1983). Several earlier studies, however, had commented on the prevalence of penicillin-resistant viridans streptococci associated with oral penicillin prophylaxis (Krumweide, 1949; Garrod & Waterworth, 1962; Doyle et al., 1967; Stirland & Shotts, 1967; Sprunt, Redman & Leidy, 1968; Phillips et al, 1976; Parillo et al., 1979; Spencer et al., 1970). A careful review of the literature by Levin et al. (1982) also revealed that even before the 1980s, the presence of penicillin-resistant viridans streptococci was not unusual or confined primarily to those patients receiving penicillin prophylaxis. Drucker & Jolly (1969) reported that 27% of routine dental patients who were not receiving nor had recently received antibiotics carried penicillin-resistant oral streptococci. Wolfe & Johnson (1974) showed that overall 14.6% of viridans streptococci isolated from patients with endocarditis during the time periods 1944-1947 and 1967-1971 had penicillin MICs ≥ 0.1 μg/ml. Roberts et al. (1979) reported that the incidence of penicillin-resistant viridans streptococci isolated from patients with endocarditis at the Cornell Medical Centre, New York, between 1944 to 1951 was 18%, from 1952 to1960, 20% and from 1970 to1978, 17%. In a study by Bourgault, Wilson &

Washington (1979) 19% of the viridans streptococci recovered from blood specimens at the Mayo Clinic, Minnesota, were shown to have penicillin MICs of ≥ 0.1 μg/ml. Pulliam & Hadley (1979) on examining viridans streptococci from blood cultures in San Francisco found 30% of the strains resistant to penicillin with MICs 0.12-1.0 μg/ml.

Since 1980 reports on the prevalence of penicillin resistance in viridans streptococci have been increasing. Hess, Holiway & Dankert (1983) reported 16% of the viridans streptococci isolated from the gingival sulcus of children were resistant to penicillin. Faber et al. (1983) found 10 multiply resistant viridans streptococci isolated from the oral cavity amongst a group of penicillin-resistant *S. pneumoniae* isolates from South Africa. A South African survey on children admitted to Baragwanath hospital in 1987, showed 76% of oropharangeal viridans streptococci isolated had penicillin MICs of ≥ 0.25 μg/ml, and an alarming 95% of the viridans strains isolated from children receiving antibiotics while in hospital were resistant to penicillin (Van den Berg, 1990). Seventeen percent of the viridans streptococci isolated from cancer patients in Rome during 1988 had penicillin MICs ≥ 0.25 μg/ml (Venditti et al., 1989). Spanish studies have shown an increase in penicillin-resistant viridans streptococci isolated from clinically significant infections (Escribano et al., 1990; Loza et al., 1990; Alcaide et al., 1993). The number of viridans streptococci from blood cultures in Madrid with penicillin MICs ≥ 0.2 μg/ml, increased from 47.8% in 1988 to 51.5% in 1989 (Loza et al., 1990). Another study reported that the incidence of penicillin resistance in viridans streptococci isolated from blood in Barcelona increased from 39% in 1988 to 60% in 1989 (Escribano et al., 1990). In a further study from Barcelona 33.7% of viridans streptococci recovered from blood cultures between 1990 and 1992

were shown to be resistant to penicillin (Alcaide et al., 1993). A Dutch study showed that 40-60% of healthy children under the age of 5 years harboured penicillin-resistant viridans streptococci in the oral cavity, while 20% of children over the age of 15 years were colonised with penicillin-resistant viridans streptococci with MICs of 2-8 μg/ml (Guiot, Corel & Vossen, 1993). In an English study 31.3% of the viridans streptococci isolated from the blood of neutropenic patients were found to be resistant to penicillin (McWhinney et al., 1993). Furthermore, apart from these surveys anecdotal reports of individual species of viridans streptococci resistant to penicillin are also frequently encountered in the literature (Levin et al., 1982; Rahman, 1982; Goldfarb, Wormser & Glaser, 1984; Boenning, Nelson & Campos, 1988; Quinn et al., 1988). In addition to penicillin resistance, viridans streptococci have been reported to be multiply resistant to other antibiotics (Rahman, 1982; Faber et al., 1983b; Goldfarb, Wormser & Glaser, 1984; Venditti et al., 1989; Escribano et al., 1990; Van den Berg, 1990).

For patients allergic to penicillin, or for patients who have received long-term penicillin prophylaxis and are at risk of harbouring penicillin-resistant viridans streptococci, erythromycin has been recommended before and after certain dental or surgical procedures (Bisno et al., 1989; Durack, 1990; Parillo, 1979). The reasons given for caution in using erythromycin as a prophylactic are, a) the bacteriostatic action of erythromycin (Glauser & Francioli, 1982), b) the prevalence of erythromycin-resistant viridans streptococci (Sprunt, Leidy & Redman, 1970; Southall et al, 1983; Longman et al, 1991), c) the transfer of erythromycin resistance amongst various species of streptococci (Horodniceanu et al., 1982; Horaud, Le Bouguenec & Pepper, 1985;

Courvalin & Carlier, 1986; Clermont & Horaud, 1990) and d) reports of failure of erythromycin in preventing endocarditis in animals (Durack & Petersdorf, 1973) and humans (Eng, Wolff & Smith, 1982).

High-level streptomycin and kanamycin resistance amongst viridans streptococci was first described in 1982 (Horodniceanu et al., 1982) and subsequently high-level streptomycin-resistant strains were isolated in South Africa (Faber et al., 1983). *In vitro* and *in vivo* studies with streptomycin-resistant strains of viridans streptococci showed loss of penicillin/streptomycin synergy while synergistic killing with a combination of penicillin and gentamicin was achieved in these cases (Faber et al., 1983; Enzler et al., 1987; Faber & Yee, 1987). The variability in response of viridans streptococcal strains to β-lactam/aminoglycoside synergy is implicit in the published data, but has not been stressed. In a study on the synergistic effect of various combinations of β-lactams/ aminoglycosides on viridans streptococci, 20% of the strains isolated from patients with endocarditis showed no penicillin/streptomycin synergy, and 15% no penicillin/gentamicin synergy (Watakunakorn & Glotzbecker, 1977). None of the strains were resistant to penicillin with MICs ≥ 0.125 but they showed different degrees of resistance to aminoglycosides (Watakunakorn & Glotzbecker, 1977). For penicillin-resistant viridans streptococci lack of penicillin/streptomycin synergy had been described for both streptomycin-resistant isolates (Parillo et al., 1979) and for streptomycin-sensitive isolates (Levin et al., 1982). Similarly, failure of combined penicillin/gentamicin therapy in cases of penicillin-resistant isolates have been reported (Levin et al., 1982; Rahman, 1982; Goldfarb, Wormser & Glaser, 1984; Boenning, Nelson & Campos, 1988), and an *in vitro* study on viridans streptococci with penicillin MICs between 2 and 8

μg/ml, showed no penicillin/gentamicin synergy (Guiot, Corel & Vossen, 1993). It has been suggested that β-lactam/aminoglycoside synergy in viridans streptococci might be species (Snyder, Wilkowske & Washington, 1975; Miller et al., 1986) or even strain specific (Miller et al., 1986). Unfortunately methods for studying synergy have not been standardized and some of the discrepant findings reported in the literature may be due to lack of agreement between methological approaches.

Antibiotic resistance results in increasing mortality and health care costs. In conjunction with susceptibility monitoring, knowledge of resistance mechanisms is important if resistance is to be understood and prevented.

.2.3 *Penicillin and aminoglycoside resistance mechanisms.*

.2.3.1 *Penicillin resistance.*

β-Lactam antibiotics bind to specific targets located in the bacterial cell membrane to exert their inhibitory effect. These target proteins are identified by their ability to covalently bind isotope-labelled penicillin and are called penicillin-binding proteins (PBPs) (Malouin & Bryan, 1986). PBPs have been found in all bacterial species examined thus far and are enzymes involved in the final stages of cell wall peptidoglycan synthesis (Bryan & Godfrey, 1991). Penicillin inhibits PBPs by acting as a substrate analogue of the acyl-D-alanyl-D-alanyl component of peptidoglycan (Bryan & Godfrey, 1991). PBPs have been extensively studied in *Escherichia coli* where the higher-molecular-weight PBPs, the transpeptidases, are essential for bacterial function and are therefore lethal targets, while the lower-molecular-weight PBPs the D-alanine carboxypeptidases are not

(Spratt, 1983). This is with the exception of the *E. coli* PBP 7, which has been shown to act as a transpeptidase in stationary cells (Tuomanen & Schwartz, 1987). Resistance to penicillin is due to alterations in the essential PBPs. Modifications of a target PBP result in reduced binding affinity for penicillin whilst enzyme activity is retained. PBPs of many bacteria and PBP alterations involved in β-lactam resistance development have been extensively reviewed in the literature (Blumberg & Strominger, 1974; Spratt, 1983; Waxman & Strominger, 1983; Bryan & Godfrey, 1991). Because the relative ease of inducing mutations or other genetic manipulations pertaining to *E. coli*, is lacking in Gram-positive bacteria determination of detailed specific functions of PBPs in the latter organisms has not proceeded at the same rate as for Gram-negative bacteria (Bryan & Godfrey, 1991).

Penicillin resistance in viridans streptococci is generally regarded as being due to alterations in their penicillin-binding proteins (Faber et al., 1983; Massida & Daneo-Moore, 1988; Quinn et al., 1988; Zito & Daneo-Moore, 1988). Relatively few limited studies have, however, been done on PBPs in viridans streptococci. Faber et al. (1983) studied the PBP profiles of 5 clinical isolates of *S. mitis* , and Quinn et al. (1988) the PBP profiles of one clinical isolate of *S. mitis* and one of *S. intermedius*. Major PBPs associated with penicillin resistance were reported to be the 72 kDa PBP 4 in *S. sanguis* (Massida & Daneo-Moore, 1988), and the 76 kDa PBP 3 in *S. mutans* (Zito & Daneo-Moore, 1988). Both these studies were done using laboratory mutants only (Massida & Daneo-Moore, 1988; Zito & Daneo-Moore, 1988).

The PBPs of *S. pneumoniae* have been extensively studied (Hakenbeck, Tarpay & Tomasz, 1980; Percheson & Bryan, 1980; Zigelboim & Tomasz,

1981; Williamson & Tomasz, 1985; Handwerger & Tomasz, 1986a; Hakenbeck et al., 1986; Hakenbeck, Tornette & Adkinson, 1987; Chalkley & Koornhof, 1988; Chalkley, Van den Berg & Koornhof, 1989; Chalkley & Koornhof, 1990; Chalkley & Koornhof, 1991). Based on molecular size the *S. pneumoniae* PBPs have been assigned to three major groups 1, 2, and 3 and further divided into sub-groups 1a, 1b; 2x(2a'), 2a and 2b (Zigelboim & Tomasz, 1981; Laible et al., 1989). Low-molecular-weight PBP 3 , a D,D-carboxypeptidase (Hakenbeck & Kohiyama, 1982) seems to be dispensible *in vitro* (Williamson, Hakenbeck & Tomasz, 1980), while a decrease in penicillin affinity of PBPs 1 and 2 is associated with increased in resistance (Percheson & Bryan, 1980; Hakenbeck, Tarpay & Tomasz, 1980; Zigelboim & Tomasz, 1980). Special significance has been assigned to PBP 2b, as peptidoglycan synthesis was shown to be dependent on the amount of PBP 2b that remained unacylated by penicillin (Williamson & Tomasz, 1985) and it was shown that lysis does not occur if β-lactams failed to bind to PBP 2b (Hakenbeck, Tornette & Adkinson, 1987). A reduction in penicillin binding-affinity of PBP 2b was shown to be associated with increased resistance in transformation experiments (Zigelboim & Tomasz, 1981; Chalkley & Koornhof, 1988; Chalkley, Van den Berg & Koornhof, 1989) and subsequently identified as a killing target for penicillin by Tuomanen & Sande (1989). Similarity in the size of the *S. pneumoniae* PBP 2b (77 kDa) (Hakenbeck, Briese & Ellerbrok, 1986) and PBP 3 (76 kDa) (Zito & Daneo-Moore, 1988) in *S. mutans* and PBP 4 (72 kDa) (Massida & Daneo-Moore, 1988) in *S. sanguis* and studies on the development of penicillin resistance in the PBP 2b genes of *S. oralis* and *S. sanguis* (Dowson et al., 1989) as well as results from transformation experiments (Chalkley & Koornhof, 1990) suggested the possibility of genetic homology and

intergenetic transfer of penicillin resistance between viridans streptococci and *S. pneumoniae*.

1.2.3.2 *Aminoglycoside resistance.*

Resistance to aminoglycosides is due to one or a combination of the following mechanisms including ribosomal resistance, inactivation by aminoglycoside-modifying enzymes and impermeability (Shannon & Phillips, 1982).

Ribosomal resistance

Mutations in the genes coding for ribosomal proteins result in the production of altered ribosomal proteins which have lower binding affinities for the aminoglycosides (Shannon & Phillips, 1982). Ribosomal resistance for example in *Enterococcus faecalis* usually confers high-level streptomycin resistance with MICs $> 16\,000\,\mu$g/ml (Elioupolus et al., 1984). In viridans streptococci, ribosomal-mediated resistance to streptomycin has been observed although MICs have only been reported as $\geq 2\,000\,\mu$g/ml (Faber et al., 1983).

Inactivation

Aminoglycosides can be inactivated by aminoglycoside- modifying enzymes at various modification sites. Three classes of aminoglycoside-modifying enzymes are known: *O*-phosphotransferases (APH), *O*-nucleotidyltransferases (ANT) or adenylyltransferases (AAD) and *N*-acetyltransferases (AAC). Aminoglycoside-modifying enzymes are often located on plasmids and are therefore transferable (Shannon & Phillips, 1982).

High-level streptomycin-resistant viridans streptococci were first found in Paris in 1982 (Horodniceanu et al., 1982). High-level streptomycin and

high-level kanamycin resistance, mediated by aminoglycoside-modifying enzymes have been reported amongst viridans streptococci from South Africa (Faber & Yee, 1987). No plasmids could be detected in these isolates which suggested that the resistance determinants were located on a transposon integrated into the chromosome (Faber & Yee, 1987), as has been reported for *S. pneumoniae* (Courvalin & Carlier, 1986).

Resistance to gentamicin in enterococci and staphylococci is due to a bifunctional enzyme with 6'-acetyltransferase and 2"-phosphotransferase activities (Eliopoulos et al., 1988; Feretti, Gilmore & Courvalin, 1986; Ubukata et al., 1984). The gene encoding high-level gentamicin resistance resides on transposon Tn*5281* in *Enterococcus faecalis* and has been shown to be similar to the staphylococcal transposons Tn*4001* and Tn*4031* (Hodel-Christian & Murray, 1991). This gene encoding high-level gentamicin resistance in enterococci has been shown to reside on transposons (Hodel-Christian & Murray, 1991), the chromosome (Rice et al., 1991) and on conjugative as well as nonconjugative plasmids (Patterson & Zervos, 1990). A similar gene has recently been located on the chromosome of a single *Streptococcus agalactiae* strain with high-level gentamicin resistance (Kaufhold et al., 1992). High-level gentamicin resistance amongst viridans streptococci was first described for a single oropharyngeal South African isolate in 1990 (Van den Berg, 1990), but no further investigation into the resistance mechanism was reported.

Impermeability

Resistance to aminoglycosides can also involve diminished uptake of the compound (Shannon & Phillips, 1982). Moellering & Weinberg, (1971) showed that the synergistic killing of the combination of penicillin and streptomycin was associated with stimulation of streptomycin uptake by

penicillin in enterococci and concluded that damage to the cell wall by penicillin promoted the penetration of streptomycin. Yee, Faber and Mates, (1986) similarly showed penicillin and vancomycin to promote uptake of streptomycin and tobramycin into the viridans streptococci. However, this promotion of uptake was observed in a strain resistant to penicillin and ribosomally resistant to streptomycin, indicating that the mechanism of increased uptake was not dependent on the cell wall disruptive effect of penicillin, but rather that the increased aminoglycoside uptake was as a result of direct stimulation of a transport process (Yee, Faber & Mates, 1986).

.2.3.3 *Transfer of penicillin resistance.*

Viridans streptococci and *S. pneumoniae* are normally isolated from the upper respiratory tract of man and very high carrier rates have been reported (Austrian, Howie & Ploussard, 1977; Van den Berg, 1990). Faber et al. (1983b) studied 10 multiply resistant viridans streptococci isolated in South Africa and commented on the close proximity of these strains to the presence of multiply resistant *S. pneumoniae* isolates in the oropharynx. Penicillin resistance in both the viridans streptococci (Levin et al., 1982; Goldfarb, Wormser & Glaser, 1984; Escribano et al., 1990; Van den Berg, 1990) and *S. pneumoniae* (Klugman, 1990) has been shown to be increasing worldwide. As early as 1957 transformation studies on streptomycin resistance transfer from various streptococci into *S. pneumoniae* was reported to occur under laboratory conditions (Bracco et al., 1957; Ravin & De Sa, 1964). Transfer of macrolides-lincosamines- streptogramin B (MLS), tetracycline, streptomycin and kanamycin resistance genes on transposons, using filter mating techniques was reported between viridans

streptococci and other streptococcal species (Horodniceanu et al., 1982; Courvalin & Carlier, 1986; Clermont & Horaud, 1990). In addition plasmid transformation studies have shown transfer of MLS and chloramphenicol-MLS resistance (Horaud, Le Bouguenec & Pepper, 1985). Transfer of several antibiotic resistance genes between viridans streptococci and *S. pneumoniae* appeared to be possible, however, these earlier studies did not include transfer of penicillin resistance.

Penicillin resistance in *S. mitis* and *S. pneumoniae* is due to alterations in the binding affinity of the PBPs for penicillin (Percheson & Bryan, 1980; Hakenbeck, Tarpay & Tomasz, 1980; Zigelboim & Tomasz, 1980; Faber et al., 1983; Quinn et al., 1988).

The extent of PBP variability in penicillin-resistant isolates of *S. pneumoniae* (Chalkley & Koornhof, 1988; Chalkley, Van den Berg & Koornhof, 1989; Markiewicz & Tomasz, 1989; Hakenbeck et al., 1991) similarities in the sizes of target PBPs associated with increased penicillin resistance in virdans streptococci and *S. pneumoniae* (Hakenbeck et al., 1986; Massidda & Daneo-Moore, 1988; Zito & Daneo-Moore, 1988) transformation of penicillin resistance from isolates of *S. sanguis* II and *S. mitior* to *S. pneumoniae* (Chalkley & Koornhof, 1990) and the presence of PBPs in penicillin-susceptible strains of *Streptococcus pyogenes* and *S. oralis* that cross-react with antibodies raised against *S. pneumoniae* PBP 1a and/or PBP 2b (Hakenbeck et al., 1987; Hakenbeck et al., 1991) suggested transfer of homologous genes amongst a variety of streptococcal species.

In clinical isolates of *S. pneumoniae* resistant to penicillin PBPs are the products of mosaic genes (Dowson et al., 1989; Laible, Spratt & Hakenbeck, 1991). The PBP genes of susceptible *S. pneumoniae* strains

have been replaced by homologous gene fragments as a result of the transfer of genes from related species with subsequent recombination into the chromosomal PBP gene (Dowson et al., 1989; Laible, Spratt & Hakenbeck, 1991; Martin, Sibold & Hakenbeck, 1992). Studies of the mosaic structures of the *S. pneumoniae* PBP 2b genes indicated that at least two different sources were involved (Dowson et al., 1989), while analysis of the *S. pneumoniae* PBP 2x and 1a genes suggested that at least four different sources were involved (Laible, Spratt & Hakenbeck, 1991; Martin, Sibold & Hakenbeck, 1992).

As result of DNA hybridization and DNA sequence analysis, it was originally proposed that *S. pneumoniae* transferred PBP 2b resistance genes to *S. oralis* and *S. sanguis* (Dowson et al., 1990; Coffey et al., 1993). Dowson et al. (1993) has since shown that PBP 2b genes from many penicillin-resistant isolates of *S. pneumoniae* contain blocks of nucleotides originating from *S. mitis*. Concerning PBP 2x, (Sibold et al., 1994) has shown that gene transfer from *S. oralis* into various genetic lineages of *S. pneumoniae* has occurred, including sequences from an unidentified source.The sequence data and resistant gene blocks of *S. oralis* and *S. mitis* recently reported by Coffey and Dowson (Coffey et al., 1993; Dowson et al. 1993) will be discussed fully in conjunction with results obtained in this study on further *S. mitis* and *S. oralis* isolates in Chapter 6.

The possibility of mutual exchange of PBP genes between different species has significant implications in the search for the primary source of mosaic genes. Further studies of PBP sequences in related species are therefore essential in order to fully understand the direction of gene flow and all the partners involved.

CHAPTER 2

IDENTIFICATION OF VIRIDANS STREPTOCOCCI

2.1 Introduction

Despite considerable effort by many microbiologists from as early as 1906 (Andrewes & Horder, 1906), to classify the medically important viridans streptococci, international concensus on classification and nomenclature has still not been obtained (Coykendall, 1989b; Kilian, Mikkelsen & Henrichsen, 1989a; Colman, Efstratiou & Morrison, 1991; Facklam & Washington, 1991). All these efforts produced a large amount of information and several taxonomic ideas but conflicting data from different investigating groups also contributed to the confusion (Coycendall, 1989b). Generally lack of distinguishing phenotypic traits have left these problems unsolved (Welborn et al., 1983; Gilmour et al., 1987; Kilian, Mikkelsen & Henrichsen, 1989a).

When this study was initiated at the end of 1989 there were two major, generally accepted, identification schemes; that proposed by Facklam (Facklam, 1977) and that of Colman and Williams (Colman & Williams, 1972). Both these schemes required the use of many time-consuming physiological tests which made them impractical for screening large numbers of strains. Furthermore a large number of exceptions to the rule were evident in these identification schemes with a number of strains remaining unidentified no matter which identification scheme was employed (French et al., 1989). In addition biochemical tests employed in these schemes needed to be standardized as small variations in a test could give different results (French et al., 1989; Kilian, Mikkelsen & Henrichsen, 1989a).

The nomenclature of the viridans streptococci was, however, a major problem for researchers whose studies required definitive identification for comparative studies. The approved Lists of Bacterial Names published in

1980 included the following oral species: *Streptococcus sanguis, Streptococcus mitis, Streptococcus salivarius, Streptococcus anginosus, Streptococcus constellatus* and *Streptococcus intermedius* (Skerman, McGowan & Sneath, 1980). The literature, however, abounded with reasons for either splitting or combining some of these species (Coykendall, 1989b).

It became clear therefore that a realistic identification of a large number of viridans streptococci to species level that is universally accepted would be extremely difficult. There seemed however, to be general agreement on five main groups within the viridans streptococci and an identification scheme that would at least separate these major groups from each other was therefore employed for the screening of the 211 clinical isolates (Facklam & Washington, 1991).

Isolates selected for further studies were initially classified according to French et al. (1989) in order to conform, at the time of publishing transformation data (Potgieter & Chalkley, 1991), to European nomenclature. These isolates were then recently re-classified according to Kilian, Mikkelsen & Henrichsen (1989a).

2.2 Materials and Methods

2.2.1 *Strains.*

Two hundred and eleven viridans streptococcal strains isolated from blood were collected from three Johannesburg hospitals between June 1988 and March 1991. Thirty-one nasopharyngeal viridans streptococcal strains isolated from children admitted to Baragwanath hospital during 1988 were kindly provided by I. van den Berg. All these strains had previously been identified as viridans streptococci, therefore excluding other α-haemolytic streptococci. Nevertheless all isolates were again checked for certain characteristics: all were resistant to optochin and sensitive to vancomycin using a zone, no-zone criterion for screening, no strains grew on bile-aesculin medium or 6.5% NaCl and none fermented arabinose or mannitol.

2.2.2 *Biochemical tests.*

Conventional biochemical tests were based on the methods of MacFaddin (1980) and Waitkins, Ball & Fraser (1980).

Production of acids from carbohydrates was determined in a semi-solid phenol red medium (MacFaddin, 1980). pH of the uninoculated medium was 7.2-7.4 and a pH of between 4.0 and 6.2 was considered indicative of a positive reaction.

The hydrogen peroxide test was performed as described by Whittenbury (1964).

Production of acetoin (VP test) and arginine hydrolysis were demonstrated as described by Waitkins, Ball & Fraser (1980). Aesculin hydrolysis was detected by the method proposed by Kilian, Mikkelsen and Henrichsen

(1989a) and the aesculin-bile test perfomed according to the method described by MacFaddin (1980).

Growth in 4% and 6% NaCl was determined by addition of the relevant concentrations of NaCl to Todd-Hewitt broth and, after inoculation, incubation for 7 days before examining for the presence or absence of growth.

The dextran test of Hehre & Neill (1945) was performed as described by French et al. (1989). Cultures were incubated in sucrose broths for 7 days and then centrifuged at 3000 rpm for 10 min; 0.5 ml of the supernatant was placed in a test tube, and 4.5 ml of 10% sodium acetate (w/v) was added; 1.2 volumes of absolute alcohol was added and mixed well. Formation of a precipitate within 3 h indicated production of dextran.

The API-20STREP and API ZYM systems (API Systems, S.A. France) were inoculated and read as described by the manufacturer.

2.3 Results and discussion.

2.3.1 *Identification and classification of the 211 viridans streptococci isolated from blood using conventional tests.*

Identification was performed according to the presumptive identification scheme proposed by Facklam & Washington (1991). According to this scheme the *S. sanguis* / *S. mitis* group which does not produce acetoin is distinguished from the *S. mutans* / *S. salivarius* / *S. anginosus* group which produces acetoin (Voges-Proskauer test). Arginine-positive aesculin-positive isolates from the *S. sanguis* / *S. mitis* group were classified as *S. sanguis*, while arginine-negative, aesculin-negative isolates were classified as *S. mitis*. Arginine-positive, aesculin-negative isolates were included in the *S. sanguis* group, while arginine-negative, aesculin-positive isolates were included in the *S. mitis* group according to Price et al (1986). *S. anginosus* isolates could be differentiated from *S. salivarius* isolates by their ability to hydrolyse arginine. When characterized by this scheme 108 (51 %) of the isolates were classified as *S. mitis*, 91 (43 %) as *S. sanguis* and 12 (5%) as either *S. salivarius* or *S. anginosus*. No *S. mutans* isolates were found.

The reasons why these strains were only placed in general groups instead of being specifically speciated have been fully discussed in Chapter 1.2.7. In summary the main reasons were: a) differentiation between these five groups in routine laboratories could be done without any problem. There was at the time, end 1989, general agreement on the five main groups within the viridans streptococci, but no international concensus had been reached on further sub-divisions of the strains within the groups (Coykendall, 1989b; Kilian, Mikkelsen & Henrichsen, 1989a; Beighton,

Hardie & Whiley, 1991; Colman, Efstratiou & Morrison, 1991; Facklam & Washington, 1991). b) Lack of standardized tests still remained a problem for the phenotypic differentiation of the various species within the groups. Differentiation of *S. oralis* from *S. mitis* was problematic due to lack of distinguishing phenotypic characteristics; the two conventional tests proposed by Kilian, Mikkelsen & Henrichsen (1989a) to distinguish between these two species, growth in 4% salt and production of dextran are both time-consuming (7 days) and not always easy to interpret. The dextran test also depends on the extraction system used and in any case showed too many exceptions (French et al., 1989). The IgA1 protease test also proposed by Kilian, Mikkelsen & Henrichsen (1989a) requires specialised equipment. The definitive β-glucosaminidase API ZYM test to differentiate *S. oralis* from *S. mitis* although easy to perform and certainly more standardized than conventional tests was too expensive to screen large numbers of strains, especially since only one of the twenty tests included in the kit was required. The only two conventional tests used by Kilian, Mikkelsen & Henrichsen (1989a) to distinguish between *S. sanguis* and *S. gordonii*, the tests for the fermentation of amygdalin and the production of alkaline phosphatase, still showed too many exceptions, and the additional IgA1 protease and API ZYM required to resolve this problem are not readily performed or are too expensive.

2.3.2 *Identification of 52 strains isolated from blood cultures and nasopharyngeal specimens using the API-20S system.*

Twenty-one viridans streptococci isolated from blood cultures and an additional 31 isolates from nasopharyngeal specimens were selected for PBP and genetic studies.

As mentioned in Chapter 1.2.7 standardization of conventional biochemical tests is still a major problem in the differentiation of the viridans streptococcal species, as small variations in a test may give different results (French,et al., 1989; Kilian, Mikkelsen & Henrichsen, 1989a). The API-20STREP system was therefore used for selected isolates in an attempt to obtain more reproducible and definitive identification results and to retain a profile number for future reference. Because of the cost involved, however, only isolates selected for PBP and genetic studies were identified using the API-20STREP system.

The scheme as proposed by French et al. (1989) showed a definite separation of the *Streptococcus sanguis* and *Streptococcus mitis/mitior/sanguis* II groups, based on the hydrolisis of arginine and aesculin which was supported by the cell wall composition of these two groups of bacteria (Price et al, 1986). As the scheme of French et al. (1989) was employed for strains used in the transformation experiments, the name *S. mitior* was initially used in the publication (Potgieter & Chalkley, 1991). The *S. mitior* isolates used in the transformation studies, Chapter 4 (Potgieter & Chalkley, 1991) and the *S. mitis* isolates used in the PBP studies, Chapter 5 (Potgieter, Koornhof & Chalkley, 1992) have since in (1993) been re-classified according to Kilian, Mikkelsen & Henrichsen (1989a) employing the API ZYM system (Table 2.1).

The sub-division of the *S. mitis* group into *S. mitis* and *S. oralis* has recently become an issue concerning the origins of penicillin resistance genes in streptococci with the publications of Dowson et al. (1989) and Dowson et al. (1993). The isolates used to investigate this aspect have now been identified according to Kilian, Mikkelsen & Henrichsen (1989a), and the direction of PBP gene transfer *S. mitis* ↔ *S. pneumoniae* or *S. oralis* ↔ *S.*

pneumoniae will be discussed in conjunction with the findings of Dowson and his associates (Dowson et al., 1989; Dowson et al. 1993) in Chapter 6.

Table 2.1. *S. mitis* isolates selected for PBP analysis and genetic studies classified according to Kilian, Mikkelsen & Henrichsen (1989a).

Blood isolates		Nasopharyngeal isolates	
Strain No.	Identification	Strain no.	Identification
E2	*S. oralis*	I1	PN
E3	*S. mitis*	I2	PN
E4	*S. mitis*	I3	*S. oralis*
E7	*S. mitis*	I4	ND
E9	*S. oralis*	I5	PN
E10	*S. oralis*	I9	PN
E11	*S. mitis*	I11	ND
E12	*S. oralis*	I13	S. oralis
E14	*S. mitis*	I14	PN
E23	PN	I16	*S. mitis*
E35	PN	I17	*S. oralis*
E52	PN	I19	ND
E63	*S. oralis*	I20	PN
E76	PN	I21	*S. oralis*
		I22	PN
		I23	PN
		I24	PN
		I25	PN
		I28	*S. mitis*
		I29	*S. mitis*
		I31	*S. mitis*
		I32	*S. oralis*
		I33	*S. mitis*
		I35	PN
		I36	PN
		I39	*S. mitis*
		I42	PN

ND = not determined; PN = not determined as isolates were PCR negative (Chapter 6).

CHAPTER 3

IN VITRO ANTIMICROBIAL SUSCEPTIBILITY OF VIRIDANS STREPTOCOCCI ISOLATED FROM BLOOD.

3.1 Introduction

Viridans streptococci had been considered to be susceptible to penicillin until 1978 when ten nasopharyngeal viridans streptococci isolated in South Africa were found to be multiply resistant to β-lactam antibiotics (Faber et al.,1983).

Reports of increasing antibiotic resistance in viridans streptococci isolated from clinically significant infections have come from America and Spain (Goldfarb, Wormser & Glaser, 1984; E. Escribano et al., 1990). In a South African antibiotic resistance survey conducted in 1987 on oropharyngeal viridans streptococci isolated from children admitted to Baragwanath Hospital 76% of the isolates were shown to be resistant to penicillin (MICs $\geq 0.25\ \mu$g/ml) (Van den Berg, 1990). From this group of isolates a high-level gentamicin-resistant (MIC $>2560\ \mu$g/ml) viridans streptococcus was also identified.

The possibility of penicillin resistance being coupled to gentamicin resistance in oral strains of viridans streptococci is cause for concern. It is likely that such strains (which could give rise to serious infections) would no longer respond to penicillin/gentamicin combination therapy. This has been found with enterococcal endocarditis (Moellering, 1991).

The present survey was therefore undertaken during 1988 to 1991 to investigate antimicrobial susceptibility of 211 South African strains of viridans streptococci, isolated from blood.

3.2 Materials and Methods

3.2.1 *Strains.*

Two hundred and eleven viridans streptococci isolated from blood were collected from three Johannesburg hospital laboratories between June 1988 and March 1991. Strains were identified using conventional biochemical tests (Cowan, 1974) as described in Chapter 2 and were generally classified according to the criteria of Facklam and Washington (1991). Aesculin-positive and arginine-negative strains were included in the *S. mitis* group and aesculin-negative and arginine-positive strains incorporated into the *S. sanguis* group as described by Price et al. (1986).

3.2.2 *MIC determination.*

Antimicrobial activity was determined by the agar dilution method as described by the National Committee for Clinical Laboratory Standards (NCCLS) (1990). The antimicrobial agents were obtained as standard reference powders from the respective manufacturers. Five millilitre Mueller-Hinton (Oxoid, UK) broth was inoculated and cultures incubated at 37°C until turbid, 3-5 h. These logarithmic growth phase cultures were then diluted to approximately $1x10^7$ cfu/ml, (Mc Farland 0.5) and inoculated onto Mueller-Hinton agar (Oxoid, UK) plates, supplemented with 5% horse blood and containing appropriate antibiotic concentrations. A multipoint inoculator (Mast), which delivered 1 μl of the diluted culture, was used for this purpose. Plates were incubated aerobically at 35°C for 18 h. MICs of the antimicrobial agents listed above were determined using antibiotic concentrations ranging from 0.03 to128 μg/ml. Strains with gentamicin MICs of ≥64 μg/ml were further tested at concentrations of

250, 500, 1 000 and 2 000 μg/ml. In addition streptomycin, kanamycin, amikacin and tobramycin MICs were determined on the strains, with gentamicin MICs $\geq$64 mg/ml, at concentrations of 1 000 and 2 000 μg/ml. One strain for which the streptomycin MIC was $>$2 000 μg/ml was tested for ribosome-associated resistance at 32 000 μg/ml. Strains for which gentamicin MICs were 64 and 128 μg/ml were described as exhibiting increased resistance and strains with MICs of $\geq$500 μg/ml were described as showing high-level resistance, as defined for enterococci (NCCLS, 1990). The susceptibility breakpoint concentrations of the antibiotics used were in accordance with recommended values (NCCLS, 1990), although it should be noted that the NCCLS cephalosporin breakpoints are not specific for viridans streptococci.

3.2.3 *Synergy studies.*

Synergy studies were performed using the time-kill method as described by the NCCLS (NCCLS, 1987). Twenty-five millilitre flasks containing 9 ml of cation-supplemented Mueller-Hinton broth, were inoculated with an overnight broth culture to give an initial culture viability of about $3x10^5$ cfu/ml. Cultures were incubated at 35^{o}C on a rotary shaker at 150 rpm. After 1.5 h when the cells had reached the exponential growth phase (Washington, 1991) antibiotic additions (1 ml) were made, this being zero time for the time-kill method. One flask recieved 1 ml of broth as the culture control. At time zero and after 2, 4 and 6 h incubation, 500 μl of culture was withdrawn for dilutions and standard plate counts were performed (Miles & Misra, 1938). Cell death was observed after 24 h in the control cultures not exposed to antibiotics, even when the broth was supplemented with 5% lysed horse blood. These 24 h cultures were turbid

in appearance, indicating growth had occured although viability was $< 10^3$ cfu/ml. This phenomenon requires further investigation. For bactericidal assessments using time-kill studies, NCCLS (1990) recommendations prefer readings to be taken during the first 6 h. Synergy has also been shown to occur by 6 h using penicillin plus aminoglycosides against viridans streptococci (Miller et al., 1986). Antibiotic combinations of penicillin and gentamicin were tested against three representative *S. mitis* isolates (strains 56, 256 and 192) showing increased/high-level resistance to gentamicin. In addition time-kill studies were also conducted on one *S. mitis* strain (strain 157) which was susceptible to both penicillin and gentamicin. Synergy was tested using bacteriostatic concentrations of both penicillin and gentamicin over a period of 6 h.

3.3 Results

The MICs of *S. mitis*, *S. sanguis*, *S. salivarius* and *S. milleri* are presented in Table 3.1. Forty percent of the *S. mitis* and 33% of the *S. sanguis* strains had penicillin MICs of ≥0.25 μg/ml (Table 3.1). Overall 29% of the isolates were resistant to the intermediate resistance range of concentrations of penicillin (MIC 0.25-2 μg/ml) and 9% of the isolates were resistant with penicillin MICs ≥4 μg/ml (Table 3.2). A further breakdown of the penicillin MICs (Table 3.2) shows that 14% of the *S. mitis* strains but only 2% of the *S. sanguis* strains had penicillin MICs of ≥4 μg/ml.

All the isolates were susceptible to the cephalosporins according to NCCLS breakpoints, but nine strains had MICs at the susceptibility breakpoint concentration. Cefotaxime and ceftriaxone were more active than penicillin against all strains except three. Imipenem showed excellent activity as can be seen by the MIC_{90} concentrations (Table 3.1).

Combined tetracycline and erythromycin resistance (MICs ≥16 μg/ml and 8 μg/ml respectively) was found in 7% of the viridans streptococci studied. Resistance to tetracycline alone was 34% compared with only 0.9% for erythromycin resistance alone.

Sixty-four percent of the *S. mitis* and 13% of the *S. sanguis* strains had gentamicin MICs of ≥8 μg/ml (Table 3.1). Scrutiny of an extended breakdown of gentamicin MICs shows that 10 *S. mitis* strains had MICs of 32 μg/ml and five strains MICs ≥64 μg/ml. No *S. sanguis* strain isolated had a gentamicin MIC >16 μg/ml (Table 3.3). The susceptibility of the five gentamicin-resistant (MICs ≥64 μg/ml) *S. mitis* strains mentioned above to additional aminoglycosides is shown in Table 3.4. Three strains isolated at Baragwanath Hospital, 56, 256 and 265 showed a similar aminoglycoside resistance pattern. Strain 160, however, had a higher MIC to streptomycin

(>32000 μg/ml) than strains 56, 256 and 265. Strain 160 also differs from the other Baragwanath isolates in that it is resistant to tetracycline. All the Baragwanath isolates exhibiting elevated/high-level resistance to gentamicin, kanamycin, tobramycin and streptomycin (one strain) were also resistant to penicillin. The Johannesburg Hospital isolate, strain 192 was susceptible to penicillin.

It is noteworthy that no consistent high-level cross-resistance involving the five aminoglycosides tested was demonstrated amongst the five strains examined. Also only the Johannesburg Hospital strain exhibited high-level resistance to amikacin at an MIC of 1000 μg/ml, while four of the strains were resistant to tobramycin at this level.

Results of the synergy studies performed on strains 56, 256 and 192 are shown in Figures 3.1a, 3.1b, 3.2a, 3.2b, 3.3a and 3.3b. Combinations of penicillin and gentamicin were not synergistic (nor even additive) against these isolates. In addition to the gentamicin-resistant and penicillin-resistant/penicillin-susceptible strains 56, 256 and 192 mentioned above, one strain (strain 157), susceptible to both gentamicin and penicillin was also examined using synergy time-kill studies. Synergy between penicillin and gentamicin was also not demonstrated with the susceptible strains (Figures 3.4a and 3.4b).

Table 3.1: Susceptibility of 211 strains of viridans streptococci.

Strain	Antimicrobial agent	MIC (μg/ml)		Breakpoint concentration (μg/ml)	Percent susceptible at breakpoint concentration
		MIC_{90}	Range		
Streptococcus mitis (n = 108)	Penicillin	4	≤ 0.03 - 32	0.12	60
	Imipenem	0.5	≤ 0.03 - 4	4	100
	Cefotaxime	2	≤ 0.03 - 8	8	100
	Ceftriaxone	2	≤ 0.03 - 8	8	100
	Vancomycin	1	0.5 - 2	4	100
	Gentamicin	32	1 - > 128	4	36
	Erythromycin	4	≤ 0.03 - > 128	0.5	59
	Tetracycline	64	0.25 - 128	4	50
Streptococcus sanguis (n = 91)	Penicillin	1	≤ 0.03 - 32	0.12	67
	Imipenem	0.25	≤ 0.03 - 1	4	100
	Cefotaxime	1	≤ 0.03 - 8	8	100
	Ceftriaxone	2	≤ 0.03 - 8	8	100
	Vancomycin	1	1 - 2	4	100
	Gentamicin	8	0.5 - 16	4	87
	Erythromycin	4	≤ 0.03 - > 128	0.5	67
	Tetracycline	64	0.06 - > 128	4	57
Streptococcus salivarius *Streptococcus milleri* (n = 12)	Penicillin	2	≤ 0.03 - 4	0.12	42
	Imipenem	0.25	≤ 0.03 - 0.5	4	100
	Cefotaxime	1	≤ 0.03 - 2	8	100
	Ceftriaxone	1	≤ 0.03 - 2	8	100
	Vancomycin	2	1 - 2	4	100
	Gentamicin	4	0.25 - 4	4	100
	Erythromycin	4	≤ 0.03 - 4	0.5	83
	Tetracycline	128	0.25 - 128	4	75

Table 3.2. MICs of viridans streptococci to penicillin.

Strain	Number of strains with following MIC (μg/ml)										
	≤ 0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32
Streptococcus mitis (n = 108)	40	14	11	12	3	8	5	3	7	4	1
Streptococcus sanguis (n = 91)	25	21	15	4	5	14	5	1	0	0	1
Streptococcus salivarius *Streptococcus milleri* (n = 12)	4	0	0	1	4	0	1	1	0	0	0

Table 3.3. MICs of *S. mitis* and *S. sanguis* strains to gentamicin.

Strains	Number of strains with following MIC (μg/ml)										
	0.25	0.5	1	2	4	8	16	32	64	128	> 128
Streptococcus mitis (n = 108)	0	0	10	17	12	19	35	10	2	1	2
Streptococcus sanguis (n = 91)	0	4	12	36	27	9	3	0	0	0	0

Table 3.4. Susceptibility of five gentamicin resistant *Streptococcus mitis* isolates.

Strain Number	Hospital	MIC (μg/ml)							
		Penicillin (0.12)*	Erythromycin (0.5)*	Tetracycline (4)*	Gentamicin (4)*	Streptomycin (4)*	Kanamycin (16)*	Amikacin (16)*	Tobramycin (4)*
56	Baragwanath	16	4	0.5	1000	64	>2000	≤64	1000
160	Baragwanath	16	2	64	1000	>32000	>2000	≤64	1000
265	Baragwanath	16	0.5	0.5	128	≤64	>2000	≤64	1000
256	Baragwanath	32	1	0.5	128	≤64	>2000	≤64	1000
192	Johannesburg	≤0.03	0.125	1	64	1000	1000	1000	128

* Susceptibility breakpoint concentration.

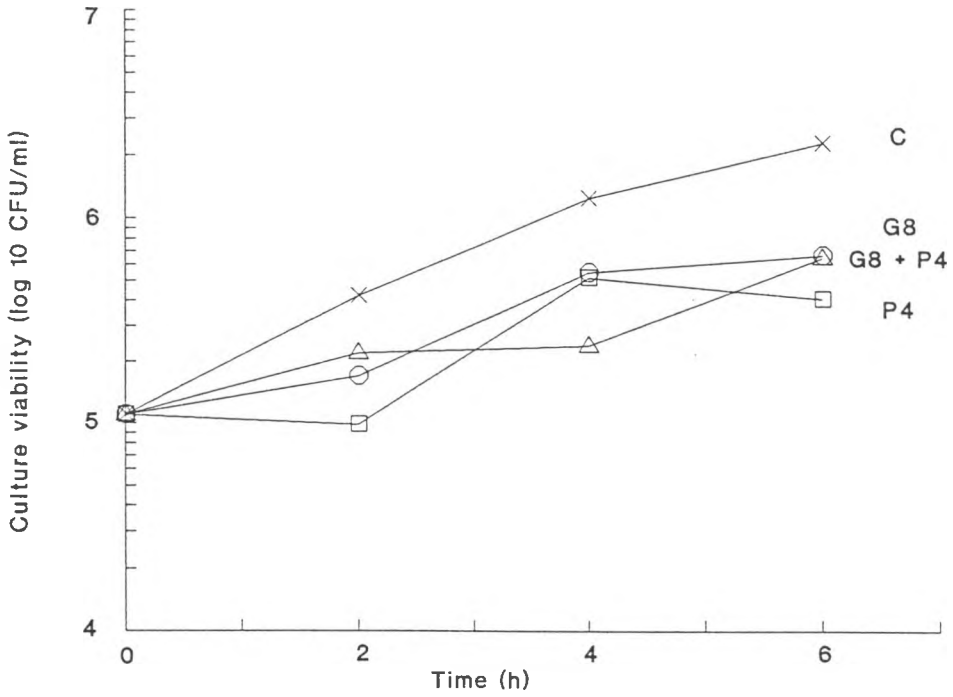

Figure 3.1a. *In vitro* effect of penicillin (P) and gentamicin (G) alone and in combination against *S. mitis* 56. Penicillin is at a bacteriostatic concentration. Numbers next to P or G indicate concentration of antibiotic in μg/ml. C = control (no antibiotics present).

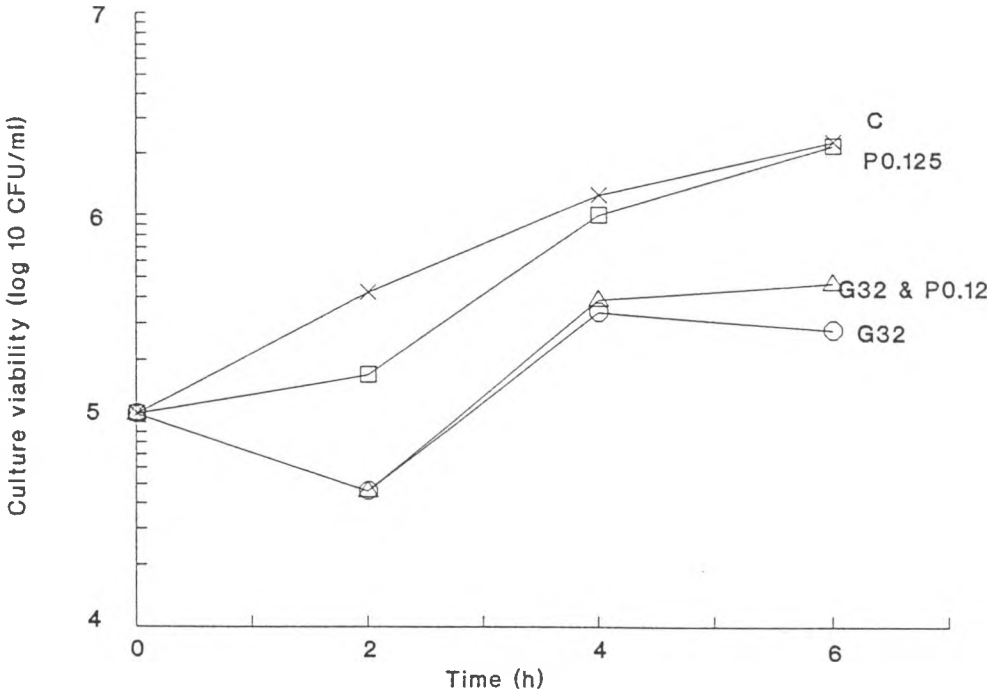

Figure 3.1b. *In vitro* effect of penicillin (P) and gentamicin (G) alone and in combination against *S. mitis* 56. Gentamicin is at a bacteriostatic concentration. Numbers next to P or G indicate concentration of antibiotic in μg/ml. C = control (no antibiotics present).

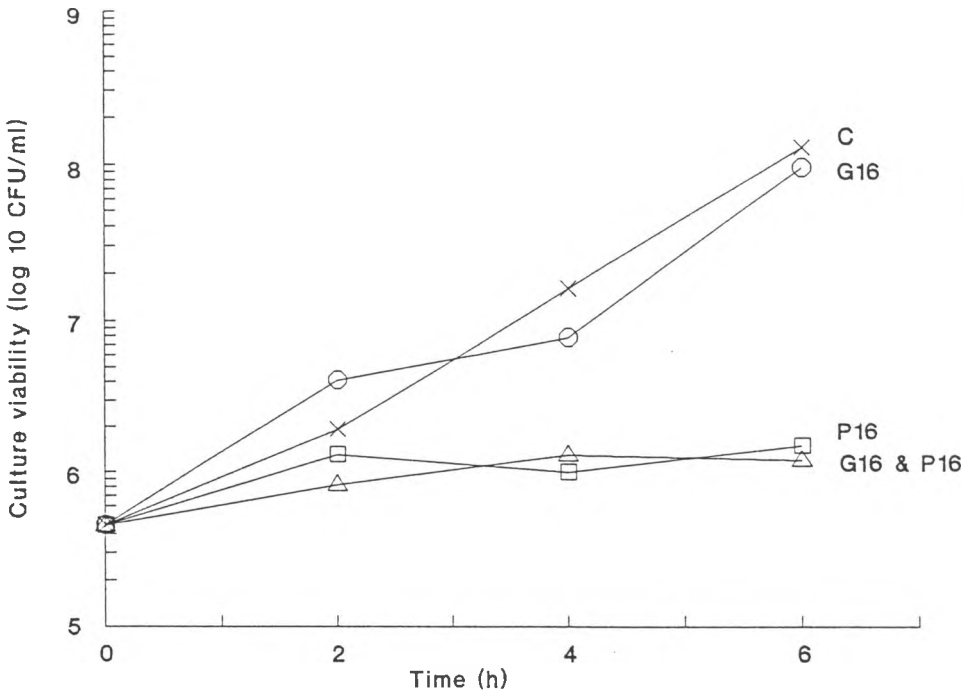

Figure 3.2a. *In vitro* effect of penicillin (P) and gentamicin (G) alone and in combination against *S. mitis* 256. Penicillin is at a bacteriostatic concentration. Numbers next to P or G indicate concentration of antibiotic in μg/ml. C = control (no antibiotics present).

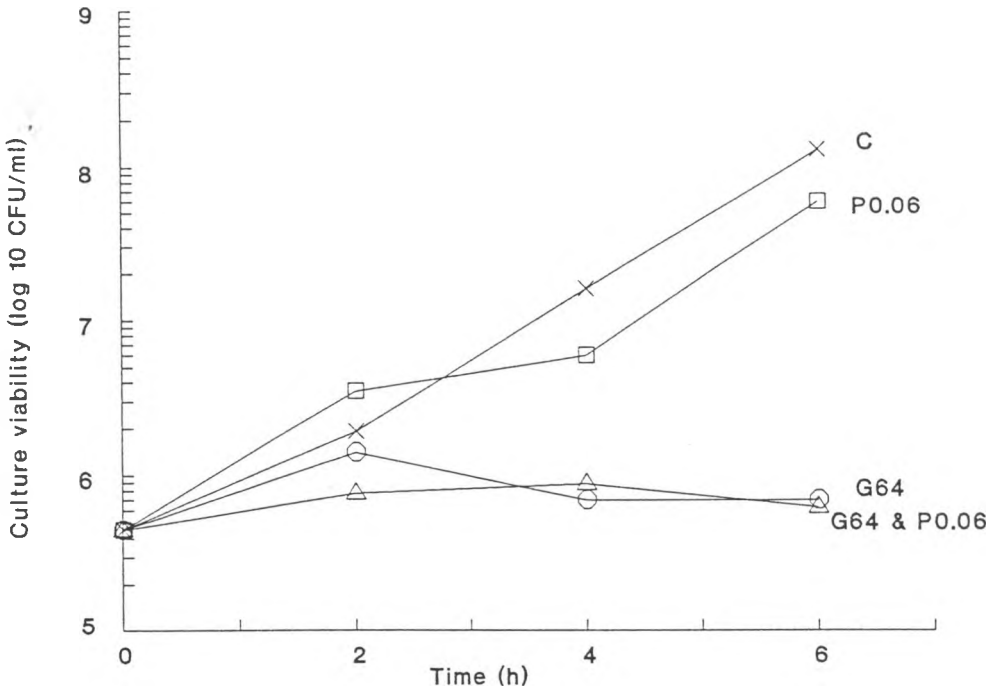

Figure 3.2b. *In vitro* effect of penicillin (P) and gentamicin (G) alone and in combination against *S. mitis* 256. Gentamicin is at a bacteriostatic concentration. Numbers next to P or G indicate concentration of antibiotic in μg/ml. C = control (no antibiotics present).

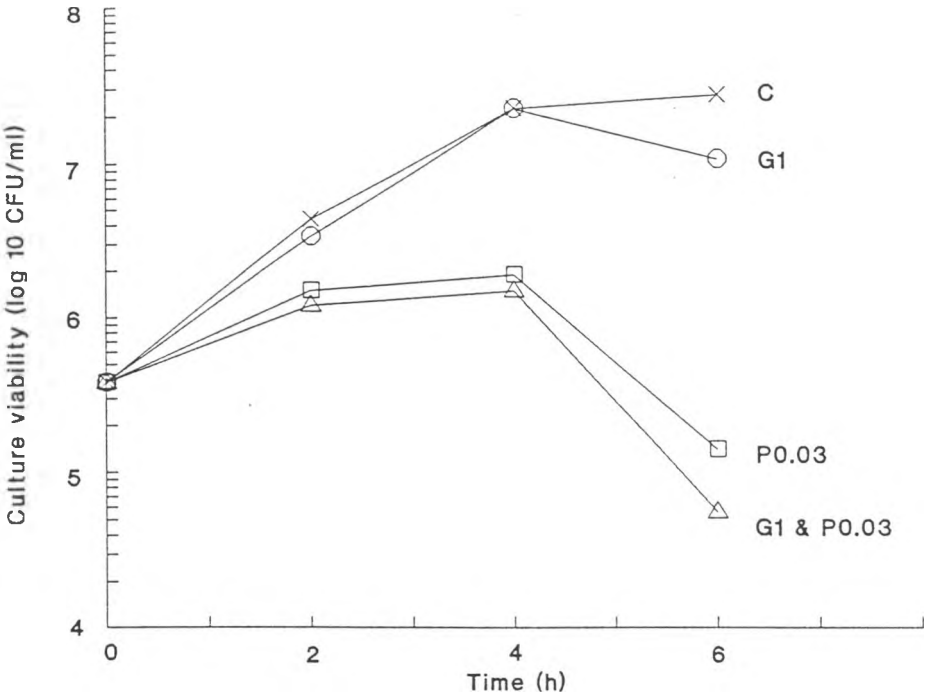

Figure 3.3a. *In vitro* effect of penicillin (P) and gentamicin (G) alone and in combination against *S. mitis* 192. Penicillin is at a bacteriostatic concentration. Numbers next to P or G indicate concentration of antibiotic in μg/ml. C = control (no antibiotics present).

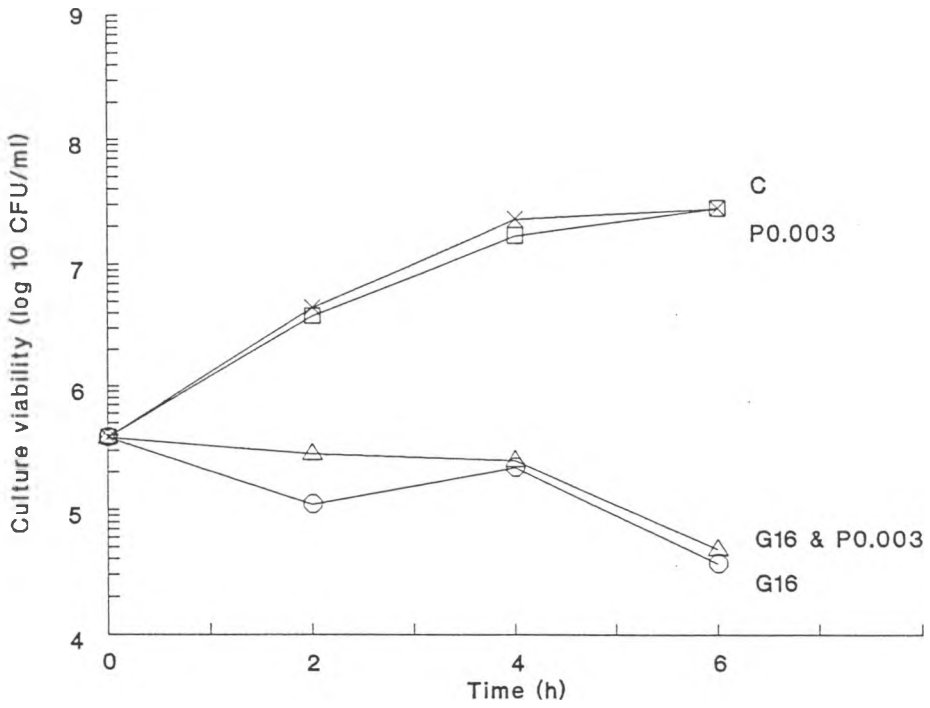

Figure 3.3b. *In vitro* effect of penicillin (P) and gentamicin (G) alone and in combination against *S. mitis* 192. Gentamicin is at a bacteriostatic concentration. Numbers next to P or G indicate concentration of antibiotic in μg/ml. C = control (no antibiotics present).

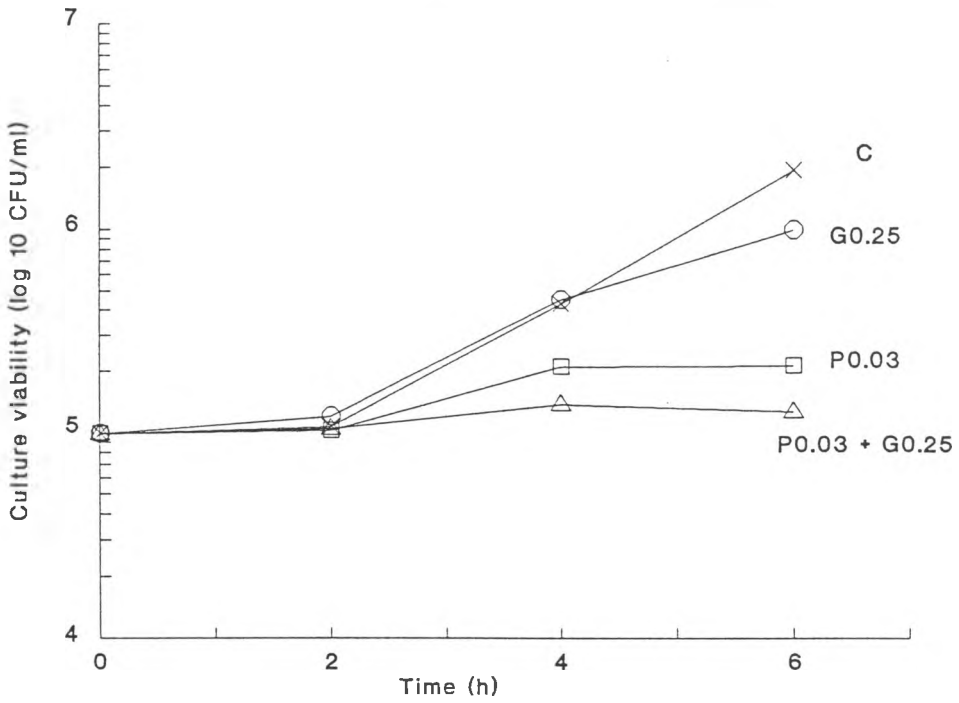

Figure 3.4a. *In vitro* effect of penicillin (P) and gentamicin (G) alone and in combination against *S. mitis* 157. Penicillin is at a bacteriostatic concentration. Numbers next to P or G indicate concentration of antibiotic in μg/ml. C = control (no antibiotics present).

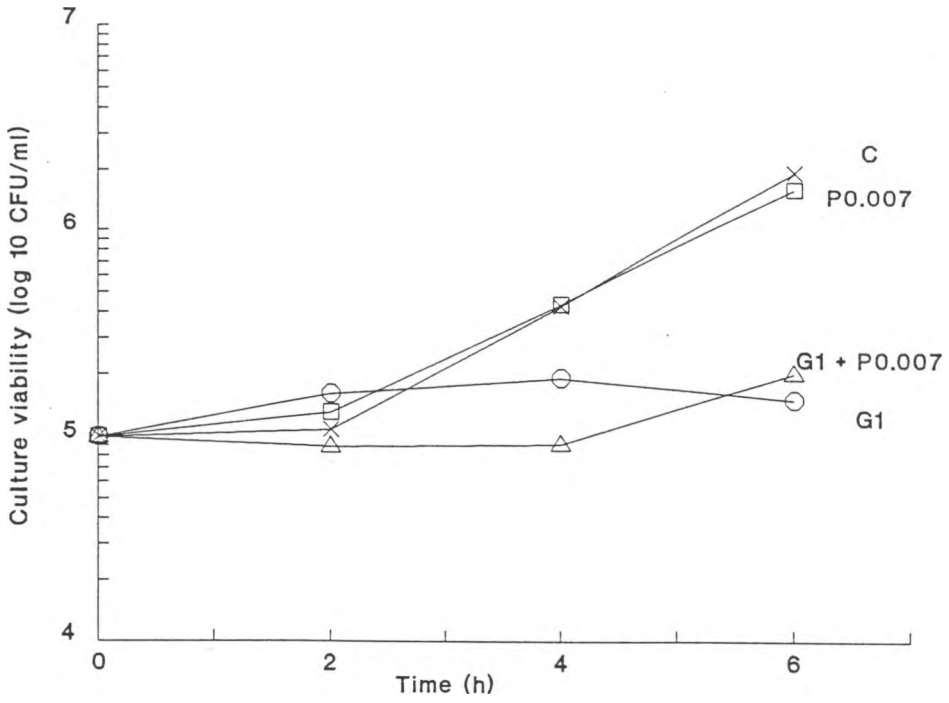

Figure 3.4b. *In vitro* effect of penicillin (P) and gentamicin (G) alone and in combination against *S. mitis* 157. Gentamicin is at a bacteriostatic concentration. Numbers next to P or G indicate concentration of antibiotic in μg/ml. C = control (no antibiotics present).

3.4 Discussion

In the present study 29% of the 211 viridans streptococcal isolates obtained from blood between 1988-1991 were resistant to intermediate levels of penicillin (MICs 0.25-2 μg/ml), and a further 9% were resistant with penicillin MICs of $\geq$4 μg/ml. On comparing penicillin MICs of the South African viridans streptococci, isolated from clinically significant infections in this study, with the values obtained from studies conducted in Europe, similarities as well as differences are evident. A Dutch study of 200 isolates of viridans streptococci, isolated from patients with endocarditis between 1986 and 1988, reported that most of the isolates were susceptible to penicillin, the MIC_{90} for penicillin being 0.04 μg/ml (Van der Meer et al., 1991). A Spanish study conducted on 142 isolates of viridans streptococci isolated from blood, during the time period 1990-1992, showed 26% of the isolates were resistant to intermediate levels of penicillin, and 7.7% resistant to penicillin MIC $\geq$4 μg/ml. Penicillin-resistant viridans streptococci (31.3% of 47 strains) isolated from blood from neutropenic patients, have also been found in England (McWhinney et al., 1993). The incidence of penicillin resistance amongst blood isolates from London, Spain and South Africa therefore seem to be very similar. Cefotaxime and ceftriaxone were found to be more active against the strains investigated than penicillin, similar to results reported from Spain. Imipenem showed excellent activity against the South African, Spanish and English viridans streptococci.

The viridans streptococci from this study showed similar tetracycline and erythromycin resistance patterns to those of streptococcal isolates investigated elsewhere (Winstanley, Wilcox & Spencer, 1991). Resistance to tetracycline alone was also reported to be more common than

erythromycin resistance alone or the combination of tetracycline and erythromycin resistance (Winstanley, Wilcox & Spencer, 1991). This situation should be monitored as co-transfer of these resistance markers on transposons has been documented in streptococci (Horaud, Le Bouguenec & Pepper, 1985) and resistance may therefore increase in due course. However, tetracycline resistance alone, in blood isolates of *S. pneumoniae* isolated in South Africa during the period 1983-1986, was found to occur at the same frequency as combined tetracycline-erythromycin resistance (1.3%) (Klugman & Koornhof, 1988). Tetracycline resistance is of less importance in viridans streptococci as bacteriostatic tetracycline is not used for the treatment of infective endocarditis.

Viridans streptococci resistant to erythromycin should be viewed with concern as such bacteria have been implicated in the failure of prophylaxis against endocarditis (Eng, Wolff & Smith, 1982). Longman et al. (1991) investigated the incidence of erythromycin resistance in 65 patients at risk from infective endocarditis. They reported that 29% of the isolates obtained from patients receiving antibiotic prophylaxis were resistant to erythromycin (MIC >3.5 μg/ml), compared with 22% resistance in a control group obtained from patients not at risk from infective endocarditis. In the present study, overall 17.5% of the isolates were found to be resistant to erythromycin at MICs ≥ 3.5 μg/ml. Whether these were isolated from patients receiving erythromycin prophylaxis or not, is not known.

In the present study strain 160 had a streptomycin MIC of >32000, which is indicative of a ribosomal mutation (Eliopoulos et al., 1984).

In addition to the report of a high-level gentamicin-resistant viridans streptococcus isolated from the oropharynx (Van den Berg, 1990), two

further high-level gentamicin-resistant (MIC 1000 μg/ml) *S. mitis* isolates from blood cultures have now been identified and 3 *S. mitis* isolates were shown to exhibit gentamicin MICs of 64 & 128 μg/ml (Potgieter et al., 1992).

The time-kill method, because of its dynamic representation of antimicrobial action and interaction with time is considered to be the best method for determining synergy (NCCLS, 1992; Eliopoulos & Moellering, 1991). Time-kill synergy studies were performed in accordance with NCCLS (1992) and Eliopoulos & Moellering (1991) specifications. For each strain antibiotic concentrations are independently selected in order to conform to time-kill synergy requirements. In one direction: aminoglycoside alone inactive (little to no growth difference when compared with the control culture) plus penicillin at a bacteriostatic concentration (holds the culture close to the original number of viable cells without causing a pronounced bactericidal effect) (Eliopoulos & Moellering, 1991). For each experiment antibiotic concentrations are therefore standardized according to their effect on the culture, being either inactive or bacteriostatic, from which on combining the two antibiotics bactericidal synergy ($\geq$ 2-$\log_{10}$ decrease in cfu/ml between the combination and the most active constituent) may then be assessed. Synergy between gentamicin and penicillin was not demonstrated with the susceptible *S. mitis* isolates. Synergy using penicillin and the aminoglycoside gentamicin does not appear to be as evident as previously documented (Watakunakorn & Glotzbecker, 1977; Levin et al., 1982; Giout, Corel & Vossen, 1993). A. van den Berg & L.J. Chalkley (personal communication) also using recent South African *S. mitis* isolates found only 2 of 6 strains (3 susceptible, 2 penicillin resistant only and one gentamicin resistant only)

showed penicillin/gentamicin synergy using the checkerboard technique. When these 2 strains were further tested for synergy using the time-kill method neither strain showed synergy. This is not unusual as discrepancies on comparing results obtained from checkerboard and time-kill methods has been known for years (Moellering, 1979). Although synergy was not demonstrated with the susceptible isolates as synergistic combinations have been found to be highly variable from strain to strain (Heineman & Lofton, 1978; Miller et al., 1986) it was still necessary to demonstrate lack of synergy with the resistant isolates. Synergy between penicillin and gentamicin was not demonstrated with the high-level gentamicin- and penicillin-resistant strain 56. Of importance, two strains that were resistant to only 64 μg/ml (strain 192) and 128 μg/ml (strain 256) gentamicin failed to show synergy with penicillin. Strain 192 which although resistant to gentamicin at MIC 64 μg/ml, was susceptible to penicillin also failed to show penicillin/gentamicin synergy. In addition synergy studies on penicillin resistant, gentamicin susceptible *S. mitis* and *S. sanguis* strains, also showed loss of *in vitro* synergism with gentamicin (Guiot, Corel & Vossen, 1993). Resistance to either penicillin or gentamicin can therefore result in loss of synergy in viridans streptococci (Potgieter et al., 1992; Guiot, Corel & Vossen, 1993). The findings of viridans streptococci which based on laboratory findings may no longer respond to combined penicillin-gentamicin therapy are of great concern.

Resistance to gentamicin in the enterococci is due to the bifunctional aminoglycoside-modifying 6-acetyltransferase-2"-phosphotransferase (6AAC-2"-APH) enzyme (Eliopoulos et al., 1988; Ferretti, Gilmore & Courvalin, 1986 and Ubukata et al., 1984). The gene encoding for high-level gentamicin resistance has been shown to reside on transposons

(Hodel-Christian & Murray, 1991) the chromosome (Rice et al., 1991) and on conjugative as well as nonconjugative plasmids (Patterson & Zervos, 1990) in the enterococci.

The four *S. mitis* isolates (strains 56, 160, 256 & 265) with gentamicin MICs of 128 and 1000 μg/ml were investigated in a collaborative study with A. Kaufhold of Germany using oligonucleotide probes which correspond to part of the gene encoding the 6'-acylating activity of the 6AAC-2"-APH enzyme (AAC probe, Kaufhold et al., 1992). The gentamicin-resistant genes in these strains were found to be chromosomally mediated and in two strains the gene was located on identical restriction fragments of genomic DNA (Kaufhold & Potgieter, 1993). It has been shown that the gene encoding for the production of the bifunctional aminoglycoside-modifying 6'AAC-2"-APH enzyme of enterococci and a group B streptococcal strain are similar (Kaufhold et al., 1992) and this gene has now appeared in *S. mitis*. It is thought at this stage that the genes in the *S. mitis* strains might also reside on a transposon (Kaufhold & Potgieter, 1993), as has been found in other streptococcal species (Hodel-Christian & Murray, 1991) in which case this resistance trait would have the potential of further spread amongst the viridans streptococci.

The emergence of high-level gentamicin resistance in addition to the high incidence of penicillin resistance in viridans streptococci isolated from blood in South Africa is of concern. This retrospective study suggests that on initiating combined penicillin and gentamicin therapy for the treatment of viridans streptococci, both penicillin and gentamicin MICs should be determined, synergy tests be performed and clinical outcome monitored.

CHAPTER 4

RECIPROCAL TRANSFER OF PENICILLIN RESISTANCE GENES BETWEEN *STREPTOCOCCUS PNEUMONIAE* AND THE VIRIDANS STREPTOCOCCI

4.1 Introduction

S. pneumoniae strains are normally isolated from the nasopharynx of man and very high carrier rates have been reported (Austrian, Howie & Ploussard, 1977). This places pneumococci in an environment with the viridans streptococci which may, as has been shown in South Africa be resistant to penicillin (Van den Berg, 1990; Potgieter et al., 1992). Reciprocal transfer of streptomycin resistance between streptococcal species was reported as early as 1957 (Bracco et al., 1957). With the emergence of increasing numbers of penicillin-resistant streptococci, investigations were initiated to determine whether interchange of penicillin resistance determinants could also occur (Chalkley & Koornhof, 1990).

Penicillin resistance in *S. pneumoniae* is due to the presence of altered forms of penicillin-binding proteins with decreased affinity for penicillin. (Percheson & Bryan, 1980; Hakenbeck, Tarpay & Tomasz, 1980; Zigelboim & Tomasz, 1980.) Altered PBP 2b genes in resistant isolates were reported by Dowson et al. (1989), to be products of mosaic genes in which parts of the "sensitive" sequence had been replaced by homologous fragments ("resistant" gene blocks) from related streptococci (Dowson et al., 1989). Dowson et al. (1990) was unable to identify a donor for the resistant blocks in *S. pneumoniae* and proposed that *S. sanguis* and *S. oralis* obtained resistant blocks from *S. pneumoniae*. However, in the same year transfer of penicillin resistance determinants from viridans streptococci to *S. pneumoniae* was reported (Chalkley & Koornhof, 1990). This study only investigated transfer of penicillin resistance from viridans streptococci to *S. pneumoniae* and of 9 strains studied only 2 were shown to transform the *S pneumoniae* recipient to increased penicillin resistance.

The present study was undertaken to investigate reciprocal transfer between *S. pneumoniae, S. mitis* and *S. sanguis* in order to determine the possible extent of penicillin resistance transfer between different streptococcal species.

Table 4.1. Penicillin MICs of clinical isolates of viridans streptococci.

Blood isolates			Nasopharyngeal isolates		
Strain No.	Facklam[a] 1991	MIC (μg/ml)	Strain No.	Facklam[a] 1991	MIC (μg/ml)
E2	*S. mitis*	2.0	I13	*S. mitis*	8.0
E3	*S. mitis*	0.25	I16	*S. mitis*	0.25
E4	*S. mitis*	0.25	I31	*S. mitis*	2.0
E7	*S. mitis*	1.0	I33	*S. mitis*	16.0
E9	*S. mitis*	8.0	I39	*S. mitis*	1.0
E10	*S. mitis*	4.0	I21	*S. mitis*	4.0
E11	*S. mitis*	16.0	I28	*S. mitis*	0.5
E12	*S. mitis*	4.0	I29	*S. mitis*	2.0
E14	*S. mitis*	4.0	I32	*S. mitis*	8.0
E6	*S. sanguis*	1.0	I10	*S. sanguis*	0.5
E13	*S. sanguis*	4.0	I3	*S. sanguis*	16.0
E15	*S. sanguis*	1.0	I40	*S. sanguis*	0.5
E16	*S. sanguis*	1.0	I41	*S. sanguis*	16.0
E17	*S. sanguis*	1.0	I7	*S. sanguis*	1.0
E18	*S. sanguis*	0.5			

[a] Short identification scheme, five major group differentiation (Facklam & Washington, 1991).

Table 4.2. Penicillin MICs of clinical isolates of *S. pneumoniae*.

Strain No.	MIC (μg/ml)	Source
CR101	0.25	B/CSF
31651	4.0	B/CSF
43973	2.0	B/CSF
P1	0.5	B
P2	2.0	B
P3	4.0	B
P4	0.5	CSF
P5	2.0	CSF
P6	2.0	CSF
P7	4.0	CSF
P8	0.5	N
P9	2.0	N
P10	4.0	N

NOTE:

B = Blood isolate.

CSF = Cerebrospinal fluid isolate.

N = Nasopharyngeal isolate.

4.2 Materials and Methods

4.2.1 *Strains.*

The following penicillin susceptible laboratory strains were used as recipients: *S. pneumoniae* R6 (MIC 0.006 μg/ml), *S. sanguis* Challis strain NCTC 7868 (MIC 0.015 μg/ml), *S. sanguis* NCTC 7865 (MIC 0.015 μg/ml) and *S. mitis* NCTC 10712 (MIC 0.03 μg/ml). These recipients were spontaneously mutated to streptomycin resistance and were used as donors to monitor (a) the competence state of the parent strains and (b) transformation frequency using a high efficiency marker. The streptomycin MICs of both the recipients and the mutants are shown in the results section 4.3, Table 4.3. Penicillin-resistant donors were clinical isolates comprising 13 *S. pneumoniae* strains, 18 *S. mitis* and 10 *S. sanguis* strains isolated from blood culture, CSF and nasopharyngeal specimens between 1987-1991. Their penicillin MICs ranged from 0.25-16 μg/ml as shown in Table 4.1 and 4.2.

4.2.2 *Identification of the clinical isolates.*

The *S. pneumoniae* isolates were identified using optochin discs and were serogrouped using group sera obtained from Statens Serum Institute, Copenhagen, Denmark. The *S. mitis* and *S. sanguis* isolates were identified as described in Chapter 2 according to Facklam & Washington, (1991). Initially physiological properties of these strains were determined by the API-20 STREP system (API System S.A. France) with strains being classified according to French et al (1989). Therefore, in the publication of Potgieter & Chalkley (1991) the strains were originally classified as

S. mitior, S. sanguis I and *S. sanguis* II. For a detailed explanation concerning the problems and reclassification of the viridans streptococci in 1993 refer to Chapter 2.

4.2.3 *Transformation.*

Preparation of donor lysates.

Crude DNA preparations of *S. pneumoniae* strains were prepared as described by Saunders & Guild (1980). Cultures were grown to a density of $1x10^8$ cfu/ml in Todd Hewitt broth (Oxoid) containing 1% (w/v) yeast extract. Cells were harvested from 10 ml of culture by centrifugation at 3500 g for 10 min. The cells were resuspended in 0.2 ml lysis buffer [0.1% (w/v) sodium deoxycholate, 0.01% (w/v) sodium dodecyl sulfate (SDS), 0.15 M sodium citrate] and incubated for 5 min at 37°C. To the cell lysate 1.8 ml SSC [0.15 M NaCl, 0.015 M sodium citrate] was added. For *S. mitis* and *S. sanguis* strains lysis was performed according to Chalkley & Koornhof (1990), however, culture lysis at 37°C was extended to 40 min and the 65°C incubation period was increased to 50 min. Half of the DNA preparation was tested for sterility and the remainder stored at -70°C. The same DNA preparation from each donor strain was used throughout the transformation experiments.

Transformation of the S. pneumoniae R6 recipient.

Transformation was performed as previously described (Chalkley & Koornhof, 1988). To 1 ml of a competent *S. pneumoniae* R6 culture 100 μl of the crude DNA preparation was added. The culture was incubated for 30 min at 30°C after which time 3 ml Todd Hewitt broth containing 1% (w/v) yeast extract was added. Incubation was continued for a further 2 h at 37°C. Having allowed for phenotypic expression, neat samples and culture

dilutions were incorporated into 5% (v/v) blood agar plates containing various concentrations of penicillin or streptomycin. *S. pneumoniae* transformants were scored after 48 h incubation at 37°C. The transformation frequency was calculated as the number of transformants divided by the number of viable cells at the time culture samples were incorporated into the selection media and expressed as a percentage. Transformation frequencies were calculated for those concentrations of penicillin or streptomycin on which no spontaneous mutants were detected (detection limit 10^{-8}). Using the *S. pneumoniae* recipient, four individual experiments were performed with each donor which was shown to transform the recipient to increased penicillin resistance. Only three donor *S. pneumoniae* clinical isolates were investigated for intraspecific transfer as this is well documented (Chalkley & Koornhof 1988, Handwerger & Tomasz 1986 and Zigelboim and Tomasz 1980).

Transformation of the S. sanguis and S. mitis recipients.

Transformation was performed as described by Potgieter & Chalkley (1991). An overnight culture grown in Todd Hewitt broth (Oxoid) supplemented with 1% (w/v) yeast extract (Oxoid) was diluted with fresh medium to approximately $5x10^6$ cfu/ml. After 2 h incubation at 37°C for the *S. sanguis* and *S. mitis* strains and after 3 h for the *S. sanguis* Challis strain, DNA (100 μl) was added to 1 ml of each culture and the mixture incubated for a further 2.5 h. Transformants were scored after 48 h incubation at 37°C in candle jars. Two independent transformation experiments were performed with each donor strain shown to transform a recipient to increased penicillin resistance.

Table 4.3. Intra- and inter-specific transfer of streptomycin resistance.

RECIPIENTS		DONORS			
		Streptomycin transformation frequencies[a]			
		S. pneumoniae (>1 000)[b]	*S. sanguis* Challis (>1 000)	*S. sanguis* (>1 000)	*S. mitis* (500)
S. pneumoniae	(≤ 50)[b]	$3x10^{-2}$	$7x10^{-6}$	$7x10^{-6}$	0
S. sanguis Challis	(≤ 50)	$6x10^{-1}$	3	2	$8x10^{-7}$
S. sanguis	(≤ 50)	$7x10^{-1}$	5	4	$3x10^{-5}$
S. mitis	(≤ 50)	$4x10^{-1}$	$2x10^{-1}$	$3x10^{-2}$	$2x10^{-1}$

[a] Transformation frequencies are expressed as transfer frequency % at the time of scoring.

Concentrations of streptomycin used for selection were 100 μg/ml *S. pneumoniae* and *S. mitis* and 250 μg/ml *S. sanguis* Challis strain and *S. sanguis*.

[b] Recipient and donor streptomycin MICs (μg/ml).

Table 4.4. Penicillin transformation frequencies of recipients *S. pneumoniae* (MIC 0.006 μg/ml) and *S. mitis* (MIC 0.03 μg/ml).[a]

Donor DNA	No. strains	Concentration of penicillin used for selection (μg/ml)				
		0.025	0.05	0.1	0.2	0.4
RECIPIENT *S. pneumoniae*						
S. pneumoniae	3	$4x10^{-1}$-$2x10^{-1}$[b] (3)[c]	$2x10^{-2}$-$8x10^{-3}$ (3)	$2x10^{-3}$-$8x10^{-4}$ (3)	$2x10^{-4}$ (2)	$3x10^{-5}$-$2x10^{-5}$ (2)
S. mitis	18	$3x10^{-1}$-$9x10^{-5}$ (16)	$5x10^{-3}$-$2x10^{-6}$ (15)	$8x10^{-5}$-$2x10^{-6}$ (6)	$4x10^{-6}$ (1)	0
S. sanguis	10	$4x10^{-6}$ (1)	0			
RECIPIENT *S. mitis*						
S. pneumoniae	13			$3x10^{-3}$-$3x10^{-5}$ (13)	$6x10^{-5}$-$4x10^{-6}$ (10)	$4x10^{-6}$ (1)
S. mitis	18			$3x10^{-2}$-$8x10^{-5}$ (11)	$3x10^{-2}$-$8x10^{-5}$ (7)	$2x10^{-5}$-$4x10^{-6}$ (3)
S. sanguis	10			0		

[a] Transformation frequencies are expressed as transfer frequency (%) at the time of scoring.

[b] Transformation frequency range achieved using different donors of each species.

[c] Number in parenthesis donors producing transformants at each concentration of penicillin used for selection.

Table 4.5. Penicillin transformation frequencies of recipients; *S. sanguis* Challis strain and *S. sanguis* (both MICs 0.015 μg/ml)[a].

Donor DNA		Concentration of penicillin used for selection (μg/ml)		
		0.05	0.1	0.2
RECIPIENT *S.sanguis* Challis strain.				
S. pneumoniae	13	$1x10^{-6}$-$3x10^{-7}$[b] [6][c]	$3x10^{-7}$-$2x10^{-7}$ [3]	0
S. mitis	18	$8x10^{-6}$-$3x10^{-7}$ [2]	$6x10^{-6}$ [1]	0
S. sanguis	10	$6x10^{-7}$-$4x10^{-7}$ [4]	$3x10^{-7}$ [1]	0
RECIPIENT *S. sanguis*				
S. pneumoniae	13	$6x10^{-6}$-$5x10^{-7}$ [6]	0	
S. mitis	18	$5x10^{-6}$-$3x10^{-6}$ [2]	0	
S. sanguis	10	$3x10^{-4}$-$1x10^{-5}$ [3]	$6x10^{-5}$[1]	

[a] Transformation frequencies are expressed as transfer frequency (%) at the time of scoring.

[b] Transformation frequency range achieved using different donors of each species.

[c] Number in parenthesis donors producing transformants at each concentration of penicillin used for selection.

4.3 Results

All recipients were transformed to streptomycin resistance using both homologous and heterologous DNA (Table 4.3). Although the competence state of the *S. pneumoniae* recipient was not ideal, it is noticeable that no transformants were detected using *S. mitis* donor DNA. A second competent culture of *S. pneumoniae* with a streptomycin transformation frequency of 3% (using homologous DNA) was prepared for studying the transfer of penicillin resistance.

Intra- and inter-specific transfer of penicillin resistance to the four recipients is shown in Table 4.4 and Table 4.5. Of the 18 *S. mitis* / *S. oralis* donors, 16 transformed the *S. pneumoniae* recipient to a penicillin concentration of 0.025 μg/ml. The transformation frequency range (%) was $3x10^{-1}$-$9x10.^{-5}$ Due to the higher penicillin MIC (0.03 μg/ml) of the *S. mitis* recipient the lowest concentration of penicillin that could be used for selection was 0.1 μg/ml. Fewer *S. mitis* donors were seen to transform the *S. mitis* recipient than the *S. pneumoniae* recipient. Three of the *S. mitis* donors had MICs of 0.25 μg/ml (growth on 0.125 μg/ml) which is close to the penicillin concentration used for the first selection level (0.1 μg/ml). However, lack of transfer was not due to the inability of the transformants to grow at that concentration as two of the three donors successfully transferred penicillin resistance.

Recently, with the publication of sequencing data concerning the origins of penicillin resistance determinants (Dowson et al., 1993), differentiation of the two species (*S. mitis* and *S. oralis*) within the *S. mitis* group (Kilian, Mikkelsen & Henrichsen, 1989a), has become an important issue. Table 4.6 lists the *S. mitis* strains used in the transformation study re-identified as

S. mitis and *S. oralis* according to Kilian, Mikkelsen & Henrichsen, (1989a). Of the 11 *S. mitis* isolates that transformed the *S. mitis* recipient, 8 were re-identified as *S. mitis* strains and 3 were found to be *S. oralis* strains.

Table 4.6. Transformation of laboratory strains of *S. mitis*, *S. pneumoniae* and *S. sanguis* using donor DNA of *S. mitis* isolates re-identified according to Kilian, Mikkelsen & Henrichsen, (1989a).

Blood culture isolates				Nasopharyngeal isolates			
Strain No.	Facklam[a] 1991	Kilian[b] 1989	Transformation m/p/s	Strain No.	Facklam[a] 1991	Kilian[b] 1989	Transformation m/p/s
E2	*S. mitis*	*S. oralis*	-/-/s	I13	*S. mitis*	*S. oralis*	m/p/-
E3	*S. mitis*	*S. mitis*	m/p/-	I16	*S. mitis*	*S. mitis*	m/p/-
E4	*S. mitis*	*S. mitis*	-/p/-	I31	*S. mitis*	*S. mitis*	m/p/-
E7	*S. mitis*	*S. mitis*	-/-/s	I33	*S. mitis*	*S. mitis*	m/p/-
E9	*S. mitis*	*S. oralis*	m/p/-	I39	*S. mitis*	*S. mitis*	m/p/s
E10	*S. mitis*	*S. oralis*	-/p/-	I21	*S. mitis*	*S. oralis*	-/p/s
E11	*S. mitis*	*S. mitis*	m/p/-	I28	*S. mitis*	*S. mitis*	-/p/-
E12	*S. mitis*	*S. oralis*	m/p/-	I29	*S. mitis*	*S. mitis*	m/p/-
E14	*S. mitis*	*S. mitis*	m/p/-	I32	*S. mitis*	*S. oralis*	-/p/-

[a] Short identification scheme, five major group differentiation (Facklam & Washington, 1991)

[b] Differentiation within *S. mitis* group (Kilian, Mikkelsen & Henrichsen, 1989a).

m/p/s = Donor DNA transformed *S. mitis* / *S. pneumoniae* / *S. sanguis* strain.

4.4 Discussion

Interchange of penicillin-resistant determinants was seen between the majority of *S. pneumoniae*, *S. mitis* and *S. oralis* strains. However, only one *S. sanguis* donor exhibited interspecific transfer of penicillin resistance to the *S. pneumoniae* recipient at a low frequency ($4x10^{-6}$). Penicillin transformation frequencies using the *S. sanguis* recipients were low even when using homologous DNA from recent clinical isolates of *S. sanguis*. There is considerable heterogeneity within the viridans streptococcal groups which may account for low transformation frequencies or reduced numbers of donors transforming a recipient classified as the same species. There was no correlation between the MIC of the donor, the isolation site and ability to transform recipients. The transfer of penicillin-resistance genes from recent clinical isolates to "early" laboratory recipients clearly indicates that considerable gene transfer can occur between *S. pneumoniae, S. mitis* (Potgieter & Chalkley, 1991) and *S. oralis* (Table 4.6).

In a collaborative study with L.J. Chalkley and R. Hakenbeck at the Max Planck Institute, Germany, antiserum as well as monoclonal antibodies (MAb) raised against PBPs 1a and 2b of the susceptible *S. pneumoniae* R6 strain were used to identify related PBPs in the viridans streptococci (Chalkley et al., 1991). Fourteen *S. mitior* & *S. sanguis* II donor strains (MICs ≥ 0.06 μg/ml) shown to transform *S. pneumoniae* and a further 9 susceptible *S. mitior* and *S. sanguis* II strains, contained PBPs which crossreacted with one or both of the MAbs employed. In contrast, only 4 of the 14 *S. sanguis* strains contained a PBP that reacted with the antiserum, and only one crossreacted very weakly with a MAb 2b.

Dowson et al. (1990) used oligonucleotide probes specific for penicillin-susceptible and -resistant *S. pneumoniae* PBP 2b sequences in

order to determine from which species divergent resistant gene blocks could have originated. These mosaic genes have been described for *S. pneumoniae* PBP 2b (Dowson et al, 1989) and subsequently for PBPs 2x and 1a (Laible, Spratt & Hakenbeck, 1991; Martin, Sibold & Hakenbeck, 1992), and have most probably developed as result of transformation followed by recombinational events. Dowson et al. (1990) argued that the resistant gene blocks may have been introduced from *S. pneumoniae* into *S. sanguis* and *S. oralis,* because their probes hybridised with the resistant but not with the susceptible viridans strains. Although identical epitopes, ie. identical amino acid sequences in different proteins may not necessarily correlate with identical DNA sequences, results from the monoclonal antibody study imply that PBP genes related to that of *S. pneumoniae* PBP 1a and 2b are present in susceptible viridans streptococci (Chalkley et al., 1991).

A close genetic relationship between *S. pneumoniae* and *S. sanguis* II (now classified as *S. mitis* or *S. oralis*) but not *S. sanguis* I has been documented (Kippler-Balz, Wenzig & Schleifer, 1985). *S. pneumoniae* and *S. mitis* / S. *oralis* both contain choline and ribitol in their cell walls, but these components are not present in *S. sanguis* cell walls. Correlating with this are the results from the present study; sensitive pneumococci could be transformed to penicillin resistance by using DNA from resistant *S. mitis* or *S. oralis* isolates but only one *S. sanguis* strain was able to transform the *S. pneumoniae* recipient at a very low frequency (Potgieter & Chalkley, 1991). Also whereas most of the *S. mitis* isolates contained PBPs which reacted with the MAbs raised against that of a susceptible *S. pneumoniae* strain only one *S. sanguis* isolate reacted and then only very weakly (Chalkley et al., 1991).

The high frequency with which penicillin resistance determinants have been shown to transfer between *S. mitis* / *S. oralis*, oropharyngeal commensals, and *S. pneumoniae* has important implications for the in-vivo transfer of penicillin resistance. Results clearly show that transfer of genes does not only occur in one direction, but also vice versa, including several heterogeneous species at several different levels of penicillin resistance, which can only complicate the search for the origins and subsequent transfer of penicillin resistance genes (Potgieter & Chalkley, 1991).

Very recently, Dowson et al., (1993) and Sibold et al., (1994) have shown that PBP 2b genes of susceptible *S. mitis* and PBP 2x genes of susceptible *S. oralis* isolates respectively, are similar to those genes present in resistant *S. pneumoniae* strains, which indicates that transfer occurs from *S. mitis* and *S. oralis* to *S. pneumoniae*. These findings will be discussed in Chapter 6.

CHAPTER 5

PENICILLIN-BINDING PROTEINS IN *STREPTOCOCCUS MITIS* AND *STREPTOCOCCUS ORALIS*

5.1 Introduction

Penicillin resistance in streptococci has been associated with alterations in the binding affinity of one or more penicillin-binding proteins (PBPs) (Faber et al., 1983; Zigelboim & Tomasz, 1981; Zito & Daneo-Moore, 1988). The development of intrinsic penicillin resistance has been studied in *Streptococcus pneumoniae, S. sanguis* and *S. mutans* (Massida & Daneo-Moore, 1988; Zighelboim & Tomasz, 1981; Zito & Daneo-Moore, 1988). PBP 4 (72 kDa) of *S. sanguis*, PBP 3 (76 kDa) of *S. mutans* and PBP 2b (77 kDa) of *S. pneumoniae* were shown to be the major PBPs associated with penicillin resistance (Hakenbeck et al., 1986; Massida & Daneo-Moore, 1988; Zito & Daneo-Moore, 1988). The PBP profiles of the oropharyngeal *S. mitis / S. oralis* isolates with MICs ranging from ≤ 0.015-32 μg/ml were investigated by L.J. Chalkley & I. Van den Berg (personal communication). Sequential PBP alterations with increasing resistance to penicillin were expected but no such patterns were apparent from the clinical isolates examined (Fig. 5.1, courtesy of Chalkley & Van den Berg). The extent of variation in *S. mitis / S. oralis* PBPs appears to be far greater than in *S. pneumoniae*, although the PBP profiles of *S. pneumoniae* have been shown to be more complex than initially described (Hakenbeck & Kohiyama, 1982).

The role of these proteins and their similar molecular masses prompted futher investigations into their genetic relationship. Dowson et al. (1989) suggested that penicillin resistance in *S. pneumoniae* may have been acquired by horizontal transfer of altered PBP 2b genes from closely related species; although a subsequent study by Dowson et al. (1990) failed to identify any donors. In a study by Chalkley & Koornhof (1990), however, *S. mitior* (*S. mitis / S. oralis*) strains were shown to transfer penicillin

resistance determinants to *S. pneumoniae* and later, reciprocal transfer of resistance between *S. mitior* (*S. mitis* / *S. oralis*) and *S. pneumoniae* was also shown to occur (Potgieter & Chalkley, 1991). Very recently new studies on the origins of penicillin resistance in the viridans streptococci and *S. pneumoniae* have been completed (Dowson et al., 1993; Sibold et al., 1994), and results from these studies will be fully discussed in Chapter 6.

Studies on the development of penicillin resistance in *S. mitis* and *S. oralis* are still limited and in view of increasing prevalence of penicillin resistance in clinical isolates, this study was undertaken to investigate PBP alterations in *S. mitis* which may occur as a result of mutation and intra- and inter-specific gene transfer. In the search for the origins of penicillin resistance in *S. pneumoniae*, which involves alterations to PBPs 1a, 2x and 2b it is important to study reciprocal transfer of penicillin resistance determinants between *S. mitis* and *S. pneumoniae*. Transfer of penicillin resistance from a spontaneous mutant of a *S. mitis* strain isolated in 1968, to *S. pneumoniae* was therefore also investigated.

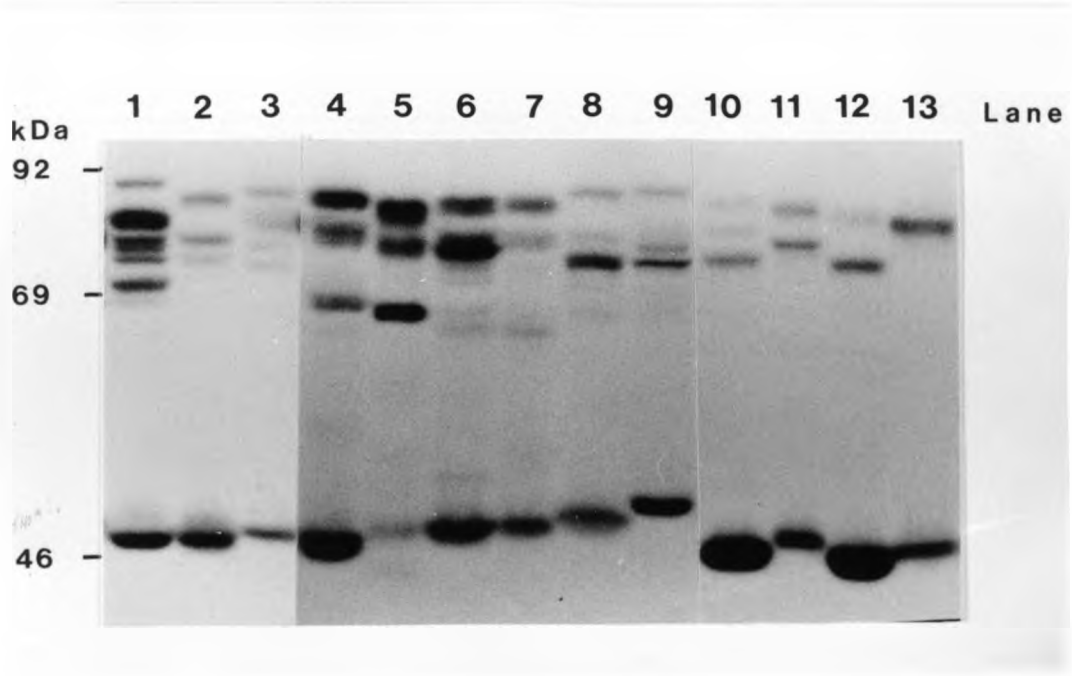

Figure 5.1. PBP profiles of clinical strains of *S. mitis* (*S. mitis* / *S. oralis*). MICs are given in μg/ml. Lanes: 1, ≤ 0.015; 2, 8.0; 3, 4.0; 4, 0.03; 5, 0.06; 6, 0.125; 7, 0.5; 8, 0.5; 9, 0.5; 10, 2.0; 11, 8.0; 13, 32.0. 5 μCi of [^{3}H]benzylpenicillin was added to each strain.

5.2 Materials and Methods

5.2.1 *Strains.*

Penicillin-susceptible and -resistant clinical strains of *S. mitis* isolated from oropharnygeal specimens were kindly provided by I. van den Berg, South African Institute for Medical Research, Johannesburg. Two penicillin-susceptible strains were used as recipients in transformation studies, *S.mitis* NCTC 10712 (MIC 0.03 μg/ml) and *S. pneumoniae* R6 (MIC 0.006 μg/ml). Four penicillin-resistant clinical isolates were used as donors in transformation experiments: *S. oralis* E9 (MIC 4.0 μg/ml), *S. mitis* E14 (MIC 8.0 μg/ml) and *S. pneumoniae* 118 (MIC 4.0 μg/ml) which were isolated from blood cultures and *S. pneumoniae* 53 (MIC 4.0 μg/ml) which was isolated from an oropharyngeal swab. The two donor viridans streptococci were identified according to Kilian, Mikkelsen & Henrichsen (1989a). The *S. oralis* isolate E9 and the *S. mitis* isolate E14 were referred to as *S. mitis* 60094 and *S. mitis* 43483 respectively in the publication by Potgieter et al. (1992). *S. mitis* NCTC 10712 was spontaneously mutated to low level penicillin resistance (MIC 0.06 μg/ml). The spontaneous mutant *S. mitis* SM1 was selected from a blood agar plate containing penicillin at a concentration of 0.05 μg/ml after 48 h incubation in a candle jar at 37^{o}C. *S. mitis* SM1 was used to transform *S. pneumoniae* R6.

The phenotypic profile of the *S. mitis* SM1 strain based on specific physiological reactions were identical to those of the original *S. mitis* NCTC 10712 isolate.

5.2.2 *MIC determination*

Antimicrobial activity was determined by the agar dilution method (NCCLS, 1987) using Mueller-Hinton agar (Oxoid) supplemented with 5% (v/v) horse blood, as described in Chapter 3.2.2. Plates were incubated aerobically at 35°C for 18 h. The NCCLS penicillin-susceptible breakpoint concentration was used to define susceptibility (NCCLS, 1987); all strains with penicillin MICs ≥ 0.25 μg/ml were considered resistant. Penicillin resistance was defined as MICs ≥ 0.25 μg/ml.

5.2.3 *Transformation.*

Crude DNA preparations of *S. pneumoniae* and *S. mitis* strains used as donors were prepared and transformation studies were perfomed as descibed in Chapter 4.2.3 according to the methods used by Chalkley & Koornhof (1988) for the *S. pneumoniae* R6 recipient and those used by Potgieter & Chalkley (1991) for the *S. mitis* NCTC 10712 recipient.

5.2.4 *PBP labeling of whole cells.*

Radiolabeling of PBPs was performed according to Laible & Hakenbeck (1987) on whole cell lysates. Cultures of the recipients *S. pneumoniae* R6 and *S. mitis* NCTC 10712 and derived transformants were grown to the same turbidity for direct comparison of PBP affinity alterations. Cell membrane preparations were not used as complications in analysing the results have been reported (Hakenbeck et al., 1988). Cultures were grown to a density of $3x10^7$ cfu/ml in Todd Hewitt broth containing 1% (w/v) yeast extract. Cells were harvested from 1 ml aliquots of the culture and cell pellets stored at -20°C. For *S. mitis* strains, cells were lysed by resuspending the pellets in 20 μl of lysis buffer (20mM sodium phosphate buffer pH 7.0,

0.2% Triton X-100) containing 7.5 μg Mutanolysin (Sigma). The cell lysates were incubated with 5 μl of appropriately diluted [^{3}H]benzylpenicillin (20.7 and 28 Ci/mmol, Amersham) for 20 min at 37^{o}C. The sample was immediately prepared for SDS-PAGE by addition of 25 μl sample buffer and boiling for 3 min. Electrophoresis and fluorography were carried out as described by Chalkley & Koornhof (1988). The electrophoresis equipment used was a Protean II (BioRad) system with gel spacers 1.5 mm. The gel composition was 4% and 8% (w/v) acrylamide in the stacking and separating gels respectively. Electrophoresis was performed at constant current; initially 25 mA/gel was applied and after 1 h this was increased to 35 mA/gel for 4-4.5 h. The gels were fixed in 7% (v/v) acetic acid overnight and afterwards treated with Amplify (Amersham) for 1 h. They were then dried and exposed to preflashed Hyperfilm-MP (Amersham) for 5-14 days at -70^{o}C. Rainbow^{TM} protein molecular weight markers ^{14}C-labelled were obtained from Amersham.

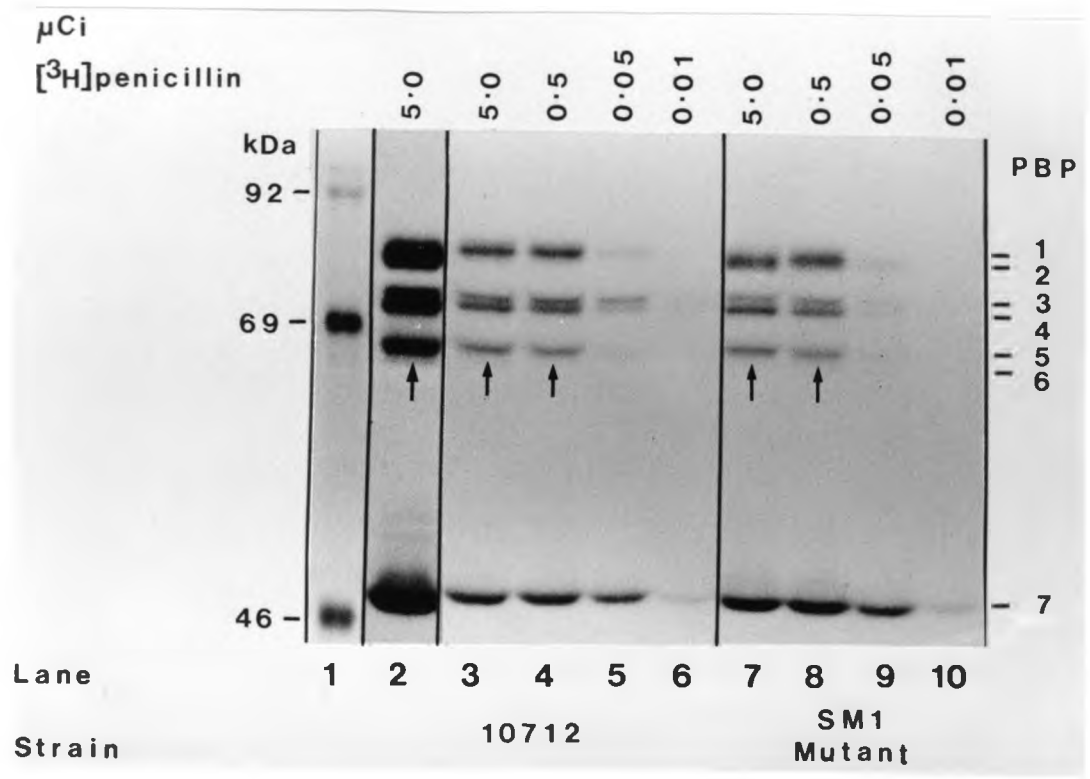

Figure 5.2. PBP profiles of *S. mitis* NCTC 10712 (MIC 0.03 μg/ml) and *S. mitis* SM1 (MIC 0.06 μg/ml). Lanes: 1, molecular mass markers; 2, *S. mitis* NCTC 10712 fluorogram 14 days exposure; 3-6, *S. mitis* NCTC 10712 fluorogram 7 days exposure; 7-10, *S. mitis* SM1 fluorogram 7 days exposure. Dilutions of [^{3}H]benzylpenicillin were added to each strain in lanes 3-10 as indicated.
↑ Denotes weak PBP 6 labelling.

Table 5.1. Frequency of transformation of *S. mitis* NCTC 10712 (MIC 0.03 μg/ml).*

Donor	Concentration of penicillin used for selection (μg/ml)			
DNA	0.1	0.2	0.4	0.8
S. oralis E9	$6.1x10^{-6}$	$3.1x10^{-7}$	$1.7x10^{-7}$	0
S. mitis E14	$4.3x10^{-6}$	$5.9x10^{-7}$	$6.9x10^{-8}$	0
S. pneumoniae 53	$1.0x10^{-5}$	$2.7x10^{-7}$	$3.6x10^{-8}$	0
S. pneumoniae 118	$2.5x10^{-5}$	$4.2x10^{-7}$	0	0

* Transformation frequency was calculated as the number of transformants per number of viable cells at the time of scoring. The results are means of two replicate determinations on seperate occasions.

5.3 Results and Discussion

Since the PBP profiles of the clinical *S. mitis* / *S. oralis* isolates were so variable, the laboratory recipient *S. mitis* NCTC 10712 was used to investigate which PBPs would vary after transfer of penicillin resistance determinants from clinical isolates of *S. mitis, S. oralis* and *S. pneumoniae*.

5.3.1 *PBP profiles of S. mitis NCTC 10712 and spontaneous mutant SM1.*

PBP affinity studies on *S. mitis* NCTC 10712 and a spontaneous mutant derived from this strain (*S. mitis* SM1) are shown in Fig. 5.2. *S. mitis* NCTC 10712 has seven major PBPs with approximate molecular masses of 82, 80, 74, 72, 67, 65 and 49 kDa. PBP 6 (65 kDa) appears to be weak but this was due to the low specific affinity of the [^{3}H]benzylpenicillin used. PBP 6 can be more clearly seen in Fig. 5.3.

Within a narrow range of penicillin concentrations, *S. mitis* SM1 was shown to be three times more resistant than the parent strain (SM1 growing in the presence of 0.05 μg/ml penicillin compared to 0.015 μg/ml penicillin for the parent strain). Comparison of PBP affinity profiles of *S. mitis* NCTC 10712 and *S. mitis* SM1 shows a decrease in penicillin affinity of PBPs 3 and 4 of the mutant strain SM1. PBPs 3 and 4 were the first PBPs to alter their affinity as spontaneous resistance developed in the *S. mitis* NCTC 10712 strain.

5.3.2 *Transformation of S. mitis NCTC 10712.*

At a penicillin selection concentration of 0.2 μg/ml, transformation frequencies of the recipient, using donor DNA from one *S. mitis,* one *S. oralis* and two *S. pneumoniae* strains ranged from 3-6x10^{-7} (Table 5.1). Twenty-four transformants using *S. mitis* / *S. oralis* donor DNA and 24

transformants from experiments using *S. pneumoniae* donor DNA were isolated from the selection plates containing 0.2 μg/ml of penicillin. The MICs of these 48 transformants were determined and found to range from 0.125-4.0 μg/ml. Of the 24 *S. mitis* / *S. oralis* - derived transformants 7 (58%) of the *S. oralis* - derived transformants and 10 (83%) of the *S. mitis* - derived transformants had MICs ≥ 1.0 μg/ml, whereas only 2 (8%) of the 24 transformants obtained with *S. pneumoniae* DNA had MICs ≥ 1.0 μg /ml.

5.3.3 *PBPs of S. mitis transformants derived from S. mitis and S. oralis donors.*

PBP affinity studies of *S. mitis* NCTC 10712 transformants derived from clinical *S. mitis* and *S. oralis* donors are shown in Fig. 5.3. Transformants with MICs ≤ 0.5 μg/ml showed reduced penicillin affinity of PBPs 3 and 5 using *S. oralis* E9 (Fig.5. 3; lanes 4-6) or PBPs 3 and 6 using both *S. oralis* and *S. mitis* as donors (Fig. 5.3; lanes 7-9 and 17-19). Transformants with MICs of ≥ 1.0 μg/ml showed additional reduction in penicillin binding of PBPs 4 and 6 (Fig. 5.3; lanes 13-15) or PBP 2 (Fig. 5.3; lanes 10-12 and 20-22). The two donor strains E9 and E14, had different PBP profiles (Fig. 5.3; lanes 16 and 23). There appeared to be no shift in PBP patterns of the transformants towards those of the corresponding donor.

Transformants of the *S. mitis* NCTC 10712 strain were seen to have similar PBP profiles using either *S. mitis* or *S. oralis* donor DNA (Fig. 5.3; lanes 7-9 and 17-19). Contrasting to this, one donor (*S. oralis* E9) produced transformants which had the same MIC but different PBP affinities (Fig. 5.3; lanes 10-12 and 13-15). This indicates that resistance can develop by alterations in different PBPs. A consistent change in the affinity of PBP 3 (74 kDa) was evident in all transformants. Other PBPs that were found to alter with increasing penicillin resistance were PBPs 2, 4, 5 and 6. There was no apparent alteration in PBP 7 (49 kDa) which appears to be similar

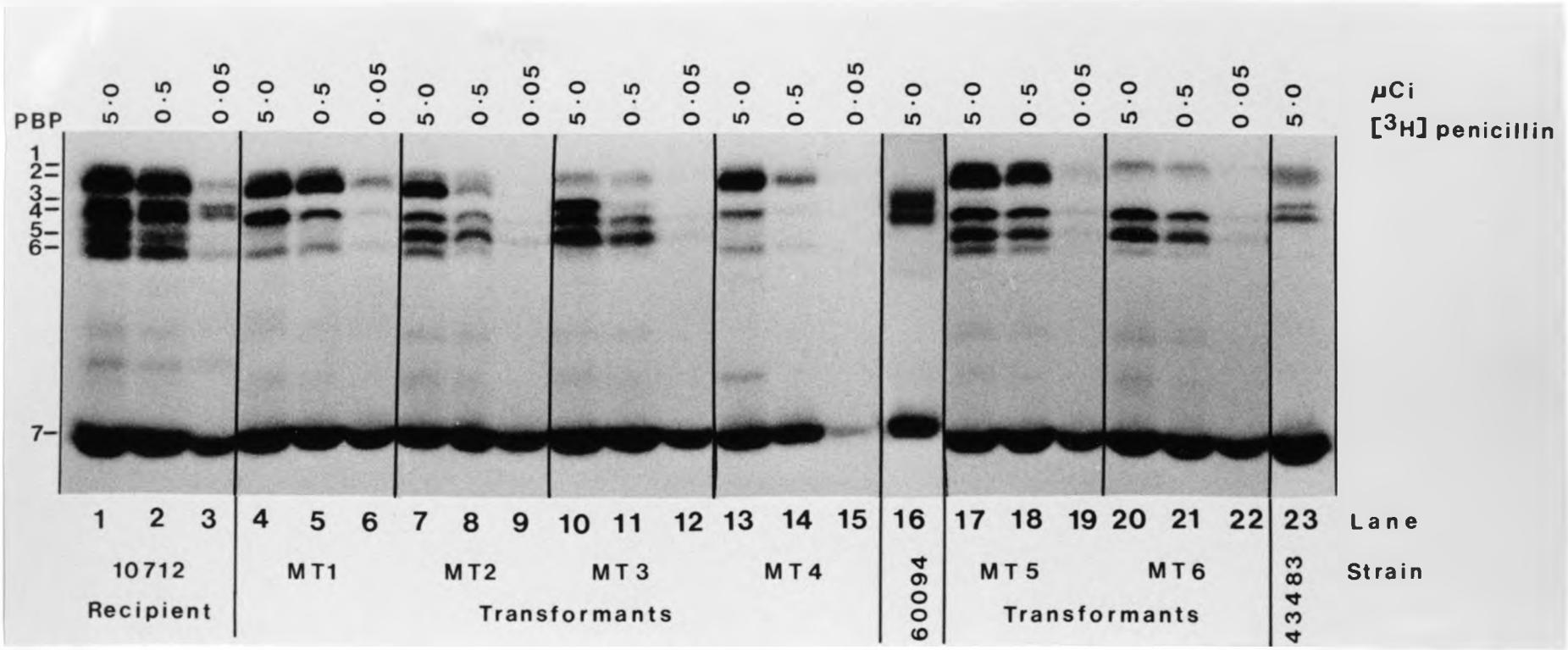

Figure 5.3. PBP profiles of *S. mitis* NCTC 10712 and transformants derived from *S. mitis* donors. MICs in µg/ml are given in brackets. Lanes; 1-3, recipient *S. mitis* NCTC 10712 (0.03); 4-6, transformant MT1 donor DNA *S. mitis* 60094 (0.25); 7-9, transformant MT2 donor DNA *S. mitis* 60094 (0.5); 10-12, transformant MT3 donor DNA *S. mitis* 60094 (1.0); 13-15, transformant MT4 donor DNA *S. mitis* 60094 (1.0); 16, *S. mitis* 60094 (4.0); 17-19, transformant MT5 donor DNA *S. mitis* 43483 (0.25); 20-22, transformant MT6 donor DNA *S. mitis* 43483 (1.0); 23, *S. mitis* 43483 (8.0). Dilutions of [^{3}H]benzylpenicillin were added to each strain as indicated. The PBP numbers assigned do not apply to the *S. mitis* donor strains. *S. mitis* 60094 and *S. mitis* 43483 was recently reclassified according to Kilian, Mikkelsen & Henrichsen (1989a) as *S. oralis* (E9) and *S. mitis* (E14) respectively (See Chapter 2).

to PBP 3 (43 kDa) of *S. pneumoniae*. Carboxypeptidase function of PBP 7 has not yet been confirmed (Hakenbeck & Kohiyama, 1982).

5.3.4 *PBPs of S. mitis transformants derived from S. pneumoniae donors*.

Two clinical *S. pneumoniae* donors (*S. pneumoniae* strain 118 and 53) were used to transform the *S. mitis* NCTC 10712 recipient. Because transformants obtained from both donors exhibited similar PBP profiles, only transformants obtained using *S. pneumoniae* strain 53 DNA are shown in Fig. 5.4. The PBPs which showed reduced penicillin affinities with increasing resistance were PBPs 3, 4, 5 and 6. The *S. pneumoniae* - derived transformants showed similar PBP affinity alterations to transformants MT1 and MT4 obtained using *S. oralis* E9 DNA (Fig. 5.3). Alteration in penicillin affinity of PBP 6 was also accompanied by an increase in molecular mass (Fig. 5.4; lanes 7 and 8). The altered M_r of this PBP was demonstrated on three separate occasions. A similar PBP band was detected in the donor *S. pneumoniae* strain (Fig. 5.4; lane 10).

5.3.5 *Transformation of S. pneumoniae R6 using S. mitis SM1 donor.*

A similarity in the recorded molecular masses of PBPs 3, 4 and 5/6 of *S. mitis* NCTC 10712 to the altered PBPs 2x, PBP 2x and PBP 2b of *S. pneumoniae* respectively can be seen in Fig. 5.5. PBP 2x has previously been referred to as PBP 2a and the altered PBP 2x as PBP 2a' (Laible et al., 1989). In addition, *S. pneumoniae* donors were found to alter the penicillin affinities of these four PBPs in the *S. mitis* recipient (Fig. 5.4). The transformation frequency of the recipient *S. pneumoniae* R6 to a penicillin concentration of 0.025 μg/ml using DNA from *S. mitis* SM1 was $8x10^{-7}$. *S. pneumoniae* R6 does not possess a detectable altered PBP 2x , however, an altered PBP 2x was demonstrated in a transformant derived

from *S. mitis* SM1 (Fig. 5.5; lanes 5 and 6). In a recent study with a clinical *S. pneumoniae* recipient which had a detectable altered PBP 2x ,a transformant derived from *S. mitis* showed reduced penicillin-binding affinity of this PBP (Chalkley & Koornhof, 1991). PBP 2x is an important PBP involved in the expression of low-level penicillin resistance in *S. pneumoniae* (Zighelboim & Tomasz, 1981). The influence therefore, of penicillin resistance genes from *S. mitis* on the alterations and binding affinity of PBP 2x may be of clinical importance in the development of penicillin resistance in *S. pneumoniae*.

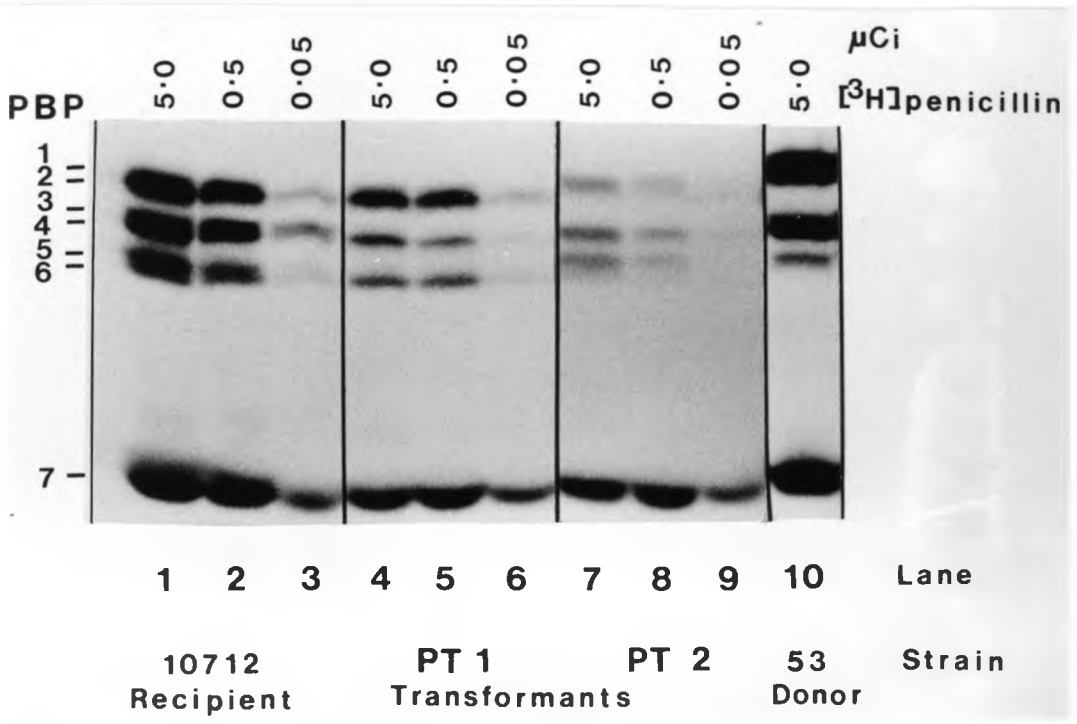

Figure 5.4. PBP profiles of *S. mitis* NCTC 10712 and transformants derived from *S. pneumoniae* strain 53. MICs in µg/ml are given in brackets. Lanes: 1-3, recipient *S. mitis* NCTC 10712 (0.03); 4-6, transformant PT1 (0.25); 7-9, transformant PT2 (1.0); 10, *S. pneumoniae* 53 (4.0). Dilutions of [^{3}H]benzylpenicillin were added to each strain as indicated. The PBP numbers assigned do not apply to the *S. pneumoniae* donor strain.

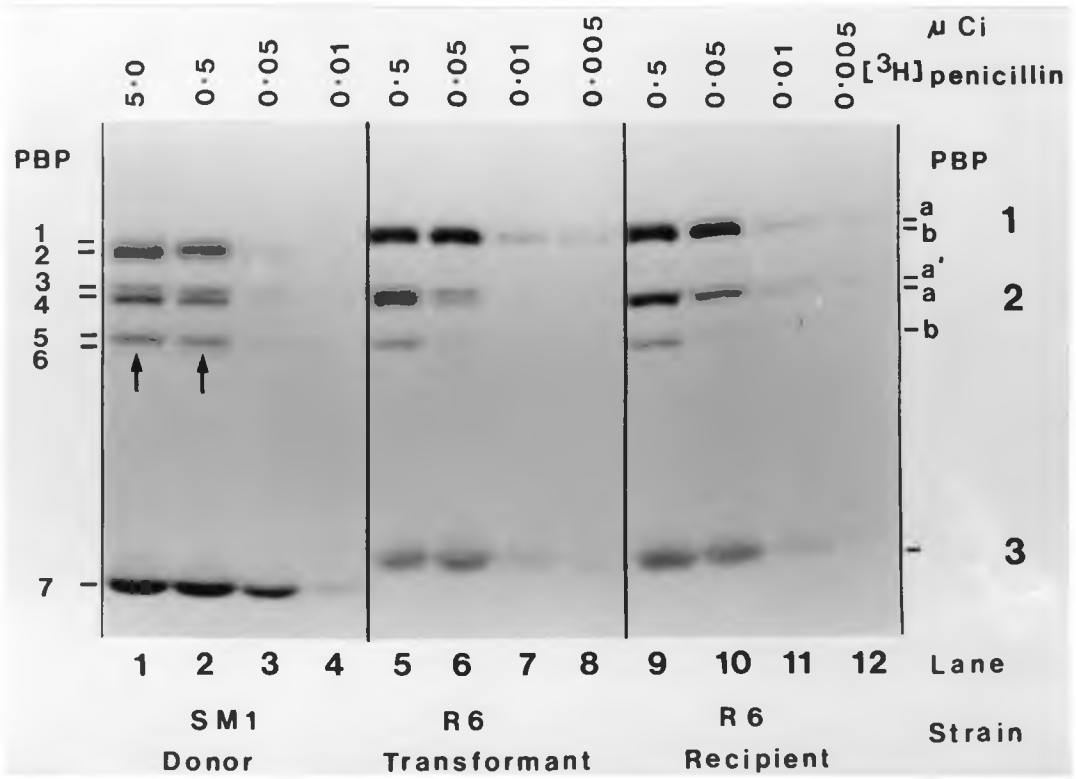

Figure 5.5. PBP profiles of *S. pneumoniae* R6 and a transformant from *S. mitis* SM1. MICs in μg/ml are given in brackets. Lanes: 1-4; *S. mitis* SM1 (0.06); 5-8, transformant of *S. pneumoniae* R6 (0.06); 9-12, recipient *S. pneumoniae* R6 (0.006). Dilutions of [^{3}H]benzylpenicillin were added to each strain as indicated. The PBP numbers indicated on the right apply to the *S. pneumoniae* recipient and the transformant strain. ↑ Denotes weak PBP 6 labelling.

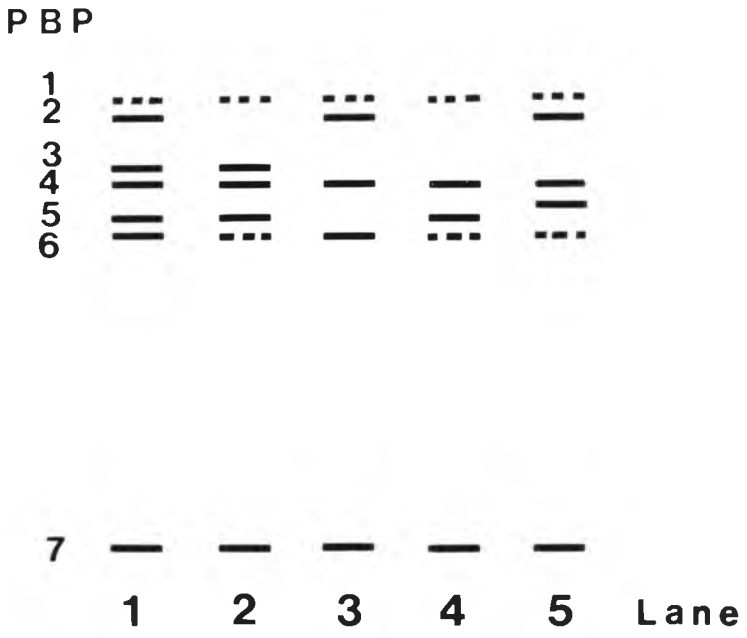

Figure 5.6. Schematic diagram of PBP profiles of *S. mitis* NCTC 10712 and selected transformants with MICs of 1 μg/ml. Lanes: 1, *S. mitis* NCTC 10712; 2, transformant MT3 donor DNA *S. oralis* E9; 3, transformant MT4 donor DNA *S. oralis* E9; 4, transformant MT6 donor DNA *S. mitis* E14; 5, transformant PT2 donor DNA *S. pneumoniae* 53. — Indicates PBP clearly visible, ... just visible, when strains were labelled with 5 μCi [^{3}H]benzylpenicillin.

The PBP profiles of clinical isolates of *S. mitis* are varied, each strain appearing to have its own individual PBP pattern. Using only four donors, transformants of the *S. mitis* NCTC 10712 strain all with MICs of 1.0 μg/ml, exhibited different PBP profiles (Fig. 5.6), indicating, perhaps how PBP variation in clinical isolates may have arisen. In *S. mitis* NCTC 10712 PBP 3 (74 kDa) is the major PBP found to alter with increasing penicillin resistance. Clearly demonstrating this, reduced penicillin binding to PBP 3 was observed in the spontaneous mutant and all transformants with higher MICs. Transformants also showed reduced penicillin-binding affinities of PBPs 2, 4, 5 and 6, but the PBP affected varied according to the donor DNA used.

Similarities in molecular masses of *S. mitis* NCTC 10712 PBPs 3,4, and 5/6 to the altered PBP 2x, PBP 2x and PBP 2b of *S. pneumoniae* are evident. PBP 6 rather than PBP 5 of the *S. mitis* NCTC 10712 strain appears to correspond to PBP 2b of *S. pneumoniae* as an increase in the M_r of PBP 6 to complement the M_r of PBP 2b in *S. pneumoniae* was observed in a *S. mitis* transformant derived from *S. pneumoniae* DNA (Fig. 5.5). Furthermore PBP 6 of the *S. mitis* NCTC 10712 strain has been shown to cross-react strongly with monoclonal antibodies raised against *S. pneumoniae* R6 PBP 2b (Chalkley et al, 1991). The presence of an altered PBP 2x in the *S. pneumoniae* R6 is of importance and R. Hakenbeck (personal communication) is continuing her genetic investigations of this PBP. The penicillin susceptible *S. mitis* NCTC 10712 strain was isolated before 1968 (NCTC catalogue reference, Colman, 1968) at which time only three penicillin-resistant clinical isolates of *S. pneumoniae* had been reported (Klugman, 1990). The findings of this study in general (Potgieter, Koornhof & Chalkley, 1992) and the demonstration of transfer of penicillin

resistance determinants from the spontaneous mutant of *S. mitis* NCTC 10712 to *S. pneumoniae* R6, a derivative of D39S isolated in 1916 (Chalkley et al., 1992), certainly opened the way to determining the direction of flow of mosaic genes from *S. mitis* to *S. pneumoniae*.

S. mitis can therefore function as a reservoir of complex penicillin resistance genes and emphasis should be placed on monitoring the development of β-lactam resistance in commensal bacteria, especially in countries such as South Africa where penicillin resistance is common and penicillin is extensively prescribed.

CHAPTER 6

RELATEDNESS BETWEEN PBP 2B GENES OF *STREPTOCOCCUS MITIS, STREPTOCOCCUS ORALIS* AND *STREPTOCOCCUS PNEUMONIAE.*

6.1 Introduction

Penicillin resistance in both viridans streptococci (Faber et al., 1983; Massidda & Daneo-Moore, 1988; Zito & Daneo-Moore, 1988; Quinn et al., 1988; Potgieter, Koornhof & Chalkley, 1992) and *S. pneumoniae* (Percheson & Bryan, 1980; Hakenbeck, Tarpay & Tomasz, 1980; Zigelboim & Tomasz, 1980) is due to altered forms of penicillin binding proteins which have reduced affinity for penicillin. The theory that these altered PBPs are the result of transfer of homologous genes from related species is supported by a number of studies; (a) great variability in the PBPs of penicillin-resistant clinical isolates of viridans streptococci (Van den Berg, 1990) and *S. pneumoniae* (Chalkley & Koornhof, 1988; Chalkley, Van den Berg & Koornhof, 1989; Markiewicz & Tomasz, 1989; Hakenbeck et al., 1991), (b) reciprocal transfer of penicillin resistance determinants between *S. pneumoniae* and *S. mitior* and *S. sanguis* II (*S. mitis*) (Chalkley & Koornhof, 1990; Chalkley & Koornhof, 1991; Potgieter & Chalkley, 1991), and (c) PBPs of *S. mitis* strains crossreacting with MAbs raised against *S. pneumoniae* PBPs 1a and 2b (Chalkley et al., 1991).

The altered PBPs are products of mosaic genes in which parts of the "sensitive" sequence have been replaced by "resistant" sequence blocks. These mosaic genes were first described for the PBP 2b genes of *S. pneumoniae*, *S. oralis* and *S. sanguis* (Dowson et al., 1989; Dowson et al., 1990) and subsequently for *S. pneumoniae* PBPs 2x and 1a (Laible, Spratt & Hakenbeck, 1991; Martin, Sibold & Hakenbeck, 1992).

The sources of the divergent gene blocks seen in streptococci have been investigated (Dowson et al., 1990; Laible, Spratt & Hakenbeck, 1991), however, definite identification of a specific donor initially proved to be very difficult (Dowson et al., 1990). Dowson et al. (1989) indicated that at

least two different sources were involved in the development of mosaic gene structures responsible for alterations of PBP 2b in *S. pneumoniae*, while analysis of *S. pneumoniae* PBP 2x and 1a genes suggested that at least four different sources were involved (Laible, Spratt & Hakenbeck, 1991; Martin, Sibold & Hakenbeck, 1992).

S. pneumoniae had been proposed as a possible donor of PBP 2b "resistant" gene blocks in *S. sanguis* and *S. oralis* (Dowson et al., 1990). The conclusions of Dowson et al., (1990) were, however, based on hybridization studies using oligonucleotide probes specific for penicillin-susceptible and resistant *S. pneumoniae* PBP 2b gene sequences. These probes were found to hybridize with PBP 2b genes of resistant but not susceptible viridans strains. In contrast, antigenic studies have shown susceptible *S. mitior* / *S. sanguis II* strains to contain PBPs that are related to PBPs of the susceptible *S. pneumoniae* R6 (Chalkley et al., 1991).

Very recently Dowson et al. (1993) proposed, using sequencing data, that PBP 2b resistance gene transfer could originally have occurred from *S. mitis* to *S. pneumoniae*. Sibold et al. (1994) demonstrated that "resistant" gene blocks of *S. pneumoniae* PBP 2x are present in susceptible *S. oralis* PBP 2x genes. In addition to sequencing data, transformation and PBP studies confirmed the direction of PBP 2x gene transfer to be from *S. mitis* to *S. pneumoniae* (Sibold et al., 1994).

Detailed studies are required in order to fully explain the emergence and spread of penicillin resistance in viridans streptococci and *S. pneumoniae*. In order to understand the direction of gene flow and the partners involved in the development of penicillin resistance it is essential to determine the relatedness of penicillin resistance determinants.

In this study DNA fingerprinting and nucleotide sequencing of PBP 2b genes of *S. mitis* and *S. oralis* were performed in order to further assess the relatedness of penicillin resistance determinants in *S. mitis*, *S. oralis* and *S. pneumoniae* isolates.

Table 6.1a. Polymerase chain reaction (PCR) products of the transpeptidase domain equivalent of *S. pneumoniae* in blood isolates of *S. mitis* and *S. oralis*.

Strain No.	ID Facklam[a]	ID Kilian[b]	MIC (μg/ml)	PCR
E2	*S. mitis*	*S. oralis*	2.0	-
E3	*S. mitis*	*S. mitis*	0.25	-
E4	*S. mitis*	*S. mitis*	0.25	-
E7	*S. mitis*	*S. mitis*	1.0	+
E9	*S. mitis*	*S. oralis*	8.0	+
E10	*S. mitis*	*S. oralis*	4.0	nsp
E11	*S. mitis*	*S. mitis*	16.0	+
E12	*S. mitis*	*S. oralis*	4.0	-
E14	*S. mitis*	*S. mitis*	4.0	+
E23	*S. mitis*		≤0.03	-
E35	*S. mitis*		≤0.03	-
E52	*S. mitis*		≤0.03	-
E63	*S. mitis*	*S. oralis*	0.03	+
E76	*S. mitis*		≤0.03	-

[a] Short identification scheme, five major group differentiation (Facklam & Washington, 1991).

b Differentiation within *S. mitis* group (Kilian Mikkelsen & Henrichsen, 1989)

+ = positive PCR product, - = no detectable PCR product, nsp = non-specific amplification product.

Table 6.1b. Polymerase chain reaction (PCR) products of the transpeptidase domain equivalents of *S. pneumoniae* in nasopharyngeal isolates of *S. mitis* and *S. oralis*.

Strain No.	ID Facklam[a]	ID Kilian[b]	MIC (μg/ml)	PCR
I1	*S. mitis*		0.06	-
I2	*S. mitis*		0.51	nsp
I3	*S. mitis*	*S. oralis*	16.0	-
I4	*S. mitis*	/	2.0	+
I5	*S. mitis*		2.0	nsp
I9	*S. mitis*		0.125	-
I11	*S. mitis*	/	0.125	+
I13	*S. mitis*	*S. oralis*	8.0	+
I14	*S. mitis*		0.5	-
I16	*S. mitis*	*S. mitis*	0.5	+
I17	*S. mitis*	*S. oralis*	0.03	+
I19	*S. mitis*	/	0.25	+
I20	*S. mitis*		8.0	nsp
I21	*S. mitis*	*S. oralis*	4.0	+
I22	*S. mitis*		1.0	-
I23	*S. mitis*		0.5	-
I24	*S. mitis*		0.03	nsp
I25	*S. mitis*		0.06	-
I28	*S. mitis*	*S. mitis*	0.5	+
I29	*S. mitis*	*S. mitis*	2.0	+
I31	*S. mitis*	*S. mitis*	2.0	-
I32	*S. mitis*	*S. oralis*	8.0	-
I33	*S. mitis*	*S. mitis*	8.0	-
I35	*S. mitis*		$\leq$0.015	-
I36	*S. mitis*		0.03	-
I39	*S. mitis*	*S. mitis*	0.5	+
I42	*S. mitis*		32.0	-

[a] Short identification scheme, five major group differentiation (Facklam & Washington, 1991).

b Differentiation within *S. mitis* group (Kilian Mikkelsen & Henrichsen, 1989)

+ = positive PCR product, - = no detectable PCR product, nsp = non-specific amplification product.

6.2 Materials and Methods

6.2.1 *Strains.*

Forty one clinical isolates of *S. mitis / S. oralis* isolated from nasopharyngeal and blood specimens were collected between 1988 - 1991. The penicillin MICs of the strains ranged from 0.03 - 16 μg/ml. The strains were identified as described in Chapter 3.2.1 and characteristics of these strains are shown in Table 6.1.

6.2.2 *Preparation of chromosomal DNA.*

Lysates were prepared by a method based on that used for *Neisseria gonorrhoeae* (Brannigan et al., 1990). Cultures were grown for 4 - 5 h to a density of approximately 1 x 10^7 cfu/ml in 10 ml Todd Hewitt (Oxoid) broth containing 1% (w/v) yeast extract (Difco). Cells were harvested by centifugation and resuspended in 1 ml of 50 mM Tris-HCl, 20 mM EDTA, pH 7.4. To the cell suspension 2.5 μl of lysozyme (10 μg/ml) and 2.5 μl of mutanolysin (10 μg/ml) were added. After 10 min at room temperature 1 ml of Triton-X (2%) and 5 μl of Proteinase K (5 mg/ml) were added and the mixture incubated at 37^{o}C for 30 min. The lysates were checked for sterility, aliquoted and stored at -70^{o}C. Five microlitres of these lysates were used for the polymerase chain reaction (PCR).

6.2.3 *Amplification of the PBP 2b gene.*

The coding region of the transpeptidase domain of the *S. pneumoniae* PBP 2b gene equivalent in *S. mitis* was amplified from chromosomal DNA using the polymerase chain reaction conditions as described by Dowson, Hutchison & Spratt (1989). The reaction mixture (100 μl) contained: 1 μg

chromosomal DNA, 50 mM KCl, 10 mM Tris - HCl, pH 8.4, 2.5 mM $MgCl_2$,1mM Pn-up (d G A T C C T C T A A A T G A T T C T C A G G T G G) and Pn-down (d C A A T T A G C T T A G C A A T A G G T G T T G G) primers, 200 μM dNTPs, 200 μg/ml gelatin and 2 Units of Taq DNA polymerase (Promega). Twenty-five PCR cycles were performed; 6 min extention at 72°C, 1 min denaturation at 94°C, and 2 min annealing at 52°C.

6.2.4 *Fingerprinting of the PBP 2b genes*

The amplified 1.5 kb PBP 2b gene fragments were purified from 0.7% agarose gel. The PBP 2b gene equivalents of *S. mitis* / *S. oralis* were identified by comparison with a PBP 2b gene PCR product of *S. pneumoniae* R6. An agarose strip containing the required band was cut from the gel and frozen at -70°C for 10 min. The DNA was then recovered by the freeze-squeeze procedure (Thuring, Sanders & Borst, 1975). The fragment was concentrated by ethanol precipitation, resuspended in 20 μl sterile distilled water and stored at -70°C until used for fingerprinting.

Gene fingerprinting based on the method of (Zhang et al., 1990) was performed as described by Coffey et al. (1991). To 20-50 ng of the 2 units of restriction enzyme *Sty*I or *Hin*fI (Boehringer Mannheim) and 1 μl of 10x appropriate restriction buffer was added in a final volume fo 10 μl. After incubating the solution for 1 h at 37°C, 5 μl of end-labelling mixture was then added to the digest. For *Sty*I digested DNA: 10 mM Tris - HCl (pH 7.4), 10 mM $MgCl_2$, 6 mM DTT, 0.1 mM dATP, 0.1 mM dTTP, 0.1 mM dGTP and 0.6 μCi of [α-^{32}P]-dCTP (Amersham, UK) and 0.1 unit of Klenow enzyme (Boehringer Mannheim) per ml; for *Hin*fI digested DNA, the radiolabelled nucleotide was [α-^{32}P]-dTTP and unlabelled dTTP was

replaced with (0.1 mM dCTP). After 10 min at room temperature 4 μl loading buffer (sucrose dye) was added and, 5 μl applied to a 6% non-denaturating polyacrylamide gel. pBR322 DNA digested with *Hpa*II (Boehringer Mannheim) and end-labeled with [α-^{32}P]-dCTP was used as a molecular size marker. The samples were run at 600 V on a 6% polyacrylamide gel 0.4 mm thick using Owl Scientific's Model S3S DNA sequencing system (Owl Scientific), until the bromophenol blue marker reached 10 cm from the bottom of the gel. The gel was transferred to Whatman No. 1 filter paper, dried under vacuum, and autoradiographed for 4 - 24 h with Hyperfilm-MP (Amersham).

6.2.5 *Cloning procedure*

Four viridans streptococcal strains were sequenced; two *S. oralis* strains I17 and E9 and two *S. mitis* strains I16 and I29. Strains E9 and I16 were cloned into pUC18 and I17 and I29 into pCR-Script SK(+).

The blunt-ended PCR fragments were Klenow-polished: To 50 μl of PCR product (approximately 200 ng/μl) 1 unit of Klenow enzyme (Boehringer Mannheim) and 5 μl 35 mM $MgCl_2$ were added. The solution was left for 30 min at room temperature. The fragments were then purified as described in Section 6.2.4 and E9 and I16 cloned into the *Sma*I site of pUC18 using the pUC18 cloning kit (Boehringer Mannheim) according to the manufacturers instructions. The purified PCR fragments were ligated with *Sma*I restricted pUC18 using T4 DNA ligase. Hybrid plasmids were then transformed into competent *E. coli* K12 JM101 and clones containing the PCR fragment were selected on Luria (LB) agar containing ampicillin (100 μg/ml) isopropyl-β-D-galactoside (IPTG) 0.5mmol/l and 0.004% X-gal

(5-bromo-5-chloro-3-indolyl-β-D-galactopyranoside). Plasmid DNA from each clone was screened by the method of Goode & Feinstein (1992).

With strain I17 repeated attempts to clone the PCR fragment into pUC18 was not succesful. Strains I17 and I29 were cloned using pCR-Script SK(+) cloning kit (Stratagene) according to the manufacturer's instructions. No problems were experienced with either strain using the pCR-Script SK(+) vector. The two cloning procedures are described in Appendices A and B.

6.2.6 *Sequence determination*

Plasmids were purified either by CsCl (Sambrook, Fritsch & Maniatis, 1989) or by using Magic^{TM} Maxipreps DNA Purification system (Promega) (Appendix D). The nucleotide sequence of the 1.5 kb insert was determined on both strands using the Sequenase Version 2.0 (United States Biochemical, Cleveland, OH) DNA Sequencing Kit. The Sequenase protocol (United States Biochemical, Cleveland, OH) was modified as described by Schuurman & Keulen (1991) (Appendix C). Occasional errors which may arise from PCR were resolved by sequencing more than one clone of each strain from independent PCR products.

6.3 Results and Discussion.

6.3.1 *Amplification of the transpeptidase domain of the PBP 2b gene by the polymerase chain reaction.*

Using the *S. pneumoniae* PBP 2b primers, the equivalent of the 1.5 kilobase fragment encoding the transpeptidase domain of *S. pneumoniae* PBP 2b in *S. mitis* and *S. oralis* was amplified by the polymerase chain reaction (PCR) from 20 of the 41 clinical viridans streptococcal isolates and these PCR products are shown in Fig. 6.1 & 6.2. There was no correlation between the site of isolation of the viridans strains or their MICs and the demonstration of PCR products (See Table 6.1). Fifteen of these 20 strains produced PCR products similar in size to that of the *S. pneumoniae* R6 PBP 2b gene Fig 6.1. The fifteen strains comprised 7 *S. mitis*, 5 *S. oralis* and 3 *S. mitis* / *S. oralis* isolates (Table 6.1). The PCR products of the other 5 strains, differed in size with that of the *S. pneumoniae* R6 Fig. 6.2 lanes 5 - 9. These 5 strains that produced non-specific amplification products would require more specific primers and were therefore not included in further studies.

Multiple PCR products were obtained with some strains. Although PCR conditions could have been changed to reduce the number of these spurious products, the PBP 2b gene equivalents of the 15 *S. mitis* and *S. oralis* strains were clearly identified when compared with the *S. pneumoniae* R6 PBP 2b gene product.

PCR products of *S. mitis* NCTC 10712 and the spontaneous mutant *S. mitis* SM1 are shown in Fig. 6.2 lanes 3 and 4. The PBP 2b PCR products of *S. mitis* NCTC10712 and the spontaneous mutant *S. mitis* SM1 strains were of slightly larger size than that of the *S. pneumoniae* R6.

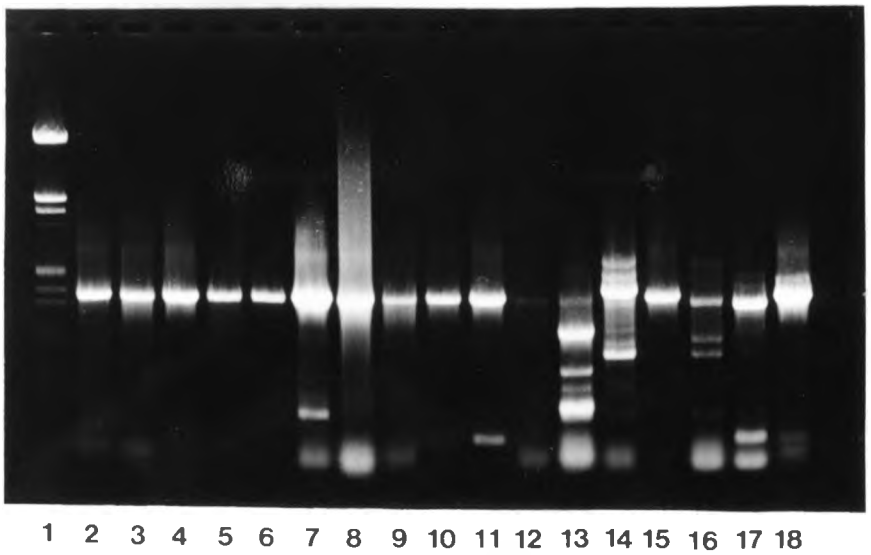

Figure 6.1. Agarose gel electrophoresis of PCR products of *S. mitis* and *S. oralis* isolates. Lanes: 1, Molecular size marker; 2, *S. pneumoniae* R6; 3, E9; 4, E14; 5, I16; 6, I19; 7, I4; 8, I13; 9, I39; 10, I28; 11, I29; 12, I17; 13, I21; 14, E63; 15, *S. pneumoniae* R6; 16, I11; 17, E7; 18, E11.

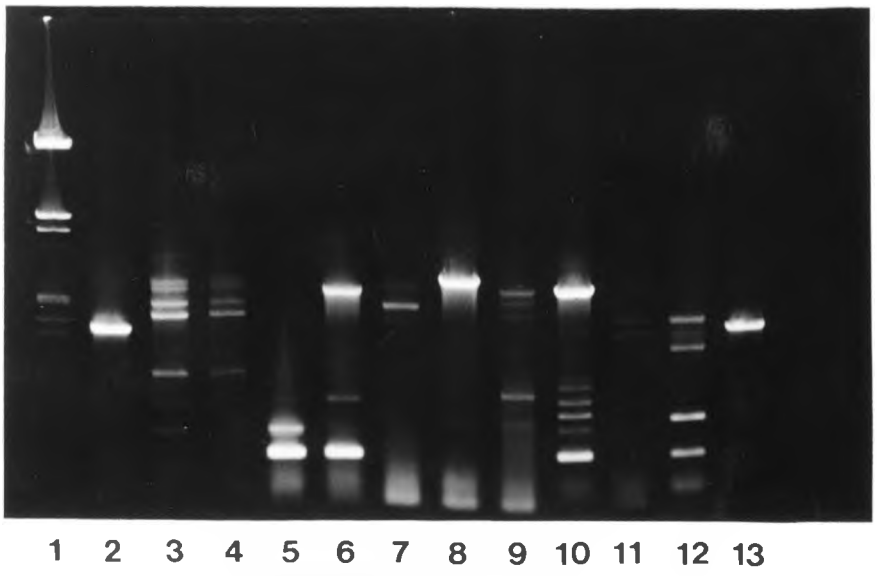

Figure 6.2. Agarose gel electrophoresis of PCR products of *S. mitis* and *S. oralis* isolates which were not similar in size to that of *S. pneumoniae* R6.. Lanes: 1, Molecular size marker; 2, *S. pneumoniae* R6; 3, *S. mitis* NCTC 10712; 4, *S. mitis* SM1; 5, I2; 6, I5; 7, I20; 8, I24; 9, E10; 10-12, *S. sanguis* strains; 13, *S. pneumoniae* R6.

6.3.2 Fingerprints of the PBP 2b genes.

Clinical strains.

On comparing the fingerprint patterns of the 15 *S. mitis* and *S. oralis* strains shown to produce PCR products of the same size as that of *S. pneumoniae* R6, the use of the *Sty*I restriction enzyme revealed, 11 different patterns (Fig. 6.3). While with the *Hin*fI restriction endonuclease, 12 different patterns were obtained (Fig. 6.4). The MICs of these strains ranged from 0.03-16.0 μg/ml. Three of the *Sty*I fingerprint patterns A, B & C as shown in Fig 6.3 were obtained in more than one strain. The 3 strains, *S. mitis* E14, *S. oralis* E9 and *S. mitis* E11 which had MICs of 4, 8 and 16 μg/ml respectively, produced fingerprints similar to the pattern A of lane 3 shown in Fig 6.3. The 3 resistant strains that produced the *Sty*I pattern A, also had the same fingerprint pattern when *Hin*fI was used (Fig. 6.5, lanes 7-9). Two strains (MICs 0.5 and 2 μg/ml) produced the same pattern B and two strains (MICs 0.5 μg/ml) gave the same pattern C (Fig 6.3 lanes 6 and 9 respectively). The strains which showed similar *Sty*I patterns B and C, did not show similar patterns with *Hin*fI. (Fig 6.5, lanes 3 &4 and 5 & 6).

Analysis of results failed to reveal correlation between the MIC and the fingerprint pattern obtained. Two *S. mitis* isolates E14 and E11 with MICs 4 and 16 μg/ml respectively had similar fingerprint patterns (Fig. 6.3 lane 3; Fig. 6.5 lanes 7 & 9), while two *S. mitis* isolates I16 and I28 both had an MIC of 0.5 μg/ml but showed different fingerprint patterns (Fig. 6.3 lanes 6 & 9; Fig. 6.5 lanes 5 & 4) with both *Sty*I and *Hin*fI restriction enzymes. The two penicillin-susceptible *S. oralis* isolates E63 and I17, both with an MIC of 0.03 μg/ml also showed different fingerprint patterns (Fig. 6.3 lanes 12 & 13; Fig. 6.4 lanes 8 & 9). This is in contrast with results found in *S.*

pneumoniae where PBP 2b fingerprint patterns of susceptible strains with MICs of ≤0.06 μg/ml all had similar fingerprint patterns (Smith et al., 1993).

PBP 2b gene fingerprint patterns of the viridans streptococci used in this study were compared with the *Hin*fI and *Sty*I PBP 2b gene fingerprint patterns of 68 *S. pneumoniae* strains with MICs ranging from ≤0.015-8 μg/ml isolated throughout South Africa between 1987-1991 (Smith et al., 1993). Both the *Sty*I and *Hinf*I PBP 2b gene fingerprint patterns of E14, E9 and E11 were identical to those of *S. pneumoniae* 52328 (Smith et al., 1993) and *S. oralis* strains 5296 and 5302 (Coffey et al., 1993). Details of these strains are presented in Table 6.2.

It is of interest that 2 *S. mitis* and 1 *S. oralis* isolates from this study, showed identical PBP 2B gene fingerprints to those of the South African *S. pneumoniae* strain 52328 (Smith et al., 1993) and those of two *S. oralis* strains isolated in England (Coffey et al., 1993), in the same year 1989.

Laboratory strains and transformants.

No mutational changes in the PBP 2b gene of the *S. mitis* SM1 were evident at the *Sty*I or *Hin*fI restriction sites as the fingerprint pattern of *S. mitis* SM1 was identical to that of the parent strain *S. mitis* 10712 (Fig. 6.6 lanes 4-5). *Sty*I and *Hin*fI fingerprint analysis of *S. pneumoniae* R6 and the *S. pneumoniae* R6 transformant (obtained using donor DNA from *S. mitis* SM1) indicated that no PBP 2b gene transfer had occurred as patterns were identical (Fig. 6.6 lanes 2-3). As possible recombination sites in the PBP 2b gene are not known, fingerprint analysis may not reveal short sequences that may have been introduced into the *S. pneumoniae* R6 transformant from the donor *S. mitis* SM1. The transpeptidase domain of

the PBP 2b gene of the *S. pneumoniae* R6 transformant was therefore sequenced and found to be identical to that of *S. pneumoniae* R6.

As the PBP 2b gene fingerprints of the *S. mitis* and *S. oralis* isolates in this study were very heterogeneous, four clinical isolates, two *S. mitis* isolates with MICs of 0.5 and 2 μg/ml and two *S. oralis* isolates with MICs of 0.03 and 8 μg/ml were therefore sequenced. The sequences were compared with the penicillin susceptible *S. pneumoniae* R6 PBP 2b gene sequence (Dowson, Hutchison & Spratt, 1989), three penicillin resistant *S. oralis* isolates (Dowson et al., 1990) and the recently published sequences of four penicillin susceptible *S. mitis* isolates (Dowson et al., 1993).

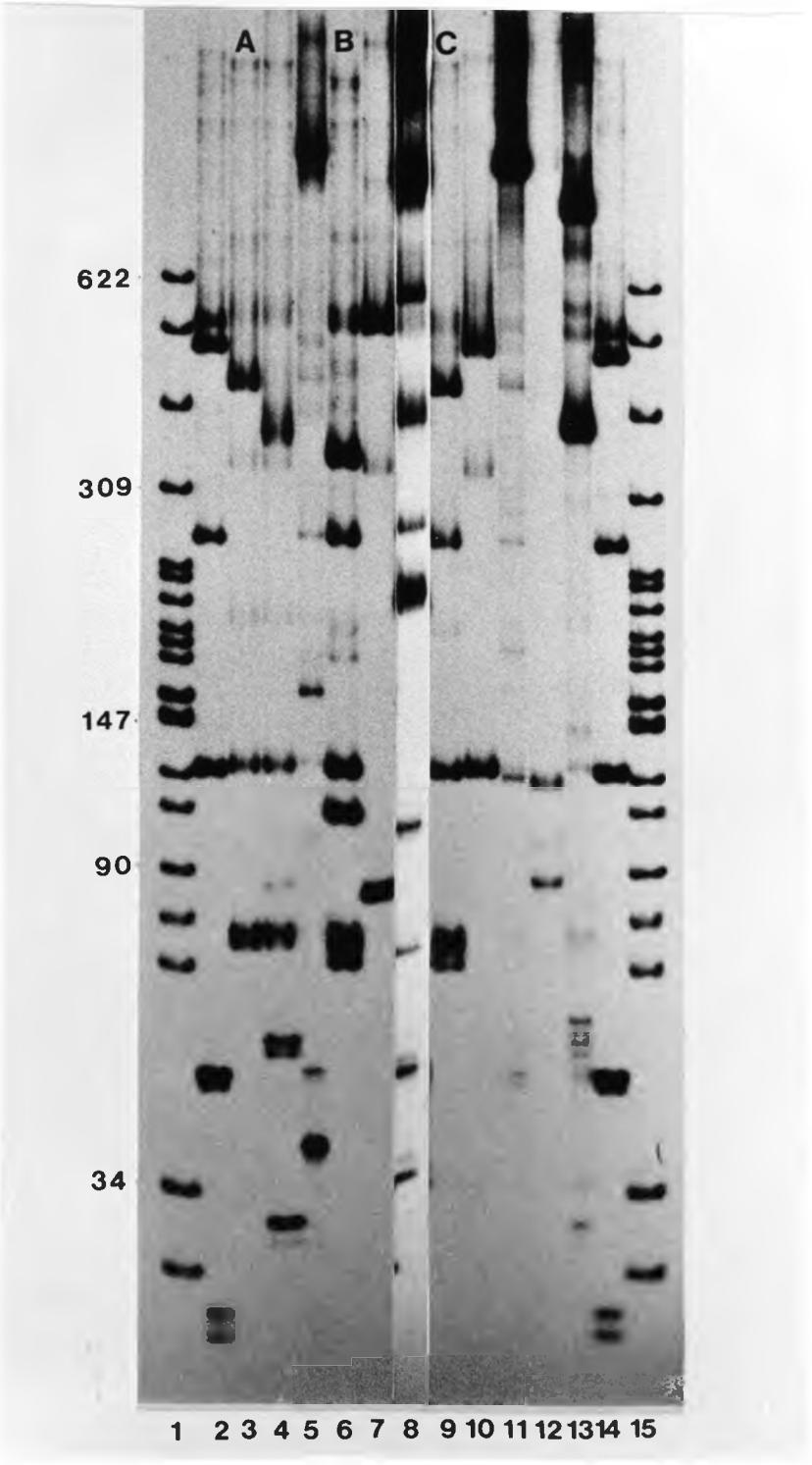

Figure 6.3. *Sty*I fingerprints of the PBP 2b gene of *S. mitis* and *S. oralis* isolates. Lanes: 1, Molecular size marker (pBR322 digested with *Hpa*II); 2, *S. pneumoniae* R6; 3, E9/E11/E14; 4, I13; 5, I21; 6, I4/I16; 7, I29; 8, E7; 9, I28/I39; 10, I19; 11, I11; 12, E63; 13, I17; 14, *S. pneumoniae* R6; 15, Molecular size marker.

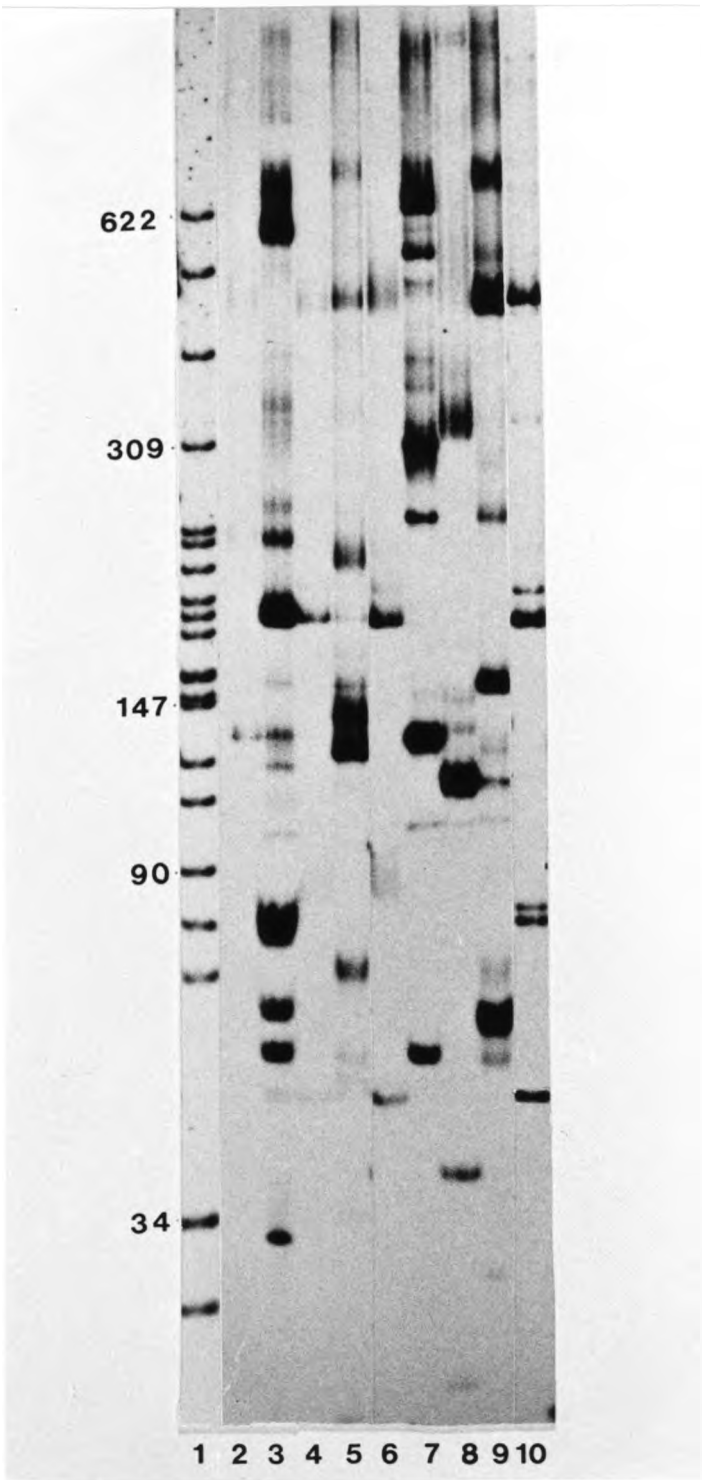

Figure 6.4. *Hin*fI fingerprints of the PBP 2b gene of *S. mitis* and *S. oralis* isolates. Lanes: 1, Molecular size marker; 2, I13; 3, I21; 4, I29; 5, E7; 6, I19; 7, I11; 8, E63; 9, I17; 10, *S. pneumoniae* R6.

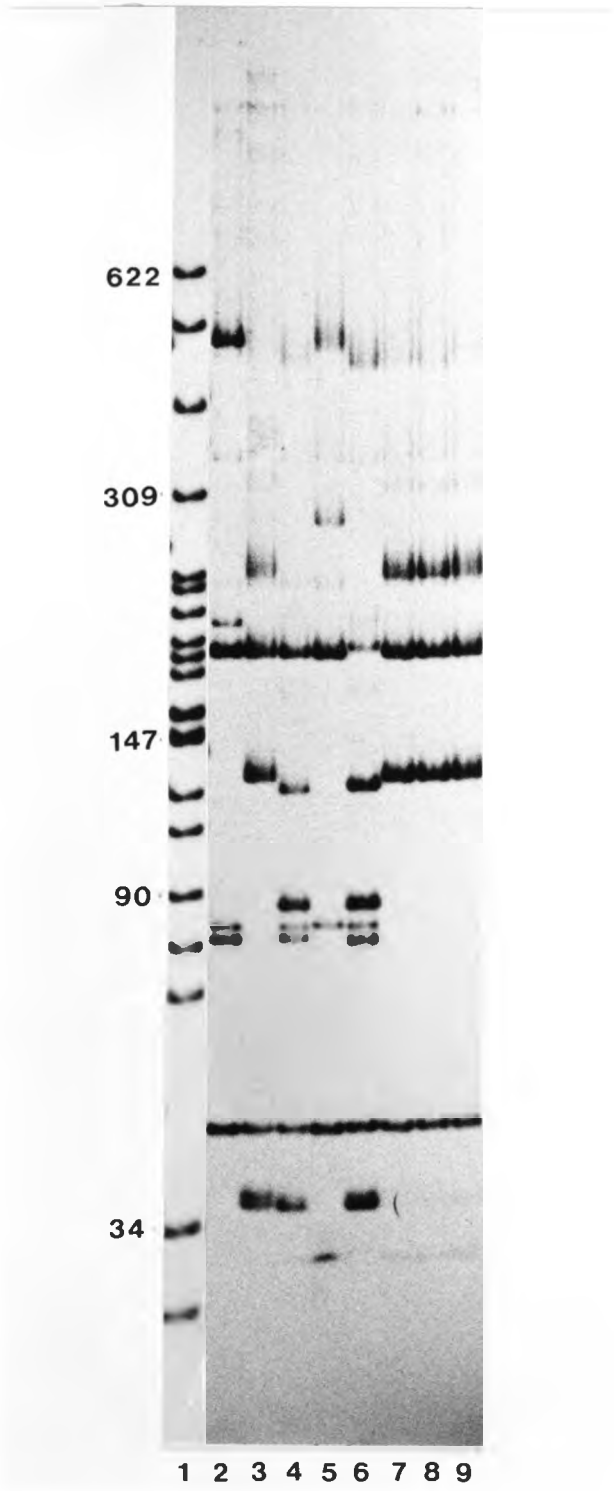

Figure 6.5. *Hin*fI fingerprints of the PBP 2b gene of *S. mitis* and *S. oralis* isolates which had similar *Sty*I fingerprint patterns. Lanes: 1, Molecular size marker; 2, *S. pneumoniae* R6; 3, I39; 4, I28; 5, I16; 6, I4; 7, E14; 8, E9; 9, E11.

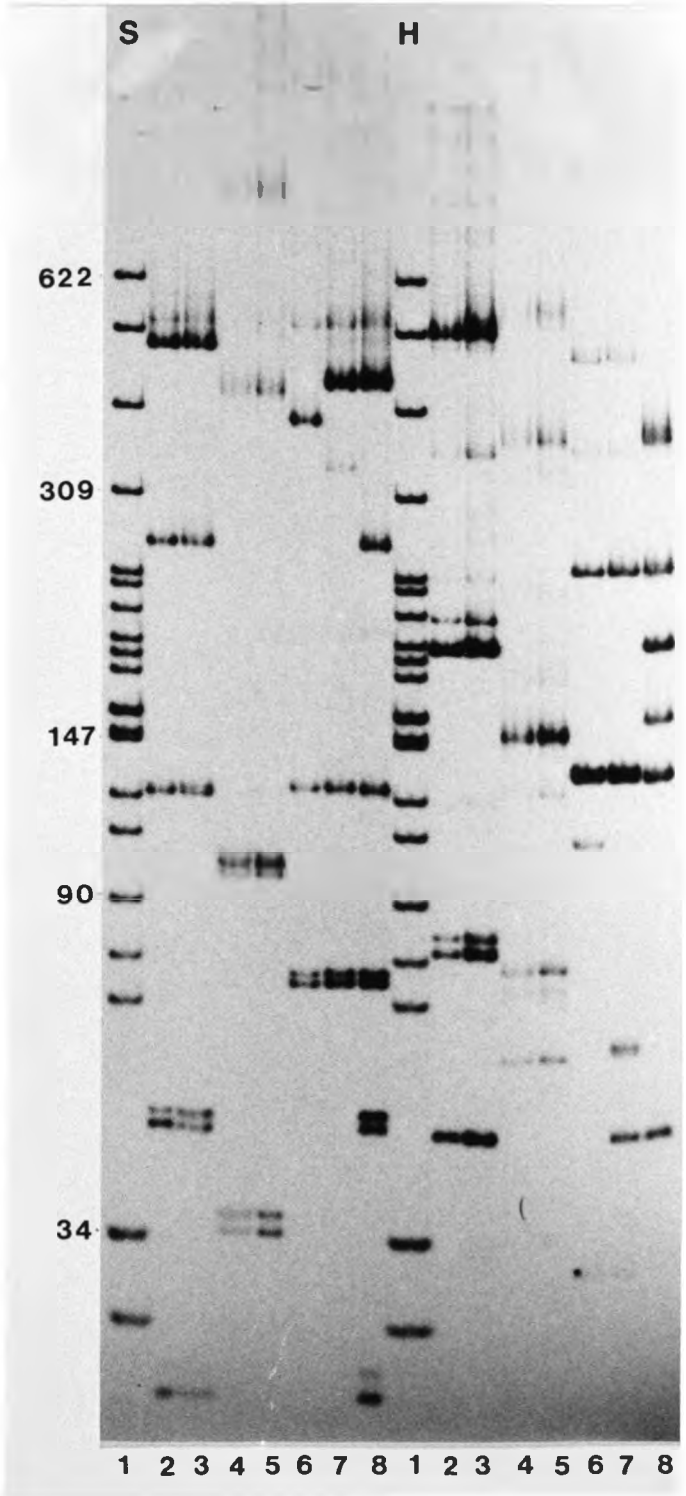

Figure 6.6. *Sty*I (lanes S 2-8) and *Hin*fI (lanes H 2-8) fingerprints of the PBP 2b genes of laboratory strains and transformants. Lanes: S1, Molecular size marker; S2, *S. pneumoniae* R6; S3, *S. pneumoniae* transformant using donor DNA from *S. mitis* SM1; S4, *S. mitis* SM1; S5, *S. mitis* NCTC10712; S6, *S. mitis* NCTC 10712 transformant using donor DNA from *S. pneumoniae* 53; S7-8, clinical isolates of *S. pneumoniae* strains 53 and 118; H1, Molecular size marker; H 2-8, fingerprints of same strains using *Hin*fI restriction enzyme.

Table 6.2. Details of selected *S. mitis* / *S. oralis* isolates with identical PBP 2b gene fingerprints to *S. pneumoniae* 52328[a] and *S. oralis* strains 5296 and 5302[b].

Strain no.	Identification	MIC (μg/ml)	Hospital	Source	Date of isolation
E9	*S. oralis*	8	Johannesburg	Blood	November 1989
E11	*S. mitis*	16	Baragwanath	CSF	December 1989
E14	*S. mitis*	4	Johannesburg	Blood	December 1989
52328[a]	*S. pneumoniae*	4	Baragwanath	Tracheal aspirate	May 1989
5296[b]	*S. oralis*	8	UK	Blood	1989
5302[b]	*S. oralis*	2	UK	Wound	1989

[a] Smith et al., 1993

[b] Coffey et al., 1993

6.3.3 *Nucleotide sequences of the PBP 2b genes of S. mitis isolates.*

Nucleotide sequencs of PBP 2b genes of one intermediately resistant strain *S. mitis* I16 (MIC 0.5 μg/ml) and one penicillin-resistant strain I29 (MIC 2 μg/ml) both isolated from nasopharyngeal specimens at Baragwanath Hospital in 1988 were determined and shown in Figure 6.7. A schematic representation of the *S. mitis* strains compared with other closely related streptococcal strains is shown in Figure 6.8. The intermediately resistant *S. mitis* (M^I) isolate I16 and penicillin-resistant *S. mitis* (M^R) isolate I29 diverged from the penicillin-susceptible *S. oralis* (O^S) strain I17 by $\leq 3.6\%$ and 11% in blocks 1 and 2 and by $\geq 20\%$ in blocks 3, 4 and 5, indicating no relatedness between the PBP 2b genes (blocks 2-5) of the two *S. mitis* isolates and that of the susceptible *S. oralis* isolate (Fig. 6.8). Block 1 of the two *S. mitis* strains differed by $\leq 3.6\%$ from that of the susceptible *S. oralis* isolate and by $\leq 3.6\%$ from that of the penicillin-susceptible *S. pneumoniae* R6 (Dowson, Hutchison & Spratt, 1989) in this area, nucleotides 837-1173 (Fig. 6.8, Table 6.3), indicating a common ancestry although the origin at this stage is unclear. I16 (M^I) and I29 (M^R) also varied extensively from that of the penicillin-susceptible *S. mitis* NCTC10712 (Dowson et al., 1993) with the exception of a 240 bp region at the end of the PBP 2b gene where I29 (M^R) varied by only 9 nucleotides (3%) and 1 amino acid alteration from that of *S. mitis* NCTC10712 (Fig. 6.7).

The PBP 2b sequences of I16 differed from that of the penicillin-susceptible *S. pneumoniae* R6 (Dowson, Hutchison & Spratt, 1989) by only 4 nucleotides (1.8%) and 2 amino acids (2.2%) in the area between nucleotides 1703-1974 and by 10 nucleotides (3.4%) and 1 amino acid (1%) between nucleotides 1975-2269 (Fig. 6.8, Table 6.3). This indicates that

these two areas (as presently defined) in this intermediately resistant *S. mitis* strain I16 had originated from *S. pneumoniae*.

Similarly, block 4 (nucleotide 1703-1974) in I29 differed by only 4% at nucleotide level and with only 2 amino acid alterations (2.2%) from that of either the penicillin-susceptible *S. pneumoniae* R6 and the penicillin-resistant *S. oralis* (E9), strongly indicating again that this block had originated from *S. pneumoniae* (Fig. 6.8, Table 6.3).

On comparing results from this study with reported sequences of PBP 2b genes of *S. pneumoniae* (Dowson et al., 1990) and viridans streptococci (Dowson et al., 1990; Coffey et al., 1993) in the areas between nucleotides 1174-1428 and 1429-1702 an obvious lack of sequence homology was found between I16 and I29 and any other *S. mitis*, *S. oralis* or *S. pneumoniae* strains studied (Fig. 6.7, 6.8 and 6.9). The origins of these two diverged blocks therefore remain unknown.

6.3.4. *Nucleotide sequences of the PBP 2b genes of S. oralis isolates.*

The nucleotide sequences of the PBP 2b genes of one penicillin-susceptible *S. oralis* strain I17, isolated from the nasopharynx in 1988 and one penicillin-resistant *S. oralis* strain E9, isolated from blood in 1989, were determined. The PBP 2b PCR product of *S. oralis* strain E63 was similar but not identical to that of *S. pneumoniae* R6. Clones containing the PCR fragment thought to be the PBP 2b gene of strain E63 gene was sequenced from both directions using pUC primers, however, the sequences obtained were not consistent with published PBP 2b genes. In addition, internal PBP 2b primers used in this study failed to determine any further sequence, indicating that the amplified product from *S. oralis* E63 was not a PBP 2b gene fragment.

The sequences of the penicillin-susceptible strain I17 and the penicillin-resistant strain E9 are shown in Figure 6.7. The sequences of the two *S. oralis* strains were compared with each other, with the penicillin-susceptible *S. pneumoniae* R6 strain (Dowson, Hutchison & Spratt, 1989) and with a penicillin-susceptible *S. mitis* strain NCTC 10712 (Dowson et al., 1993). A schematic representation of the relatedness of the PBP 2b genes when compared with each other are shown in Figure 6.9. The comparitive block structure was based on that proposed by Dowson et al. (1990).

The penicillin-susceptible *S. oralis* I17 strain contained what appeared to be unique areas with divergence of >20% when compared with *S. pneumoniae* R6 ie. from nucleotide 1428-2269 (blocks 3, 4 and 5) and with >14% divergence from nucleotide 837-1702 and 1974-2269 (blocks 1, 2, 3 and 5 respectively) when compared with *S. mitis* NCTC 10712 (Fig. 6.9 and Table 6.4).

There were no similar areas between the penicillin-resistant *S. oralis* E9 isolate and the susceptible *S. mitis* NCTC 10712 strain (Fig. 6.9).

The penicillin-resistant *S. oralis* E9 showed a mosaic structure which differed at 112, nucleotide sites resulting in 17 amino acid alterations when compared with the susceptible strain I17. The first 866 bp contained three blocks which diverged from I17 by 3.3%, 7.8% and 5.1% and this entire area differed from I17 at 45 nucleotide sites resulting in only 4 amino acid changes. Block 5 consisting of the last 290 bp showed only 2 (0.7%) nucleotide changes with no amino acid alterations. The 272 bp block between bp 1703-1974 (block 4) differed at 65 sites (23%) resulting in 13 amino acid changes. This region 1703-1974 originally termed a "susceptible" block by Dowson et al. (1990) can be seen to be very similar to the

corresponding area of *S. pneumoniae* R6 (Fig. 6.9) with only 2 amino acid alterations (Table 6.4). The penicillin-resistant *S. oralis* E9 strain therefore contained one divergent region of 272bp (block 4) which was flanked by *S. oralis* sequences.

The PBP 2b sequence of the *S. oralis* E9 isolate was also compared with two penicillin-resistant *S. oralis* strains isolated in England (Dowson et al., 1990) and a penicillin-resistant *S. pneumoniae* strain 52328 isolated in South Africa (Coffey et al., 1993). All these strains were isolated in 1989 and seen to have the same *Hin*fI and *Sty*I fingerprint patterns. *S. oralis* E9 was found to differ at only 9 nucleotide sites resulting in one amino acid change from the two penicillin resistant *S. oralis* strains 5296 and 5302 isolated in England (Dowson et al., 1990) and at 14 nucleotide sites again resulting in only one amino acid change from a resistant *S. pneumoniae* strain 52328 isolated in South Africa (Coffey et al., 1993). Coffey et al. (1993) also reported that another penicillin-resistant *S. pneumoniae* strain isolated in Spain had an identical PBP 2b sequence to that of *S. pneumoniae* 52328. The similarity in the PBP 2b sequences of *S. oralis* and *S. pneumoniae* strains isolated in the same year in South Africa, a country where the incidence of penicillin resistance amongst both the viridans streptococci (Potgieter et al., 1992) and *S. pneumoniae* is known to be high (Koornhof et al., 1992) is not surprising especially since reciprocal transfer of penicillin resistance genes between these two groups have been reported (Potgieter & Chalkley, 1991). Of interest is the presence of almost identical PBP 2b genes in penicillin-resistant *S. oralis* and *S. pneumoniae* strains isolated in South Africa, England and Spain (Dowson et al., 1990; Coffey et al, 1993).

Coffey et al. (1993) recently reported unpublished data of C.G. Dowson that showed PBP 2b gene regions 837-1173, 1420-1702 and 1975-2269, blocks 2, 3 and 5 of *S. oralis* strains 5296 and 5302 diverging from the corresponding regions of a susceptible *S. oralis* strain isolated in the 1940's by >15%. It is important to note that the above-mentioned penicillin-resistant *S. oralis* strains and the *S. pneumoniae* strains contain sequences represented by nucleotides 1429-1702 and nucleotides 1975-2269, blocks 3 and 5 that are related to the susceptible *S. oralis* I17 strain investigated in this study.

Significance of amino acid alterations between Thr-425 and Phe-431 and at Thr-445.

In penicillin-resistant *S. pneumoniae* it has been suggested that alterations of seven consecutive residues between Thr-425 and Phe-431 as well as an alteration of Thr-445 to Ala were responsible for the low affinity for penicillin of the PBP 2b Class A gene (Dowson et al., 1989). In addition to Thr/Ala, the two *S. mitis* isolates I16 intermediately resistant and I29 resistant to penicillin had 6 amino acid alterations of which five were identical to the resistant *S. pneumoniae* Class A alterations. The exception being Gly_{429}/Ala for strain I16 and Gly_{429}/Glu for strain I29.

The penicillin-resistant *S. oralis* E9 strain showed the same alterations in these regions as the resistant *S. pneumoniae* Class B gene. The susceptible *S. oralis* I17 strain also showed the same alterations indicating the presence of a resistant *S. pneumoniae* Class B gene. The presence or absence of these alterations in the development of penicillin resistance in *S. mitis* (Dowson et al., 1993) or the *S. oralis* strains in this study, remains unsolved.

Table 6.3. Variation of the *S. mitis* PBP 2b genes and their derived amino acid composition relative to those of other strains.

Strains	Blocks:Codons	Nucleotides (%) altered	Amino acids (%) altered	% Amino acid change per nucleotide
I16 / R6	1:202-314	8 (2.4)	0	0
	2:314-399	29 (8.6)	3 (3.5)	10
	3:399-491	42 (15)	11 (12)	26
	4:491-581	4 (1.8)	2 (2.2)	50
	5:581-679	10 (3.4)	1 (1)	10
I29 / R6	1:202-314	7 (2)	2 (1.8)	29
	2:314-399	10 (6)	2(2.4)	20
	3:399-491	33 (13)	10 (10.9)	30
	4:491-581	11 (4)	2 (2)	18
	5:581-679	45 (16)	7 (7.2)	16
I16 / 10712	1:202-314	60 (18)	9 (8)	15
	2:314-399	35 (14)	4 (4.7)	11
	3:399-491	67 (24)	17 (18.5)	25
	4:491-581	75 (28)	18 (19.8)	24
	5:581-679	56 (19)	13 (13.4)	23
I29 / 10712	1:202-314	61 (18.2)	10 (8.9)	16
	2:314-399	25 (10.5)	4 (4.7)	16
	3:399-491	62 (17.8)	14 (15.2)	23
	4:491-581	67 (24)	17 (18.7)	25
	5:581-679	22 (9)	6 (6)	27
I16 / I29	1:202-314	6 (1.8)	1 (0.9	17
	2:314-399	21 (8.2)	3 (3.5)	14
	3:399-491	58 (21)	15 (16.3)	26
	4:491-581	12 (4.4)	2 (2.2)	17
	5:581-679	61 (21.4)	10 (10.3)	16

Table 6.4 Variation of the *S. oralis* PBP 2b genes and their derived amino acid composition relative to those of other strains.

Strains	Block:Codons	Nucleotides (%) altered	Amino acids (%) altered	% Amino acid change per nucleotide
I17 / R6	1:202-314	12 (3.6)	1 (0.9)	8
	2:314-399	29 (11)	3 (3.5)	10
	3:399-491	60 (22)	12 (13)	20
	4:491-581	62 (23)	15 (16.5)	24
	5:581-679	67 (23)	16 (16.5)	24
E9 / R6	1:202-314	7 (2)	1 (0.9)	14
	2:314-399	18 (7)	3 (3.5)	16
	3:399-491	54 (20)	11 (12)	20
	4:491-581	3 (1.1)	2 (2.2)	67
	5:581-679	68 (23)	16 (16.5)	24
I17 / E9	1:202-314	1 (3.3)	0	0
	2:314-399	20 (7.8)	3 (3.5)	15
	3:399-491	14 (5.1)	1 (1.1)	7
	4:491-581	65 (23)	13 (14.3)	20
	5:581-679	2 (0.7)	0	0
I17 / 10712	1:202-314	62 (18.1)	9 (8)	15
	2:314-399	37 (14.5)	5 (5.9)	14
	3:399-491	44 (16)	9 (9.8)	20
	4:491-581	33 (11.8)	11 (12.1)	33
	5:581-679	69 (23)	13 (13.4)	19

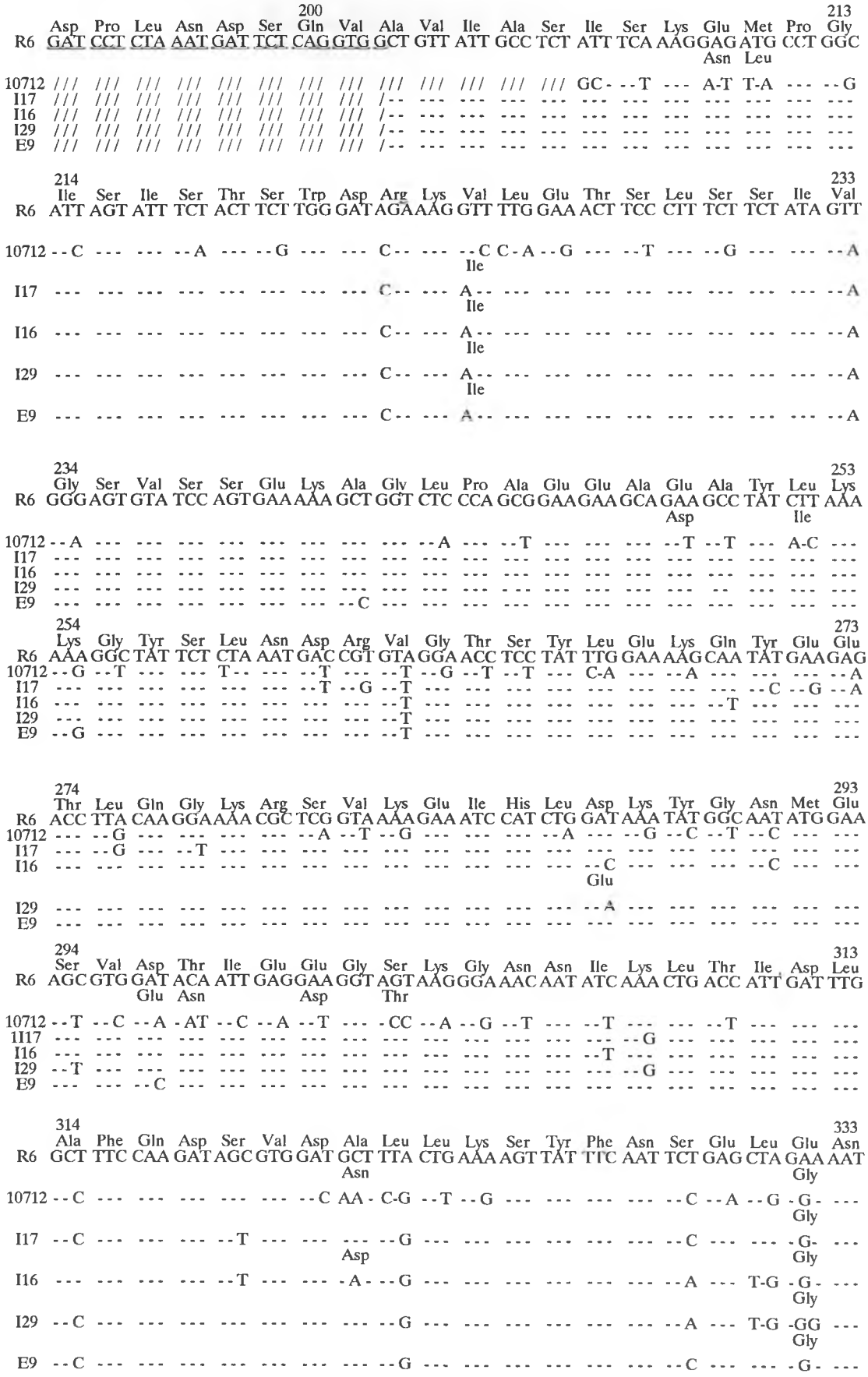

Figure 6.7. Legend page 133.

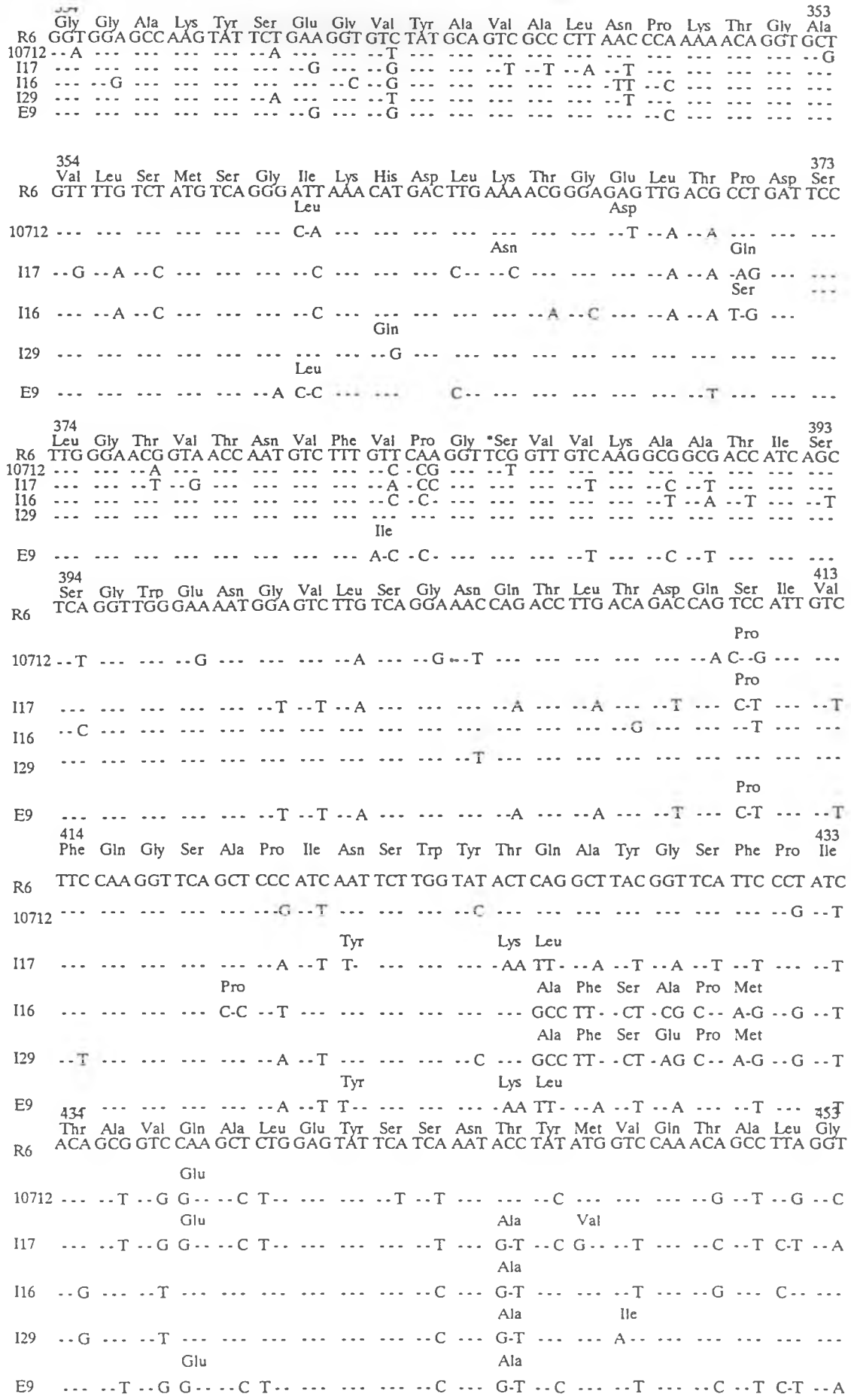

Figure 6.7. Legend page 133.

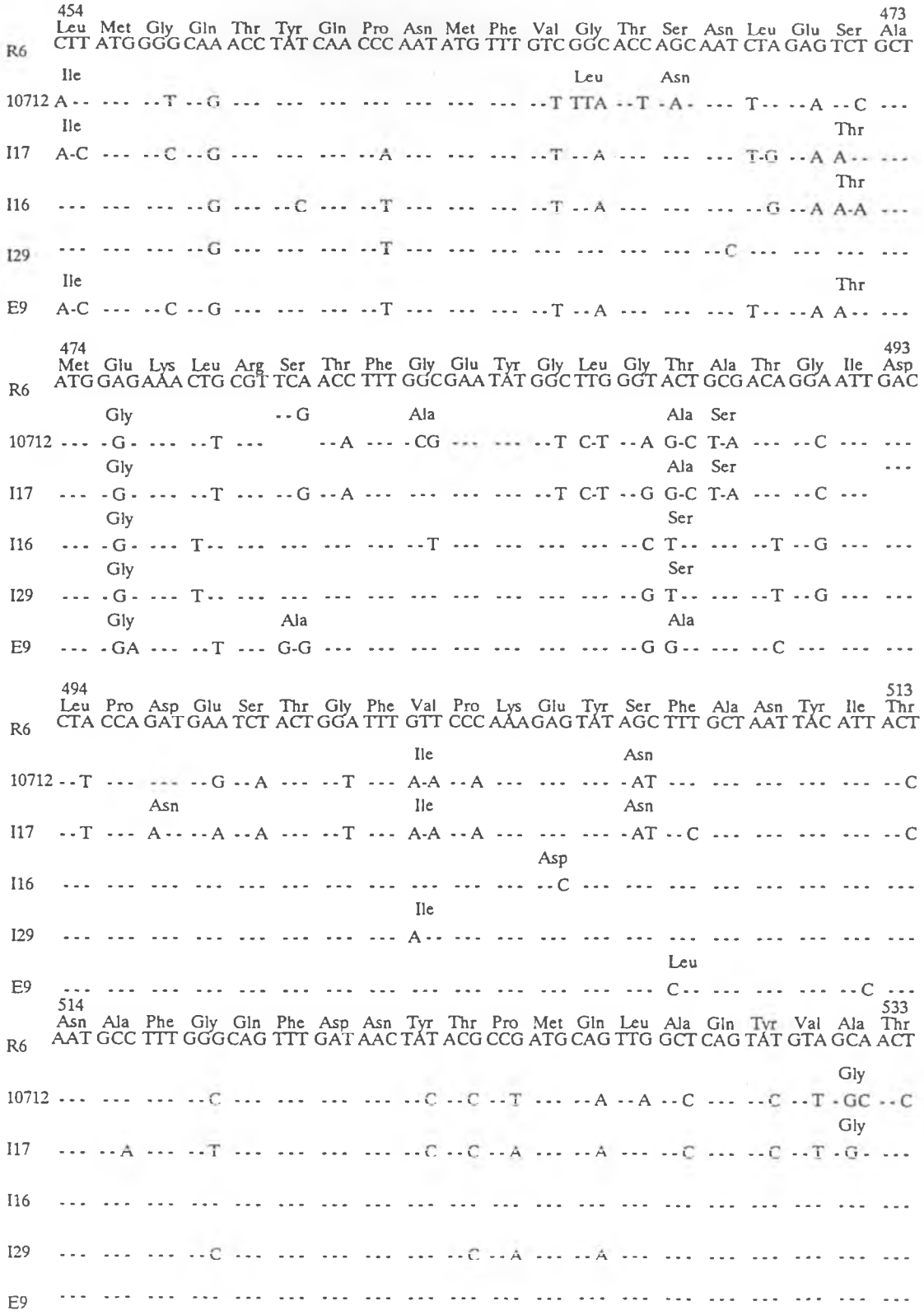

Figure 6.7. Legend page 133.

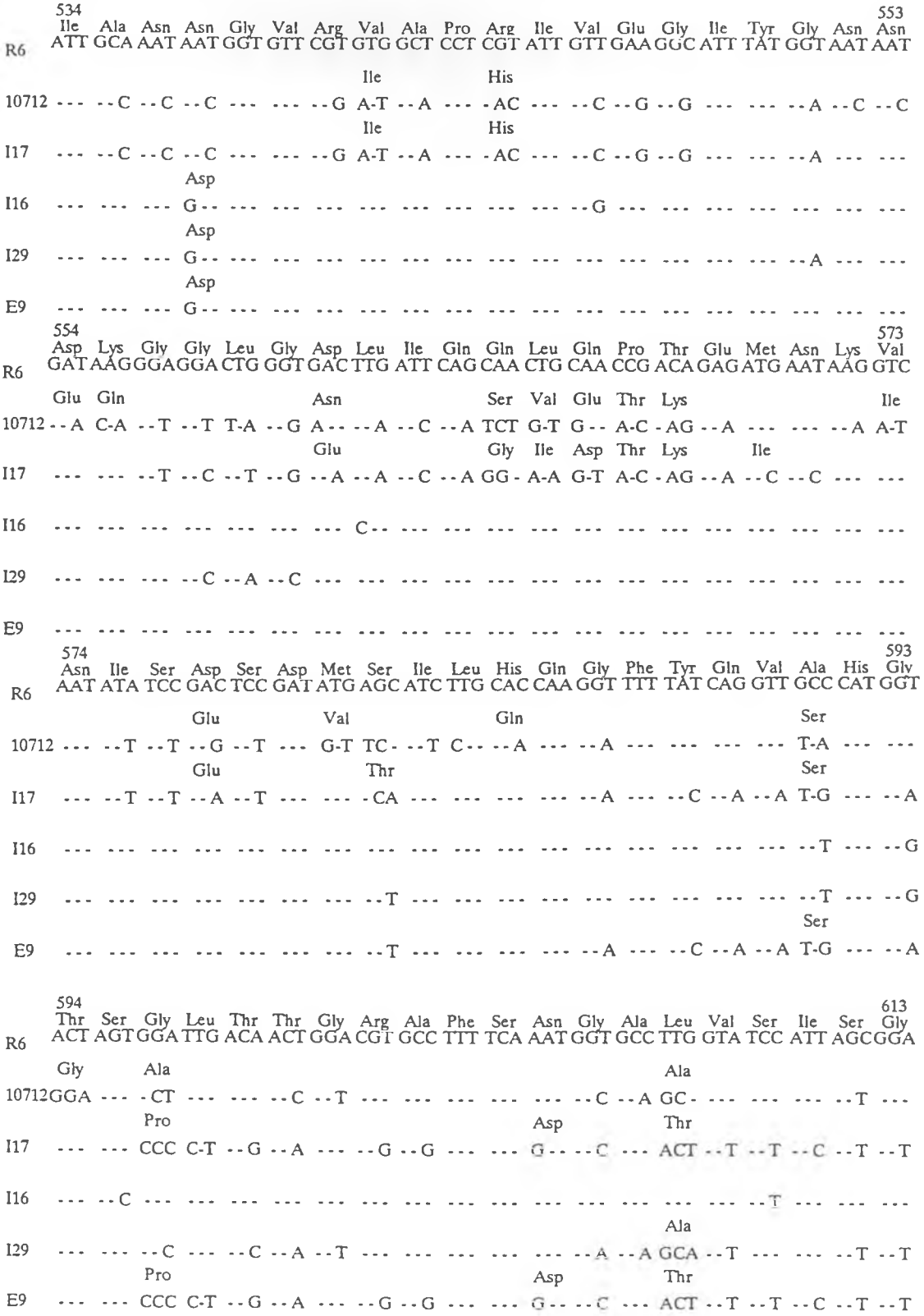

Figure 6.7. Legend page 133.

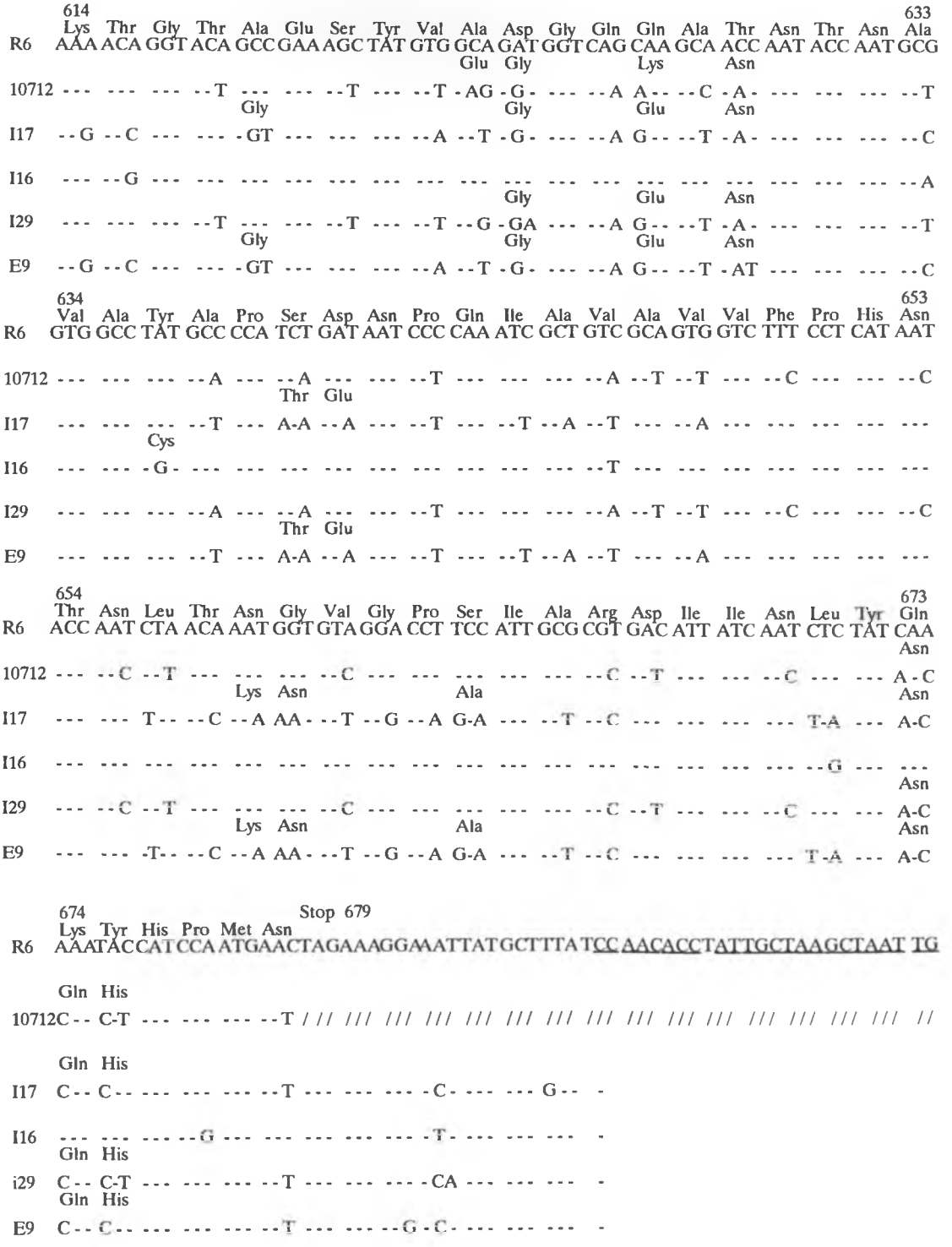

Figure 6.7. Legend page 133.

Figure 6.7. (On pages 128-132) Nucleotide sequences of part of the PBP 2b genes of two *S. mitis* and two *S. oralis* strains. The PBP 2b gene is numbered according to Dowson, Hutchison and Spratt (1989) and the sequence of the penicillin-susceptible strain *S. pneumoniae* R6 is shown in full in line R6 (Dowson, Hutchison & Spratt, 1989). Positions where the sequences of the PBP 2b genes of other strains differ from those in line R6 are shown. The positions of the PCR primers are underlined. /// Indicate unsequenced regions. Lines: 10712, penicillin susceptible *S. mitis* NCTC 10712 (Dowson et al., 1993); I17, penicillin-susceptible *S. oralis*; I16, I29, penicillin-resistant *S. mitis*; E9, penicillin-resistant *S. oralis*. *, active site serine residue.

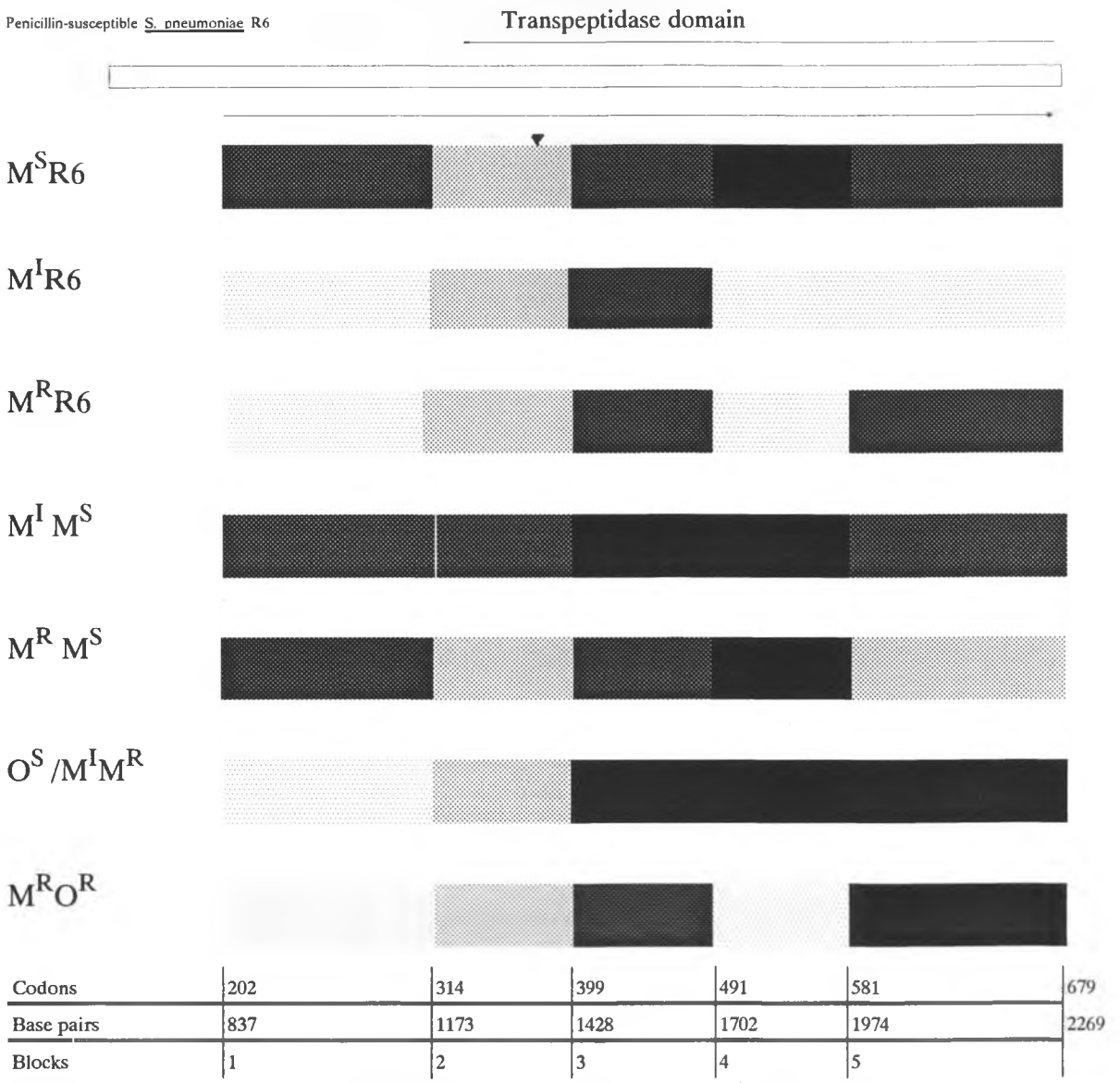

Figure 6.8. PBP 2b genes of penicillin-susceptible and penicillin-resistant streptococci.The PBP 2b gene of the penicillin-susceptible *S. pneumoniae* R6 is presented in the empty block at the top of the figure. The thin line terminating in an arrow shows the extent of the coding region. The thin line starting and terminating in an arrow shows the penicillin-susceptible transpeptidase domain.
Sequenced regions of the following strains are compared:
R6 = penicillin-susceptible *S. pneumoniae* R6 strain, O^S = penicillin-susceptible *S. oralis* strain I17, M^S = penicillin-susceptible *S. mitis* NCTC 10712 strain, O^R = penicillin-resistant *S. oralis* E9 strain, M^I = penicillin intermediately resistant *S. mitis* I16 strain, M^R = penicillin-resistant *S. mitis* I29 strain The position of the active site serine residue is marked.

= Regions of sequence which differ from each other with ≤ 5%.

= Regions of sequence which differ from each other with 6-12%.

= Regions of sequence which differ from each other with 13-19%.

= Regions of sequence which differ from each other with ≥ 20%.

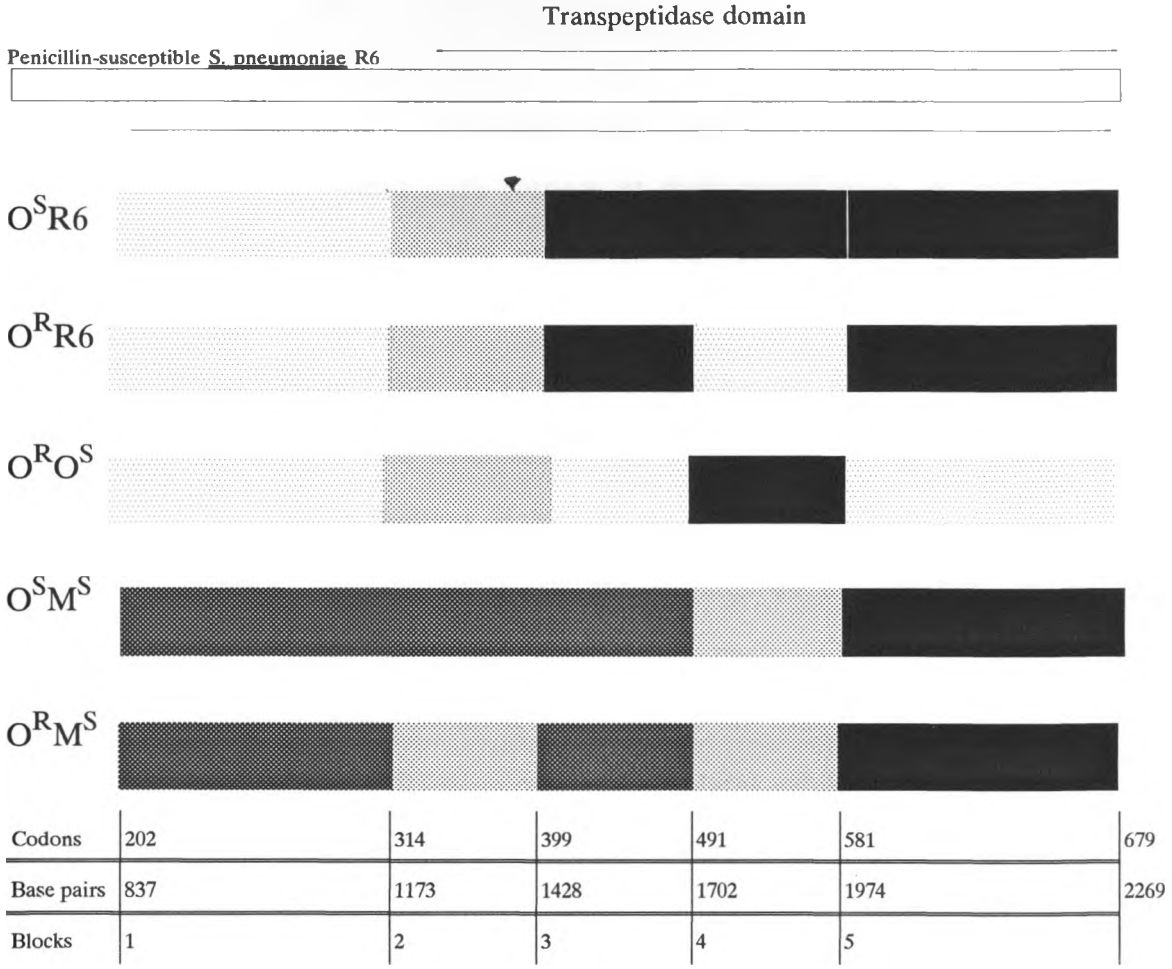

Figure 6.9. PBP 2b genes of penicillin-susceptible and penicillin-resistant streptococci.The PBP 2b gene of the penicillin-susceptible *S. pneumoniae* R6 is presented in the empty block at the top of the figure. The thin line terminating in an arrow shows the extent of the coding region. The thin line starting and terminating in an arrow shows the penicillin-susceptible transpeptidase domain.
Sequenced regions of the following strains are compared:
R6 = penicillin-susceptible *S. pneumoniae* R6 strain, O^S = penicillin-susceptible *S. oralis* strain I17, M^S = penicillin-susceptible *S. mitis* NCTC 10712 strain, O^R = penicillin-resistant *S. oralis* E9 strain. The position of the active site serine residue is marked.

= Regions of sequence which differ from each other with ≤ 5%.

= Regions of sequence which differ from each other with 6-12%

= Regions of sequence which differ from each other with 13-19%

= Regions of sequence which differ from each other with ≥ 20%

CONCLUSIONS

The approach of this section will be to summarise the main features of the present studies with respect ot their significance on the evolution and spread of penicillin resistance between viridans streptococci and *S. pneumoniae.*

The susceptibility of 211 viridans streptococci isolated in South Africa from blood, between 1988 to1991, to 8 antimicrobial agents was determined. Thirty-eight percent of the isolates were found to be resistant to penicillin (MICs $\geq$0.25 μg/ml). All the isolates were susceptible to cefotaxime, ceftriaxone, imipenem and vancomycin. Combined tetracycline and erythromycin resistance were found in 7% of the viridans streptococci studied. Five *Streptococcus mitis* isolates exhibited increased (MIC 64 and 128 μg/ml) or high-level (MIC $\geq$500 μg/ml) resistance to gentamicin. *In vitro* synergy between penicillin and gentamicin was not demonstrated with these isolates. In a collaborative study with A. Kaufhold (Germany) four of these isolates with gentamicin MICs 128 or 1000 μg/ml were shown to contain the structural gene encoding high-level gentamicin resistance, similar to that found in *Enterococcus faecalis, Enterococcus faecium* and *Streptococcus agalactiae*.

Inter- and intra-specific transfer of penicillin resistance genes between *S. mitis, Streptococcus oralis, S. sanguis* and *Streptococcus pneumoniae* was also investigated. Of the 18 *S. mitis / S. oralis* isolates selected, 16 strains were shown to transform the laboratory recipient *S. pneumoniae* R6 to increased levels of penicillin resistance (0.025 μg/ml) with transformation frequencies between $3x10^{-1}$ - $9x10.^{-5}$ All 13 *S. pneumoniae* donors tested were shown to transform the *S. mitis* NCTC 10712 recipient to 0.1 μg/ml with frequencies between $3x10^{-3}$ - $3x10.^{-5}$ None of 10 *Streptococcus sanguis*

donors transformed the *S. mitis* recipient and only one *S. sanguis* donor at low frequency was seen to transform the *S. pneumoniae* recipient. The high frequency with which penicillin resistance determinants were shown to transfer between the oropharangeal commensals *S. mitis* and *S. oralis*, and *S. pneumoniae* has significant implications for the in-vivo transfer of penicillin resistance genes.

Penicillin resistance development in *S. mitis* was investigated using penicillin-binding protein (PBP) affinity studies. *S. mitis* NCTC 10712 was used as a recipient to investigate PBP alterations which could occur as a result of spontaneous mutation and intra- and inter-specific transfer of penicillin resistance genes. *S. mitis* NCTC 10712 possesses seven major PBPs ranging in molecular mass from 49-82 kDa. Comparison of PBP affinity profiles of *S. mitis* NCTC 10712 and a spontaneous mutant (three times more resistant) showed that PBPs 3 and 4 were the first PBPs to alter their affinity as resistance develops. One *S. mitis*, one *S. oralis* and two *S. pneumoniae* penicillin-resistant clinical isolates were used as donors in transformation experiments with *S. mitis* NCTC 10712 (MIC 0.03 μg/ml) as the recipient. Transformants with MICs greater than 1 μg/ml were obtained with *S. mitis, S. oralis* and *S. pneumoniae* donor DNA. Depending on the source of the donor DNA and level of resistance achieved, transformants showed reduced penicillin-binding affinities of PBPs 2, 3, 4, 5 and 6. The most consistant PBP alteration associated with increasing resistance in *S. mitis* NCTC 10712 was seen with PBP 3 (74 kDa). Using DNA from the spontaneous mutant of *S. mitis* NCTC 10712 (MIC 0.06 μg/ml) to transform *S. pneumoniae* R6, a transformant was shown to possess a detectable altered PBP 2x. Similarities in the molecular masses of *S. mitis*, *S. oralis* and *S. pneumoniae* PBPs and PBP affinity alterations observed in transformants

exhibiting increased resistance to penicillin clearly demonstrated interchange of PBP genes between the streptococcal species.

The findings from the reciprocal transfer and PBP studies were extended to investigate PBP 2b similarities between *S. mitis, S. oralis* and *S. pneumoniae* using DNA fingerprinting and sequencing. Polymerase chain reaction (PCR) was performed on 41 penicillin-susceptible and -resistant clinical isolates of *S. mitis* and *S. oralis* using the susceptible *S. pneumoniae* PBP 2b primers (Dowson et al., 1989). PCR products were then analysed using *Hin*fI and *Sty*I restriction enzymes. Of the 41 *S. mitis* / *S. oralis* isolates 15 strains produced a PCR product of a similar size to that of *S. pneumoniae* R6. On fingerprinting these 15 strains , 11 different patterns were seen using *Sty*I restriction enzyme and 12 different patterns using *Hin*fI. The PBP 2b genes of the *S. mitis* and *S. oralis* isolates studied were found to be very heterogeneous. However, on comparing fingerprints of two penicillin-resistant *S. mitis* strains E14 and E11 and one *S. oralis* strain E9, identical *Sty*I and *Hin*fI patterns were seen. These patterns were also found to be identical to those of two further *S. oralis* strains studied by Coffey et al. (1993), a South African *S. pneumoniae* isolate (Smith et al., 1993) and a Spanish *S. pneumoniae* isolate (Coffey et al., 1993).

The *S. mitis* and *S. oralis* sequences revealed as expected mosaic PBP 2b gene structures with areas of homology and others of divergence when compared with each other and with additional related streptococcal species (Dowson et al., 1990; Coffey et al., 1993; Dowson et al., 1993). Two strains of *S. mitis* I16 and I 29 (MIC 0.5 and 2.0 μg/ml respectively) both contain regions which are related to *S. pneumoniae*. In *S. mitis* I16 the *S. pneumoniae* related region was between nucleotides 1703-2269 and in *S.*

mitis I29 between 1703-1974. The origin of the PBP 2b gene area, nucleotides 1174-1702 in both *S. mitis* strains is still unknown.

PBP 2b sequence comparison of susceptible *S. oralis* I17 with reported sequences of *S. pneumoniae* R6 (Dowson, Hutchison & Spratt, 1989) and *S. mitis* 10712 (Dowson et al., 1993) revealed what appears at this stage to be nucleotide regions unique to *S. oralis*. On comparing the penicillin-susceptible (I17) and penicillin-resistant (E9) *S. oralis* strains with each other, these regions, nucleotides 1429-1702 and 1975-2269 showed 95% and 99% similarity at nucleotide level and 99% and 100% similarity at the amino acid level respectively, strongly suggesting that these areas are specific to *S. oralis*. The penicillin-resistant *S. oralis* strain E9 contained a *S. pneumoniae* region of 272 bp (nucleotides 1703-1974) which was flanked by *S. oralis* sequences. *S. pneumoniae* has now been shown to contain PBP 2b and PBP 2x (Sibold et al., 1994) regions which could have been aquired from susceptible isolates of *S. oralis.*

On comparing *S. oralis* E9 with penicillin-resistant *S. oralis* strains isolated in England (Dowson et al., 1990) and *S. pneumoniae* strains isolated in South Africa and Spain (Coffey et al., 1993) the PBP 2b gene was found to be almost identical. These results give further evidence of interspecies and intercontinental spread of penicillin resistance genes amongst streptococci.

Considerable advances have been made during the past five years concerning the evolution and spread of penicillin resistance in viridans streptococci and *S. pneumoniae*. Further studies on PBP 2b genes from strains isolated before the introduction of penicillin are required to determine the origins of still unidentified diverse gene regions and locate recombination sites which have given rise to the mosaic structures encountered today.

APPENDIX A.

pUC CLONING

I. *Preparation of cleaved pUC vector DNA.*

1.Add the following to a sterile microfuge tube (20 μl final volume):

10 μl = 1 μg pUC 18 DNA.

2 μl 10x restriction buffer.

7 μl sterile distilled water.

2-5 units *Sma*I restriction enzyme.

2. Incubate for 1h at 25°C.

3. Add 205 μl TE buffer (Tris-HCl, 10mmol/l; EDTA, 1mmol/l; pH 8.0) and extract once with 200 μl phenol/chloroform/isoamyl alcohol, and once with 200 μl chloroform/isoamyl alcohol.

4. Add 25 μl sterile LiCl solution (4mmol/l) and 750 μl ethanol, 95%.

5. Chill for 5 min at -70°C (ethanol/dry ice mixture).

6. Centrifuge for 10 min, remove supernatant carefully and discard.

7. Add 1 ml ethanol, 70% to the precipitate.

8. Centrifuge for 5 min.

9. Remove supernatant. Dry precipitated DNA briefly under vacuum.

10. Dissolve in 10 μl sterile TE buffer.

11. Check 1 μl = 0.1 μg on an 0.8% agarose gel.

II. *Ligation.*

1. Add the following to a sterile microfuge tube on ice:

1 μl = 0.1 μg cleaved pUC DNA.

1 μl 10x ligation buffer.

1 μl = 1 unit T4 DNA ligase undiluted solution for blunt-end ligations.

2. Incubate overnight at 22°C.

III. *Preparation of competent E. coli cells.*

1. Remove a single colony of *E. coli* JM101 from a LB agar plate [1% Bacto tryptone (Difco), 0.5% Bacto yeast extract (Difco), 0.5% NaCL and 1.5% Bacto agar (Difco)] and inoculate into 5 ml LB liquid medium. Incubate the culture overnight at 37°C with shaking.

2. Inoculate 40 ml LB liquid medium with 0.4 ml fresh overnight culture and incubate with shaking at 37°C to A_{550} = 0.2-0.5.

3. Centrifuge cells for 10 min at 3000 g in a precooled rotor.

4. Resuspend pellet in 20 ml ice-cold sterile $CaCl_2$ (50mmol/l) solution and stand on ice for 30 min.

5. Centrifuge cells as described.

6. Resuspend pellet in 4 ml ice-cold sterile $CaCl_2$ solution and keep on ice.

IV. *Transformation.*

1. Mix ligated DNA with sterile Tris-HCl (50 mmol/l, pH 7.2) solution to give final volume of 50 μl.

2. Mix DNA in a sterile thin-walled test tube on ice with 300 μl competent *E. coli* cells and incubate on ice for 40 min.

3. Warm test tubes in water bath to 42°C (3 min) and let stand at room temperature for 10 min.

4. Add 1 ml LB liquid medium and shake for 30-60 min at 37°C.

5. Distribute 0.2 ml each on LB / Ampicillin (100 μg/ml) / X-gal (0.004%) / IPTG (0.5mmol/l) plates

6. Incubate plates at 37°C.

7. Choose white colonies for examination.

APPENDIX B

pCR-SCRIPT™ SK(+) CLONING

I. *Ligation.*

1. Add to a microfuge tube in the following order, to a final volume of 11 μl:

    5 μl pCR-Script vector (10 ng/μl).

    1 μl 10x Universal buffer.

    0.5 μl rATP (10mM).

    2 μl PCR product (40-200 ng).

    1 μl SrfI restriction enzyme (5U/μl).

    1 μl T4 DNA ligase

    0.5 μl dH_2O.

2. Mix gently and incubate for 1 h at room temperature.

3. Heat samples for 10 min at 65°C.

4. Store the samples on ice until transformation into competent *E. coli.*

II. *Transformation.*

1. Thaw the competent cells on ice.

2. Gently mix the cells by hand.

3. Add 0.7 μl β-mercaptoethanol (1.44M) 40 μl of bacteria, to yield a final concentration of 25mM.

4. Swirl gently. Place on ice for 10 min, swirling gently every 2 min.

5. Add 2 μl (10 ng) of DNA from step 4 of the ligation protocol to the cells and swirl gently.

6. Place on ice for 30 min.

7. Heat-pulse in a 42°C water bath for 45 seconds.

8. Place transformation mixture on ice for 2 min.

9. Add 0.45 ml of pre-heated (42°C) SOC medium, and incubate at 37°C for 1 h with shaking at 225-250 rpm.

10. Sread 100-150 μl of the transformation mixture onto

ampicillin (100 μg/ml) / X-gal (0.004%) / IPTG (0.5mmol/l) / LB agar plates.

11. Incubate overnight at 37°C.

12. Choose white colonies for examination.

SOC medium (1 l).

20 g Tryptone

5 g Yeast extract

0.5 g NaCl

H_2O to 900 ml

Autoclave

Mix separately:

2.03 g $MgCl_2$

1.2 g $MgSO_4$

3.6 g glucose

Add H_2O to a final volume of 100 ml

Filter-sterilise and add to cooled, autoclaved media

APPENDIX C

SEQUENCING DOUBLE-STRANDED DNA

1. To a precipitate of approximately 2 μg alkali-denatured plasmid DNA add the following:

    6 μl dH_2O

    2 μl DMSO (Merk)

    2 μl Sequenase buffer

    1 μl (10 ng) sequencing primer

2. Incubate the annealing mixture at 65°C for 2 min and cool to 35°C in 30-45 min.

3. Keep annealed samples on ice.

4. To the annealed mixture add:

    1 μl dithiothreitol 0.1M (DTT)

    2 μl 5x diluted labeling mixture,

    1 μl α-^{35}S-dAPT (Amersham)

    2 μl 8x diluted sequenase 2.0

5. Add 3.5 μl of this reaction mixture to 2.5 μl of each of the termination mixtures (G, A, T, C) which until then were kept on ice.

6. Allow reactions to proceed at 48°C for 10 min.

7. Add 1 μl of Klenow polymerase (0.25 U/μl) and continue to incubate at 48°C for another 3 min.

8. Add 4 μl stop solution and keep on ice.

9. Denature at 80°C for 3 min before electrophoresis.

Electrophoresis was performed using the Tris-borate-EDTA buffer system for 2-5 h on 7 M urea / 6% polyacrylamide gels.

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