CHARACTERISATION OF ANTIBIOTIC RESISTANCE IN *Streptococcus, Enterococcus* and *Staphylococcus* USING A BIOINFORMATICS APPROACH

By

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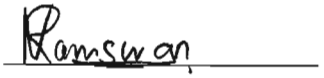
PREFACE

The experimental work described in this dissertation was carried out in the School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of Kwa-Zulu of Natal, Pietermaritzburg, from January 2005 to December 2005, under the supervision of Dr. Mervyn Beukes.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.

Signed: 

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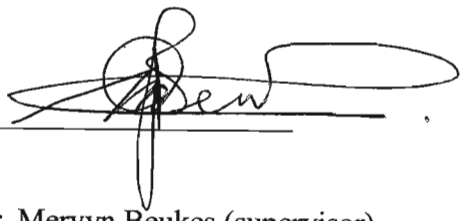
Signed: 

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ABSTRACT

The rate at which bacterial pathogens are becoming resistant to antibiotics is quite alarming, and therefore much attention has been focussed on this area. The mechanism whereby the bacterial cells acquire resistance is studied in order to determine how this process works as well as to determine if any future resistance mechanisms can be circumvented. In this study three different genera and the antibiotics that are resistant to them were used, namely, penicillin resistant *Streptococcus*, vancomycin resistant *Enterococcus* and methicillin resistant *Staphylococcus*. The results prove that the active sites SXXK, SXN and KT(S) G in the penicillin resistance *Streptococcus* plays a major role in resistance. It is seen in this study that the SXXK active site is found in all the resistant and most of the intermediate strains, therefore proving to be an important component of the cell wall resistance. It was subsequently noticed the greater the number of mutations found in the sequences the higher the resistance. Three dimensional structures showed the actives sites and their binding pockets. The results also show the change in conformation with a mutation in the active site. The results also proved that the Penicillin Binding Protein (PBP) genes essential for resistance are PBP 1a, PBP 2b and PBP 2x. The results obtained, for the vancomycin resistance in *Enterococcus* study, proved that the VanC and VanE cluster are very much alike and VanE could have evolved from VanC. There is also close similarity between the different ligase genes. The VanX 3D structure shows the position of the critical amino acids responsible for the breakdown of the D-Ala-D-Ala precursors, and the VanA ligase 3D structure shows the amino acids responsible the ligation of the D-Ala-D-Lac precursors. The analysis performed on the methicillin resistance in *Staphylococcus* study showed that the genes used to confer resistance are very similar between different strains as well as different species.

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LIST OF ABBREVIATIONS

Aux	Auxiliary
BAY	Bayesian
β-OHTyr	β-hydroxytyrosine
CSP	Competence stimulating peptide
D-Ala-D-Ala	D-Alanyl-D-Alanine
D-Ala-D-Lac	D-Alanyl-D-Lactate
D-Ala-D-Ser	D-Alanyl-D-Serine
DPGH	Dihydroxyphenylglycine
Fem	Factor essential for the expression of methicillin
GT	Glycosyltransferase
HPG	Hydroxyphenylglycine
I	Intermediate
kb	Kilo Bases
kDa	Kilo Dalton
MIC	Minimum inhibitory concentrations
ML	Maximum Likelihood
MP	Maximum Parsimony
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin sensitive *Staphylococcus aureus*
NJ	Neighbour Joining
NMR	Nuclear Magnetic Resonance
ORF	Open reading frames
PBP	Penicillin-Binding protein
R	Resistant
S	Susceptible
SCC*mec*	Staphylococcal cassette chromosome *mec*
TP	Transpeptidase
VRE	Vancomycin resistant Enterococci
μg/ml	Micrograms per millilitre
3D	Three-Dimensional

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CHAPTER ONE - INTRODUCTION

The discovery of a penicillin producing mould by Alexander Fleming in 1929 was a major breakthrough in the microbial world which signified one of the important steps in our fight against pathogenic micro-organisms. (Hakenbeck, 1999). The discovery was a great relief at the time, since it destroyed the biggest wartime killer, bacteria from infected wounds. Penicillin, the product of the soil mould *Penicillium* crippled many types of disease-causing bacteria. The fact that the mould produced – compound toxic to the bacterium was actually of great insight. However, its real importance depended on the lack of toxicity of penicillin to humans, also the isolation of penicillin for study and modification, and finally its stability which is sufficient enough to be useful (Koch, 2003).

Since the introduction of antibiotics, physicians and patients are now dependant on these drugs to treat almost everything from sore throats and urinary tract infections to meningitis and tuberculosis. Antibiotics are also used to prevent infections before, during, and after surgery (Judith, 1997). The continual use and misuse of the antibiotics has led to the increase in antibiotic resistant bacteria. When an antibiotic is used to treat an infection it kills the bacterium, but sometimes a few bacterial cells fight off the antibiotic so, even in the presence of an antibiotic, these bacteria survive and reproduce, passing on their antibiotic resistance. The more antibiotics are used, the more rapidly this process happens (Koch, 2003).

The bacteria gained resistance to the antibiotic that was used to kill them, which left scientists the task of finding new antibiotics, (Neu, 1992; Tomasz & Swartz, 1994). Research is critical to help understand the various mechanisms that pathogens use to evade drugs. Understanding these mechanisms is important for the design of effective new drugs.

Antibiotics are low-molecular weight substances that are produced as secondary metabolites by certain groups of micro-organisms, especially *Streptomyces*, *Bacillus*, and a few moulds (*Penicillium* and *Cephalosporium*) that are inhabitants of soils. Antibiotics may have a cidal (killing) effect or a static (inhibitory) effect on a range of microbes. The range of bacteria or other micro-organisms that is affected by a certain antibiotic is expressed as its spectrum of

action. Antibiotics effective against prokaryotes which kill or inhibit a wide range of Gram-positive and Gram-negative bacteria are said to be broad spectrum. If effective mainly against Gram-positive or Gram-negative bacteria, respectively they are said to have a narrow spectrum. If effective against a single organism or disease, they are referred to as having a limited spectrum (Rolinson, 1998).

Different antibiotics destroy bacteria in different ways. Some short-circuit the processes by which bacteria receive energy. Others disturb the structure of the bacterial cell wall, as shown in the illustration (refer to Figure 1.1). Still others interfere with the production of essential proteins. Examples include (i) penicillin, cephalothin, ampicillin and amoxycillin which inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly; (ii) streptomycin, gentamicin, tetracycline, clindamycin and erythromycin inhibits translation (protein translation); (iii) vancomycin and bacitracin inhibits steps in murein (peptidoglycan) biosynthesis and assembly (shown in Figure 1.1); (iv) polymyxin damages the cytoplasmic membrane and finally; (v) amphotericin inactivates membranes containing sterols (Heijenoort, 1998).

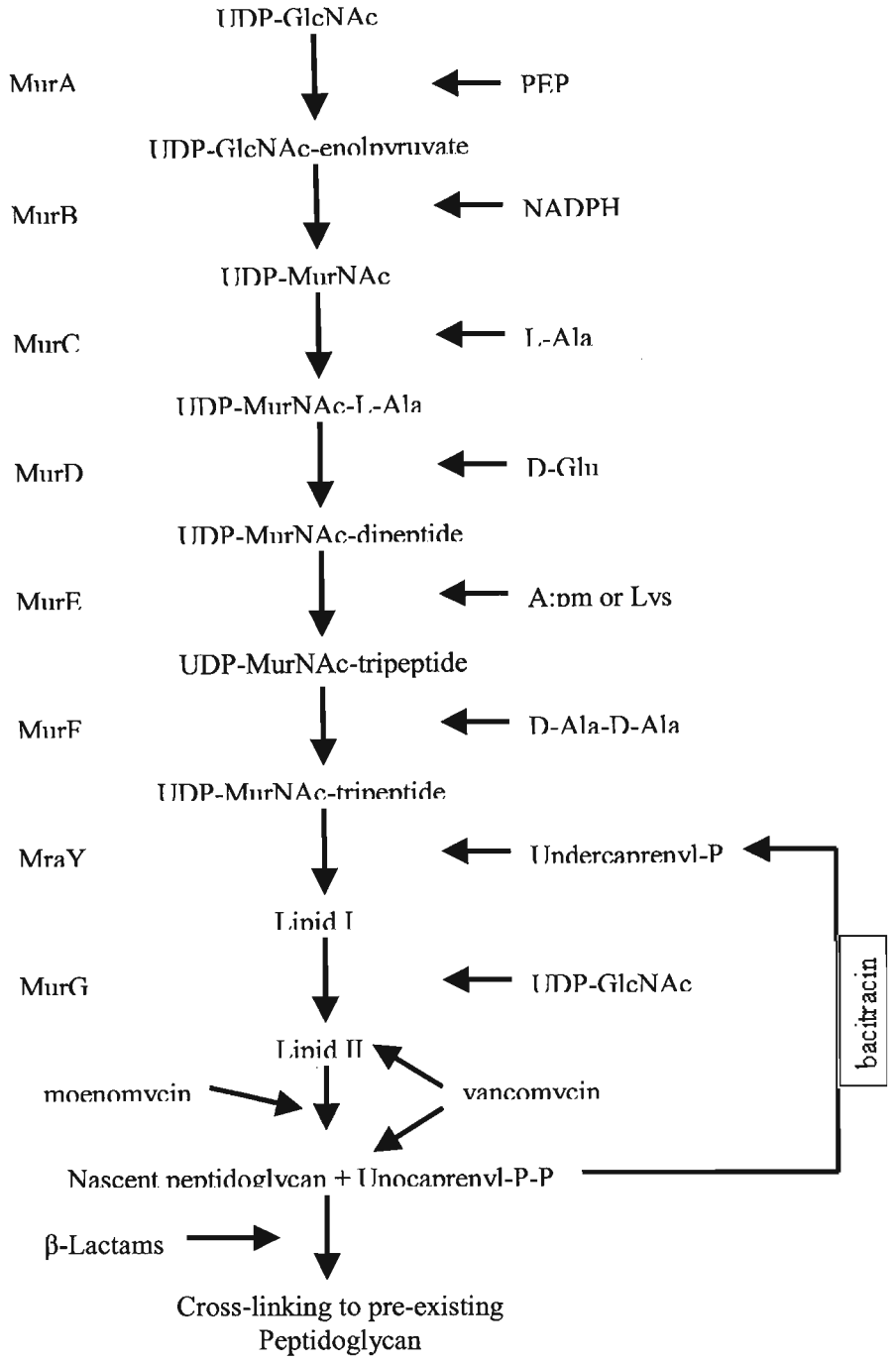


Figure 1.1. Steps involved in the production of peptidoglycan with different antibiotics and the positions of interference with polymerization. (Heijenoort, 1998).

The antibiotics which are in broad clinical use (penicillin, cephalosporin, tetracycline, macrolide-lincosamide, chloramphenicol, aminoglycoside, and glycopeptide classes) have

appeared at different stages in history (Refer to Figure 1.2) to help keep the pathogenic bacteria at bay (Rolinson, 1998). With the continued use of these antibiotics, positive-function resistance determinants enter pathogen populations, and their prevalence is the major threat to the continued success of these antibiotics (Silver & Bostian, 1993).

One of the biggest problems for successful antibiotic coverage, and hence the driving force behind the search for new therapies, is the evolution and spread of antibiotic resistance. The resistance of common or resurgent pathogens to standard antibiotic therapies is a significant nosocomial problem and is of increasing importance in community-acquired infections as well (Silver & Bostian, 1993).

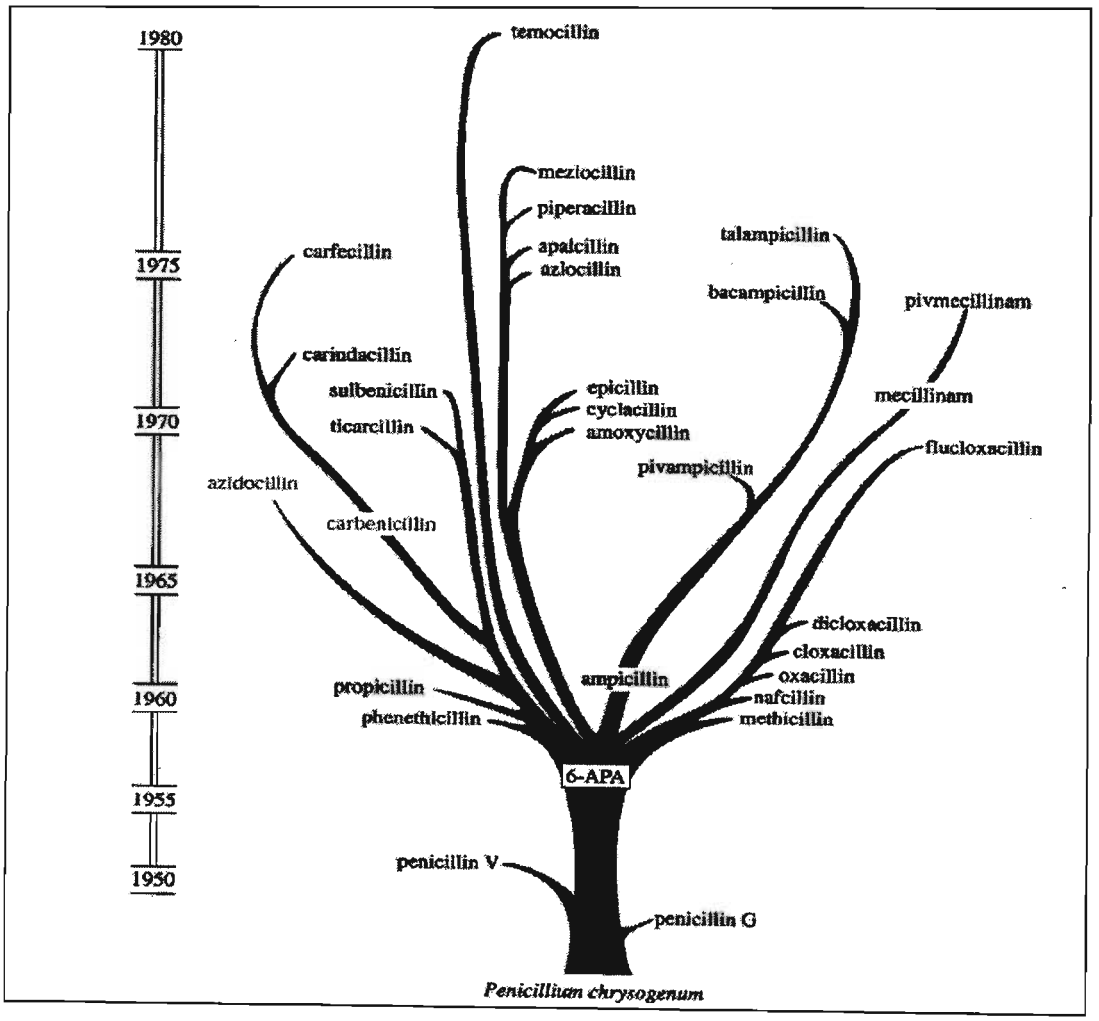


Figure 1.2. The years in which the respective antibiotics appeared, the 6-APA referring to the 6-aminopenicillanic acid (Rolinson, 1998).

Much attention has focused on the unique and quintessential element of the bacterium, namely the bacterial cell wall (Koch, 2003). The cell wall, as well as the mechanisms of the bacterial cell wall growth and function is highly sophisticated. The bacterium produces the cell wall structure to protect it against osmotic shock and other stresses and to house the cytoplasm efficiently requires conformity with the engineering principles. The peptidoglycan, which is a major component of the bacterial cell wall, also forms the murein sacculus, a giant macromolecule that surrounds the cell as a single, flexible meshwork which is closely involved in cell division (Koch, 2003).

The peptidoglycan biosynthesis is one of the favoured targets for antibiotics; due to the fact that the peptidoglycan is unique to eubacteria and stopping or blocking its synthesis leads to the bacterial cells death. The structure of this molecule predicts the shape and maintains cell integrity by protecting the cell against high internal osmotic pressure (Rohrer & Berger-Bachi, 2003). The absence of the cell wall results in a hypotonic medium and therefore lead to swelling and hence the rupture of the cytoplasmic membrane. Cells that lack the peptidoglycan structure can be maintained as protoplasts or spheroplasts, however they lose their shape and cell division is hampered and may be significantly disturbed. The main structural feature of the peptidoglycan is the linear glycan chains interlinked by short peptides (refer to Figure 1.3.) (Heijenoort, 2001).

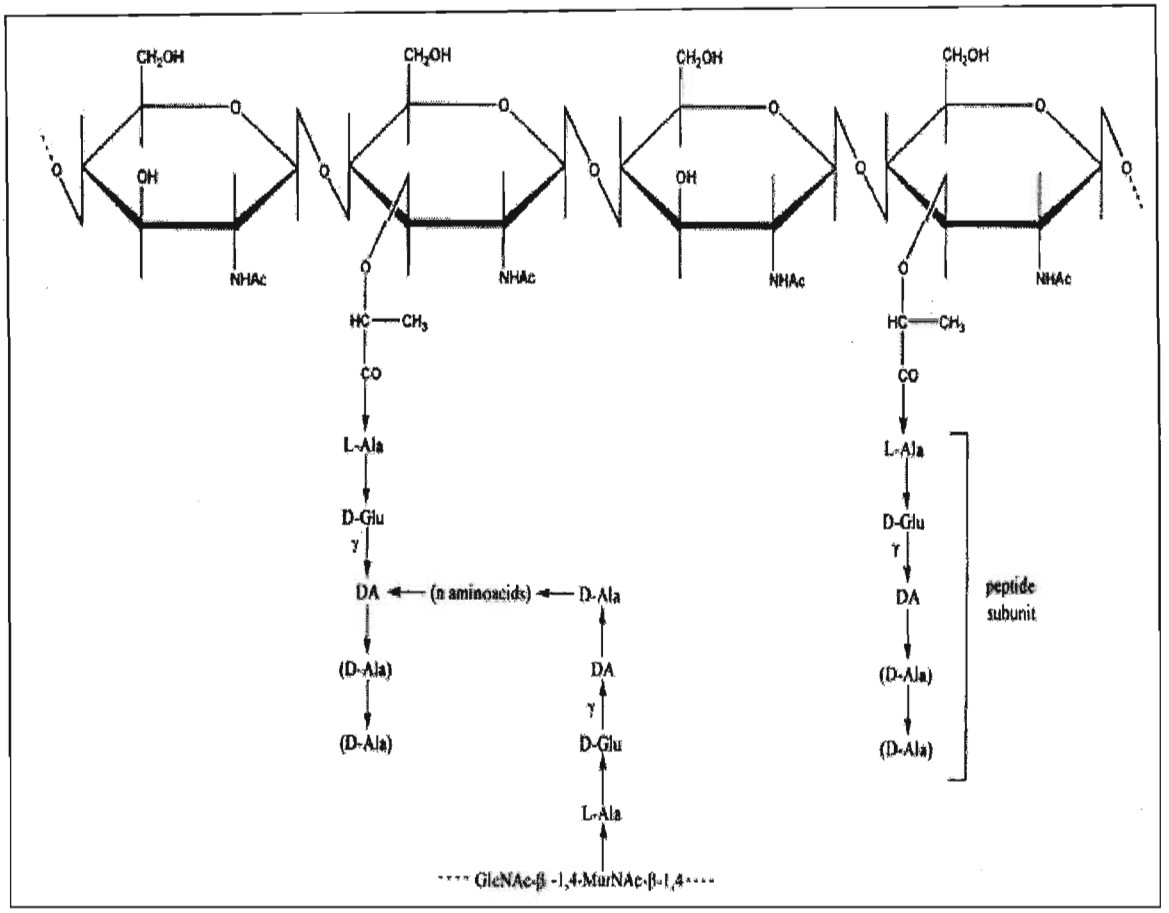


Figure 1.3. A schematic representation of the structure of peptidoglycan showing a section of the glycan chains interlinked by short peptides (Heijenoort, 2001).

1.1. Penicillin resistance in *Streptococcus*

Bacteria may have come into existence more than 3.5 billion years ago (Holland, 1997). A primary structural feature of bacteria that helped them survive is the cell wall, whose function is indispensable for the organism, providing support for the maintenance of bacterial morphology (Massova & Mobashery, 1998). In the absence of an effective cell wall, as in bacteria treated with inhibitors of cell wall biosynthesis, the bacteria will be capable of surviving only in media that match their internal osmotic pressure. Since this is generally not the case, the bacteria rely on their cell walls to perform the task of keeping their internal and the outer osmotic pressure separate (Koch, 2003).

Peptidoglycan synthesis occurs through a biosynthetic pathway that is initiated in the bacterial cytoplasm; the last steps occur on the periplasmic side of the bacterial membrane (Rohrer & Bachi, 2003). The cytoplasmic steps of the pathway are catalyzed by 'Mur enzymes' and result in the synthesis of lipid II molecules, which harbour the peptidoglycan building units and are subsequently trans-located across the cytoplasmic membrane (Heijenoort, 1998). The final steps of synthesis, namely the polymerization of the glycan chains (Figure 1.3) and their reticulation, are catalyzed by the glycosyltransferase and transpeptidase activities of Penicillin-Binding Proteins (PBPs) (Ghuysen, 1994; Di Guilmi *et al.,* 2003).

The β-lactams, which are small organic molecules with four-member strained lactam rings (Koch, 2003) and their derivatives (refer to Figure 1.4) are the most widely used chemotherapeutics in the 40 years since penicillin's discovery. The common factor between the various β-lactam antibiotics is the β-lactam ring. The most important cause of their wide application is probably due to the low toxicity of penicillins and cephalosporins to eukaryotic cells. Tipper and Strominger proposed that penicillin inhibited peptidoglycan synthesize and killed growing susceptible bacteria through the conformational similarity between penicillin and the D-Alanyl-D-Alanine part of the peptidoglycan (Tipper & Strominger, 1965).

The β-lactams include an enormous number of compounds many of which are semi-synthetic and can be divided into the bicyclic penicillins (penams, penems, carbapenems, oxapenams)

and cephalosporins (cephems, cephamycins, oxacephems, carbacephems) and the monocyclic monobactams (Georgopapadakou, 1993). β-Lactam antibiotics normally inactivate PBPs and therefore interfere with the process of transpeptidation by the formation of an ester bond between the active site serine and the carbonyl carbon of the β-lactam. Therefore the β-lactams mimic the acyl-D-Ala-D-Ala of the bacterial cell wall in their interactions with the transpeptidases (Hujer *et al.*, 2005).

The β-lactam antibiotic has shown great success in the past in combating the microbial pathogens; however, their introduction into therapy has led to the development and spread of resistance in the bacterial community (Rohrer & Bachi, 2003). Massive scale research is being done on the topic, in order to overcome the issue of bacterial resistance to the β-lactam antibiotics. Extensive use of β-lactam antibiotics has led to the selection of pathogenic streptococci resistant to β-lactams due to modifications of the PBPs (Pagliero *et al.*, 2004). The resistance to the β-lactams is caused by the presence of β-lactamases, as well as the presence of mutations in the PBPs, resulting in the reduced affinity for β-lactam antibiotics (Fluit *et al.*, 2001). The β-lactam resistance can also be attributed to the generation of mosaic PBP genes, which result from intra- and interspecies recombination with sequences from related streptococci. These genes encode PBPs harbouring several substitutions, and are spread throughout the entire length of the protein (Nagai *et al.*, 2002), which therefore leads to a loss of affinity for the antibiotics (Pernot *et al.*, 2004). The resistance is less frequently caused by reduced uptake due to changes in the cell wall or active efflux (Fluit *et al.*, 2001).

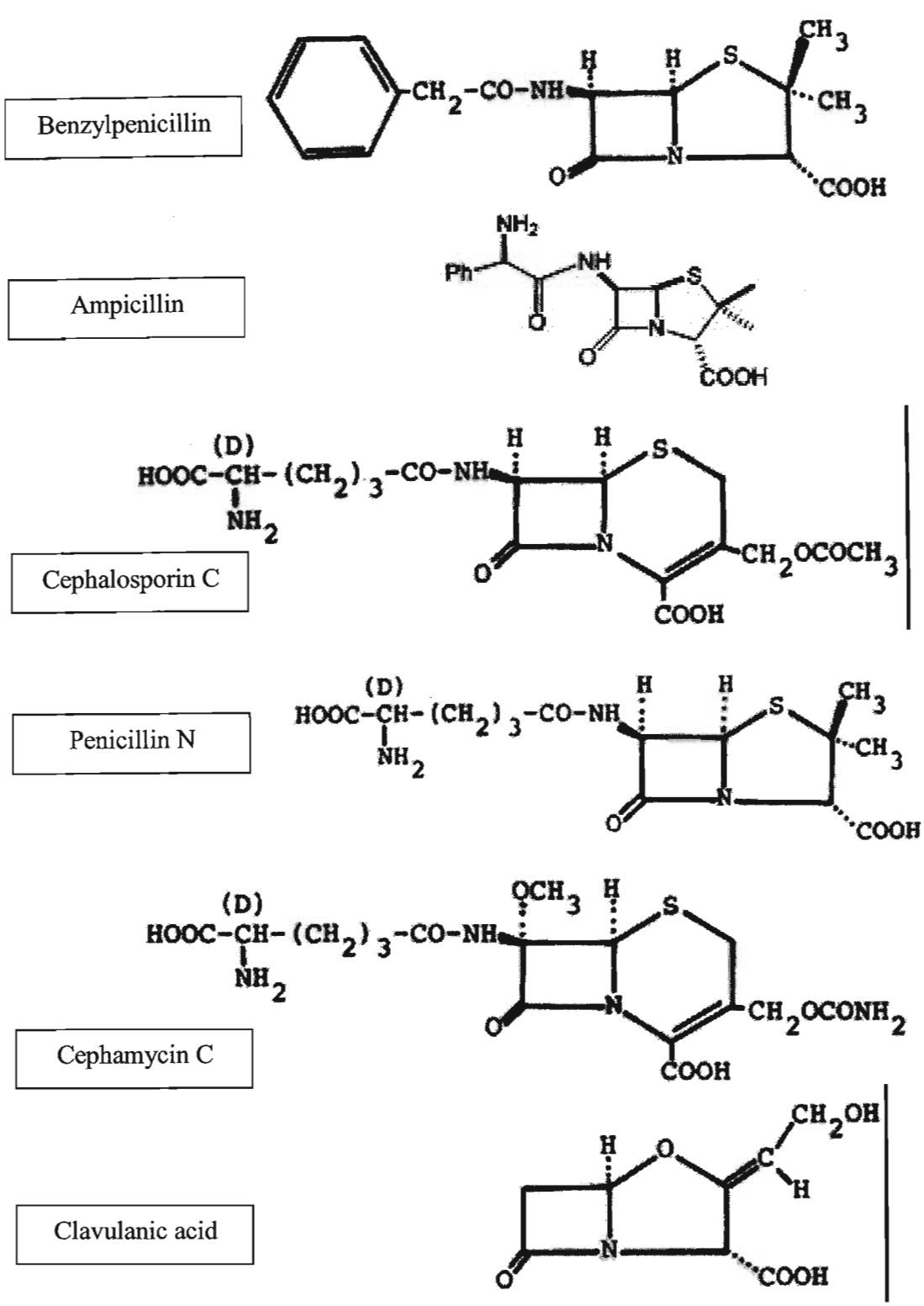


Figure 1.4. A schematic representation showing some of the common occurring Beta-Lactam antibiotics (Ogawara 1981; Hujer *et al.*, 2005).

1.1.1. β-Lactamase

The β-lactamase is the enzyme responsible for resistance to the β-lactam antibiotics. This enzyme is responsible for the breakdown of the β-lactam ring, without which the antibiotic is inactive (Ogawara, 1981). The β-lactamase does so by catalyzing the hydrolysis of the β-lactam ring of penicillin and cephalosporin. This reaction disrupts one of the most important sites for their antibacterial activity, this reaction is irreversible. Hence the β-lactamase plays a significant role in resistance to β-lactam antibiotics in the clinical field. Particularly when it is remembered that the gene that codes for β-lactamase in *Staphylococcus aureus*, is located on a plasmid and can be transferred easily to other susceptible bacteria via bacteriophages. In Gram-negative bacteria it is also located on an R-plasmid and be can transferred by conjugation (Ogawara, 1981). The β-lactamase enzyme has a common property of catalyzing the hydrolysis of the β-lactam ring of penicillin and cephalosporin. It is remarkable that although β-lactam antibiotics have various killing targets including PBPs, they are hydrolyzed to inactive compounds by only one enzyme (β-lactamase). This forms a striking contrast to many other antibiotics. Unlike most other enzymes, β-lactamase is produced by a wide range of prokaryotes. A huge number of penicillin resistant strains are present due to the presence of β-lactamases. However, there is the exception of some intrinsically resistant or tolerant strains and an example of this is that of methicillin resistance (Ogawara, 1981).

There have been several classification systems for the β-lactamases, one of the most common one is the classification scheme that is based on their nucleotide sequence, which consist of the class A, B, C and D. The class A, C and D are similar to each other in that their active sites contains a serine, while the class B enzymes has four zinc atoms at their active sites. The class A enzymes are highly active against the benzylpenicillin (refer to Figure 1.4). The other β-lactamases that also belong to this class of enzymes are the extended-spectrum β-lactamases (ESBLs) (Fluit *et al.*, 2001). This group of enzymes is also highly active against benzylpenicillin and cephalosporin (refer to Figure 1.4) and/or monobactam antibiotics. Class B β-lactamases are equally active against the antibiotics penicillin and cephalosporin and some of these enzymes also inactivate carbapenems. The class C is inducible; however mutations can lead to over expression. Finally class D is composed of the OXA-type enzymes, which

are capable of hydrolyzing oxacillin. The genes that encode for the β-lactamase are located on the plasmids and can also be found on the bacterial chromosome in both the Gram-negative and positive organisms (Fluit *et al.*, 2001). A β-lactamase could have evolved from a PBP, and for this process to occur the protein needs to acquire the ability to undergo deacylation of the acyl enzyme species. The two-step process would therefore complete the hydrolytic pathway (Massova & Mobashery, 1998).

1.1.2. Penicillin-Binding proteins

The critical sets of enzymes, which are responsible for the production of the peptidoglycan, are termed the PBPs. The reason for these proteins being called PBPs is historic. Penicillins in their active site serine residues covalently modify the proteins. The acyl enzyme species are inactive and stable in comparison to the β-lactamases (Massova & Mobashery 1998). The peptidoglycan synthesis occurs during the two major stages, the cell wall elongation and cell division, or alternatively termed septation. The cell division starts only when the bacterial homolog of tubulin, namely FstZ, polymerizes in the appearance of a contractile ring found at the future division site, thereafter approximately 12 proteins are recruited to the site of the constricting ring (Machebouf *et al.*, 2005).

The dissection of the functions of PBPs within growth and division processes has proven to be challenging in micro-organisms such as *Escherichia coli,* which has twelve PBPs, and *Bacillus subtilis,* has seven PBPs that also participates in sporulation. *Streptococcus pneumonia* has six PBPs, three of which are used for glycosyltransfer and transpeptidation of the peptidoglycan (PBPs 1a, 1b, and 2a; belong to the class A group), the other two which exclusively are used for the transpeptidation reaction (PBPs 2x and 2b; belong to the class B group), and finally the last one, the PBP 3, acts as a D, D-carboxypeptidase (Goffin & Ghuysen, 1998; Machebouf *et al.*, 2005). These PBPs are also divided according to their size: the five high-molecular-mass PBPs are PBP 1a (79.7 kDa), PBP 1b (89.6 kDa), PBP 2a (80.8 kDa), PBP 2b (82.3 kDa), PBP 2x (82.3 kDa), including the single low-molecular-mass PBP 3 (45.2 kDa) (Nagai *et al.*, 2002; Dowson *et al.*, 1989; Dowson *et al.*, 1993; Dowson *et al.*, 1994; Hakenbeck, 1999; Hakenbeck *et al.*, 1998 and Hakenbeck *et al.*, 1999). The high-molecular-mass PBPs are made up of an

N-terminal hydrophobic region, a central penicillin-binding domain, and a C-terminal domain (Nagai *et al.*, 2002).

The two PBPs involved in the elongation process are PBP 2a and PBP 2b, while the PBPs involved in division are PBP 1a and PBP 2x. It was noticed that the third class A molecule, PBP 1b, was shown to participate both in elongation and septation. However this was never noticed in the same cell. Furthermore, it was noticed that the inactivation of PBP1b together with either of the two other class A enzymes yielded viable strains with defects in the septum. This therefore suggests that there is a specific, but not fully understood, role for the class A PBPs in the cell-division process (Machebouf *et al.*, 2005).

The class A PBPs contain both glycosyltransferase (GT) and transpeptidase (TP) activities on the same polypeptide in the form of distinct domains identifiable by classic motifs. These enzymes are manufactured so as to catalyze the polymerization of *N*-acetylmuramic acid-β-1, 4-GlcNAc moieties and cross-linking of stem peptides by using the periplasmically located lipid II as substrate. The GT domain is very interesting due to the possibility of it harbouring an uncharacterised fold and thus could be a novel potential antibacterial development target. Some of the class A enzymes are also responsible for β-lactam resistance (Machebouf *et al.*, 2005).

The group of PBPs that are responsible for β-lactam resistance are, PBP 1a, 2b and 2x (Hakenbeck., 1999; Machebouf *et al.*, 2005; Massova & Mobashery, 1998; Pernot *et al.*, 2004; Nagai *et al.*, 2002; Pagliero *et al.*, 2004). These PBPs contain three conserved amino acid motifs that form the active site of transpeptidase activity, SXXK, SXN, and KT(S) G. These binding motifs occur between amino acid positions 370 to 373, 428 to 430 and 557 to 559 respectively in PBP1a, at positions 337 to 340, 395 to 397 and 547 to 549 respectively in PBP2x, and at positions 385 to 388, 442 to 444, and 614 to 616 respectively in PBP2b (Hakenbeck *et al.*, 1999a; Hakenbeck *et al.*, 1999b; Nagai *et al.*, 2002). Low-affinity variants of the PBPs are associated with changes in these motifs, or in the positions flanking these motifs (Nagai *et al.*, 2002).

The interspecies transfer of genes from a range of antibiotic resistant markers has been recognized between different streptococcal species, and all are naturally transformable (Bracco *et al.*, 1957; Hakenbeck *et al.*, 1998). It has also been noticed that identical or closely related DNA sequences of PBP genes occur in penicillin-resistant *S. pneumoniae, Streptococcus mitis, Streptococcus oralis,* and *Streptococcus sanguis* (Coffey *et al.*, 1993; Dowson *et al.*, 1990; Hakenbeck *et al.*, 1998), and the transformation of penicillin resistance from one *Streptococcus* species to another is accompanied by changes in PBPs (Chalkley *et al.*, 1991; Hakenbeck *et al.*, 1998). *S. mitis* and *S. oralis* are thought to be implicated in the origin of the PBP 2x and/or PBP 2b, which acts as resistance determinants in *S. pneumoniae* (Dowson *et al.*, 1993; Hakenbeck *et al.*, 1998).

The PBP 2x, one of the essential PBPs required for primary resistance belongs to the same class of PBPs as the *Escherichia coli* PBP 3. The gene encoding PBP 3 plays an important role in the cell division process, and is probably part of a multiprotein complex termed the divisome. A huge number of mutations that mediate resistance have been recognized in the PBP 2x. This protein is also very useful for understanding the interaction between drug and protein at the atomic level (Hakenbeck & Coyette, 1998; Hakenbeck *et al.*, 1999b). PBP 2b, the other essential PBP required for primary resistance, is related to the bacteriolytic activity of penicillins. Provided that the PBP 2b is not repressed, the process of cellular lysis does not occur and therefore the cells have a better chance of survival during the drug treatment (Hakenbeck *et al.*, 1999b).

Genetic competence is an important event during the process of β-lactam resistance development in the pneumococcus species. The *S. pneumoniae* is competent only for a short phase of the cell cycle. During the exponential growth phase, the competence is controlled by a quorum-sensing mechanism that is mediated by a peptide called the competence stimulating peptide (CSP) (Havarstein *et al.*, 1995; Hakenbeck *et al.*, 1999b). The CSP peptide is the product of the *comC* gene and is processed and secreted by ComA/B, an ABC transporter. Mutations within the operon and genes that is responsible for the functioning of this system result in increased resistance to the β-lactams when introduced into sensitive recipient cells that contain no alterations in the PBP genes (Hakenbeck *et al.*, 1999b). This is important

because it shows that the PBPs are not the only proteins that confer resistance to the β-lactam antibiotics.

The rate of acylation by β-lactam antibiotics is decreased in resistant strains. To measure the effect of this interaction between the PBPs and the β-lactams, the kinetics of the reaction is measured using the following equation (eq. 1).

$$\mathbf{E + I \underset{K^{-1}}{\overset{}{\leftrightarrows}} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + P} \qquad \text{(Eq. 1)}$$

This equation involves a three step reaction, **E** represents the enzyme (PBP), **I** represent the β-lactam antibiotics. **EI** is a non-covalent complex with dissociation constant **K**, the covalent acyl-enzyme **EI*** is formed with the rate constant $\mathbf{k_2}$. Finally the enzyme is deacylated with the rate constant $\mathbf{k_3}$, to regenerate an active enzyme and release an inactivated compound P (Chesnel *et al.*, 2003). The first step represents the formation of a noncovalent complex **EI** with the dissociation constant **K**, followed by the acylation of the active site serine by the β-lactam molecule with the rate constant $\mathbf{k_2}$. The first two steps are termed the "acylation efficiency" (Pernot *et al.*, 2004). The final step of the reaction is the deacylation characterised by the rate constant $\mathbf{k_3}$. During this step active PBP is regenerated, as well as the releasing of an inactivated compound, (Pernot *et al.*, 2004).

The penicillin resistance is classified by different levels i.e. susceptible, intermediate, and resistant. The level of resistance is determined by the minimum inhibitory concentrations (MIC) which is a fairly common technique used is most molecular biology laboratories. The bacteria is classified as susceptible if the MIC value is below 0.1 μg/ml and is classified as intermediate when the MIC value is between 0.1 and 1 μg/ml. The resistant bacteria have MIC values of greater than 1 μg/ml (Pagliero *et al.*, 2004; Nagai *et al.*, 2002; Chambers, 1999).

1.2. Vancomycin resistance in *Enterococcus*

Enterococci were originally classified as Gram-positive bacteria and afterward incorporated in the genus *Streptococcus* (Cetinkaya *et al.*, 2000; Murray, 1990). The Lancefield serological typing system in the 1930s classified enterococci as a group D *Streptococcus* and were differentiated from the nonenterococcal group D streptococci such as *Streptococcus bovis* by distinctive biochemical characteristics (Lancefield, 1933). It was further suggested by Sherman that the term "*Enterococcus*" should be used specifically for streptococci that grow at both 10 and 45°C, at pH 9.6, and in 6.5% NaCl and survive at 60 °C for 30 min (Sherman, 1937). It was also reported that, in the presence of bile, these organisms hydrolyze esculin. *Enterococcus* was removed from the *Streptococcus* genus, in the 1980s, and was once again given the genus termed, *Enterococcus* (Schleifer & Kilpper-Balz, 1984). The species such as *faecalis, faecium, durans, avium,* etc, which were once under the *Streptococcus* genus were changed and were given the genus name *Enterococcus* (Cetinkaya *et al.*, 2000).

There are only two *Enterococcus* species responsible for the majority of the human infections despite there being a dozen *Enterococcus* species identified. These two species are the *E. faecalis* and the *E. faecium*, which have the ability to survive on a variety of hospital environmental surfaces (Stosor *et al.*, 1994; Woodford *et al.*, 1995). This shows that the environment serves as a storehouse for the nosocomial infections. This led to the *Enterococcus* genus being named the nosocomial infections of the 1990s (Spera & Farber, 1992). The colonization of the bacteria is found in the gastrointestinal tract, the urethra, vagina, mouth, urinary tract and surgical wounds (Gray & Pedler, 1994). The *E. faecalis* is the predominant enterococcal species in terms of infection, accounting for 80 to 90 % of all clinical isolates. The *E. faecium* species identified as being the second largest *Enterococcus* species responsible for the majority of the human infections contributes for 5 to 15 % of the infection. The remaining *Enterococcus* species namely the *E. avium, E. durans, E. gallinarum, E. raffinosis, E. casseliflavus* and so forth, are found to cause infection at a much lower rate, and it was noticed that they account for less than 5% of the total number of clinical isolates (Gordon *et al.*, 1992; Lewis & Zervos, 1990; Moellering & Wennersten, 1983; Ruoff *et al.*, 1990; and Cetinkaya *et al.*, 2000).

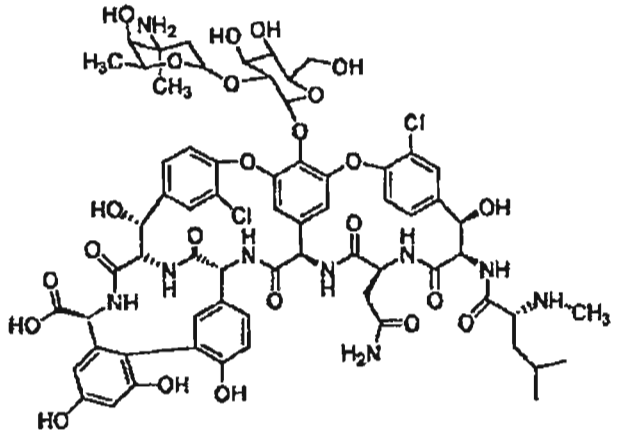
The *Enterococcus* genus has also been recognized as an important cause of endocarditis for nearly a century (Cetinkaya *et al.*, 2000). Enterococcal endocarditis provides a defined body, with mortality of about 20%, so the choice of the treatment is therefore extremely important for success (Woodford *et al.*, 1995). It was also known to be the common cause of hospital-acquired infections in the middle to late 1970s, which is probably related to the escalating use of third generation cephalosporins which enterococci are naturally resistant to (Murray, 1990; Cetinkaya *et al.*, 2000). The Enterococci are also classified as nosocomial pathogens. Resistance is acquired by either mutation or by receipt of foreign genetic material through the transfer of plasmids and transposons (Clevel, 1990). Most enterococci are tolerant to the bactericidal activity of β-lactam and glycopeptide antibiotics (Krogstad & Parquette, 1980). The synergistic bactericidal effect between the antibiotics aminoglycosides and β-lactam or glycopeptides is lost if there is high level resistance to either class of drug. Aminoglycoside modifying enzymes are used to monitor resistance to high concentrations of aminoglycoside antibiotics, which is widespread among the enterococci. Since enterococci are intrinsically resistant to β-lactams and, more specifically to penicillin, many isolates of *E. faecium* have shown to be highly resistant to penicillin which can be attributed to their PBPs having a low affinity for penicillin (Grayson *et al.*, 1991). The drug that was virtually the only drug that could be always depended on for the treatment of infections caused by the multi-drug resistant enterococci was the antibiotic vancomycin (Cetinkaya *et al.*, 2000).

One of the most important and clinical relevant member of the glycopeptide antibiotics is vancomycin. These antibiotics are critical for the treatment of serious infection caused by gram-positive bacteria. Another medically important member that fits into this group is teicoplanin (Pootoolal *et al.*, 2002). The glycopeptide antibiotic, which has several dozen members, has major use in terms of practical application against a large number of gram positive bacteria such as *Streptococci, Staphylococci, Enterococci* and *Clostridia*, which is used greatly in the hospital environment rather than in the household setting. In 1950, the crucial discovery of, vancomycin a secondary metabolite produced by *Streptomyces orientalis* (now referred to as *Amycolatopsis orientalis*) a soil bacterium, was made. Vancomycin gained popularity at a very fast rate, and therefore entered clinical use against the infectious Gram-positive bacteria. The vancomycin antibiotic was eventually largely sidetracked by the

discovery and the approval of second and third generation β-lactam antibiotics such as methicillin, which inhibited growth against penicillin-resistant bacteria: vancomycin was further restricted due to toxicity originating from non-homogenous preparations of the antibiotic. So once again vancomycin was called upon when there were strains of bacteria resistant to methicillin. The widespread nosocomial pathogen methicillin-resistant *Staphylococcus aureus* (MRSA) further escalated the use of vancomycin (Srinivasan *et al.*, 2002). In 1986, as the use of vancomycin steadily increased the first case of vancomycin resistance was reported (Kirst et al., 1998; Pootoolal *et al.*, 2002). The resistance to this class of antibiotics greatly threatened the clinicians' ability to treat serious infections, which have further resulted in spurring scientist's interest in the development of new antibacterial agents, as well as to understand the molecular basis of vancomycin resistance (Pootoolal *et al.*, 2002).

Vancomycin and the structurally similar antibiotic teicoplanin, was thought to be the last line of defence against severe infections caused by Gram-positive organisms (Arthur *et al.*, 1992; Gold, 2001). Figure 1.5 shows the structures of these two antibiotics. Containing, one of two core linear heptapeptide structures that, in turn, are modified by selective glycosylation and amino acid adjustment to create distinct compounds (Pootoolal *et al.*, 2002).

Vancomycin



Teicoplanin

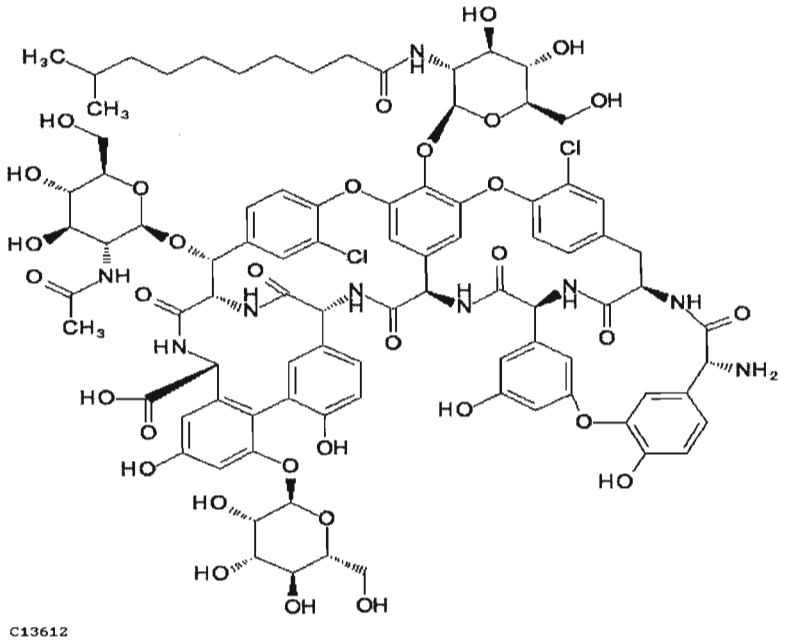


Figure 1.5. The structures of Vancomycin and Teicoplanin (Pootoolal *et al.*, 2002).

The glycopeptides incorporate unusual amino acids like 3, 5-dihydroxyphenylglycine (DHPG), β-hydroxytyrosine (β-OHTyr), and ρ-hydroxyphenylglycine (HPG). Glycopeptides consists of a heptapeptide backbone that is substituted with five to seven aromatic rings and different sugars. The vancomycin antibiotic consists of two hexoses and five aromatic rings.

These two antibiotics also serve as prototypes for the heptapeptide core structures. The mechanism by which the anti-microbial action is performed is through the inhibition of extra cellular steps in peptidoglycan biosynthesis. The glycopeptide antibiotics interact specifically with the D-Ala-D-Ala terminus of the peptidoglycan pentapeptide (N-acetyl-muramyl-L-Ala-D-Glu-Lys-D-Ala-D-Ala in most gram positive bacteria) (Nieto & Perkins, 1971; Pootoolal *et al.*, 2002). This is in direct contrast the penicillin, which directly binds and inhibits these enzymes (Healy *et al.*, 2000). Inhibition of the transglycosylase (part of a bifunctional extracellular enzyme with transpeptidase activity) by vancomycin carbohydrate residues effectively prevents growth of the peptidoglycan chain, (Ge *et al.*, 1999; Pootoolal *et al.*, 2002). Vancomycins ability to bind to the enzyme enables it to prevent both transglycosylation (incorporation into the nascent peptidoglycan) as well as transpeptidation activities (permits the formation of cross-bridges). The cell walls, therefore, become weaker due to the inability to form cross-links between peptidoglycan intermediates, which then makes the bacteria susceptible to osmotic lysis (Healy *et al.*, 2000). The ability of vancomycin to bind to the precursors inhibits their incorporation into the growing cell wall. There are, however, two factors that are of great importance for antibacterial activity including the dimerization of the vancomycin-group of antibiotics which results in an improved affinity of vancomycin cell wall analogues in free solution. Added that the chelation effect causes an increase in affinity for the bacterial cell wall peptidoglycan (Beauregard *et al.*, 1997).

1.2.1. Vancomycin resistance

For more than 30 years vancomycin had been in clinical use, with no sign of marked resistance (Centinkaya *et al.*, 2000). The first report of vancomycin resistance showed the *E. faecalis* and the *E. faecium* species being resistant to the vancomycin (Uttley *et al.*, 1988). The factor that is of serious concern is the appearance of transferable resistance to glycopeptide antibiotics in species of enterococci. The fact that vancomycin resistant enterococci (VRE) will pass on the mechanism of high-level glycopeptide resistance to more virulent genera, has the potential of generating multi-antibiotic resistance strains and thus cause immeasurable amount of damage (Pootoolal *et al.*, 2002). Since few antibiotics treatments are active against enterococci, such as penicillin-aminoglycoside combinations,

vancomycin therapy is the method of choice or the last option (Wilhelm & Estes, 1999, Pootoolal *et al.*, 2002).

The rapid appearance of vancomycin-resistant enterococci as life threatening organisms mainly in the hospital settings has brought about the concentrated investigation of the molecular basis of this glycopeptide antibiotic resistance mechanism (Leclercq & Courvalin, 1997; Murray, 1997; Barna & Williams, 1984; Bugg *et al.*, 1991). These investigations have exposed one of the most sophisticated molecular systems of acquired resistance and an example of genetic adaptation (Leclercq & Courvalin, 1997). This antibiotic resistance works by reprogramming the termini of the peptidoglycan intermediates in cell wall cross-linking steps. (Lessard & Walsh, 1999). In enterococci, the production of modified precursors ending in the depsipeptide D-Alanyl-D-Lactate (D-Ala-D-Lac) as opposed to the dipeptide D-Alanine-D-Alanine (D-Ala-D-Ala) gives the acquired resistance (Arthur *et al.*, 1996a). Figure 1.6.a shows vancomycin binding to the end termini of the D-Ala-D-Ala, which in turn blocks the transpeptidation and the transglycosylation steps. Figure 1.6.b shows the effect of the D-Ala-D-Ala being changed to D-Ala-D-Lac. This tailored peptidoglycan binds to vancomycin 1000 fold less strongly than the original D-Ala-D-Ala peptidoglycan termini; this is due to the loss of the central hydrogen bond from the NH of the D-Ala-D-Ala moiety to the vancomycin backbone carbonyl. (Bugg *et al.*, 1991; Lessard & Walsh, 1999).

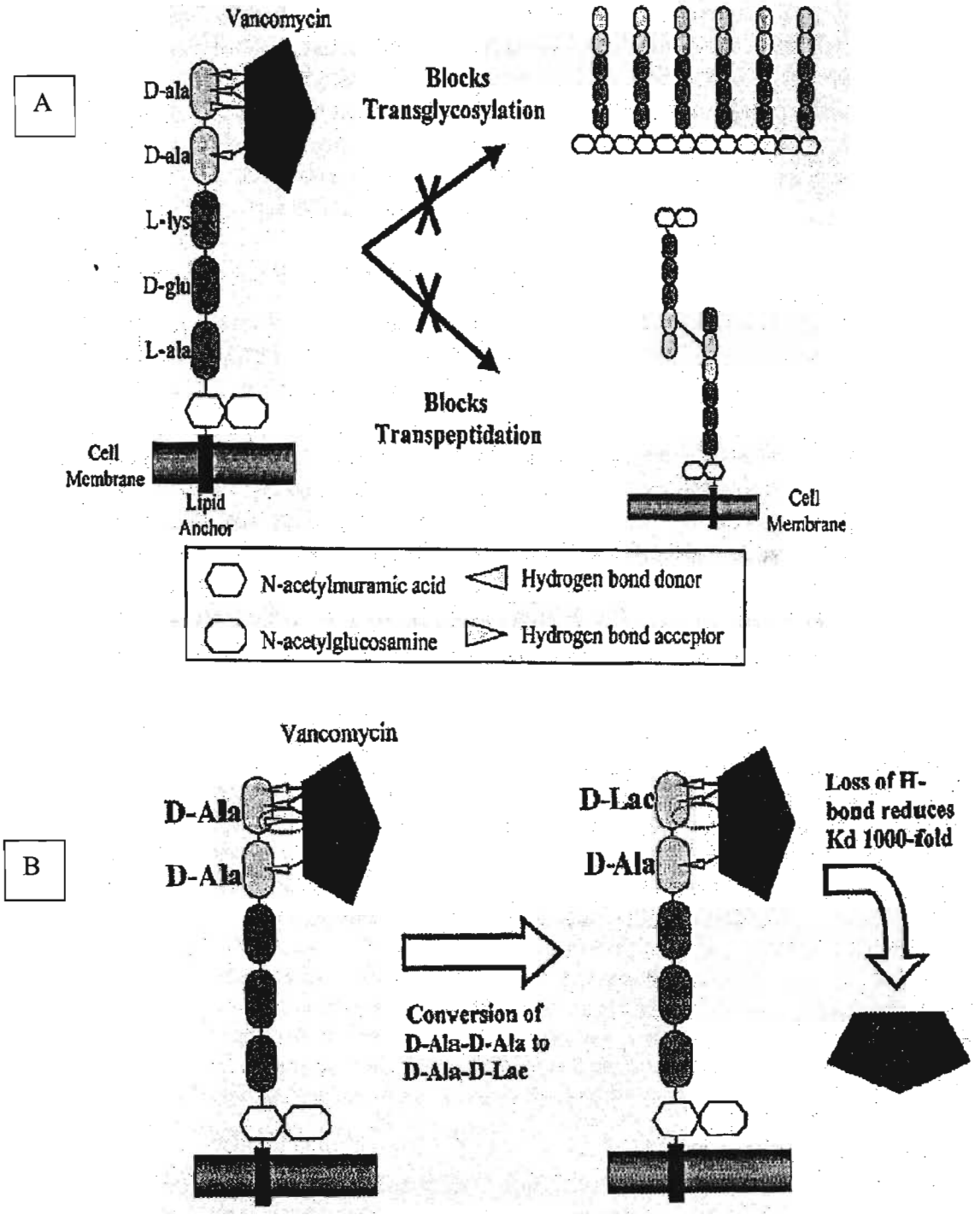


Figure 1.6. The effect of the vancomycin antibiotic on the pentapeptide ending with (a) D-Ala-D-Ala resulting in cell lysis. The effect of a pentapeptide ending with (b) D-Ala-D-Lac results in resistance (Pootoolal *et al.*, 2002).

There are at least seven distinct phenotypes of vancomycin resistance, namely the VanA, VanB, VanC, VanD, VanE, VanF, and VanG (refer to Table 1.1) (Pootoolal *et al.*, 2002). These phenotypes contain genes that play a role in resistance (refer to Figure 1.7). The genes that are found within the VanA phenotype are; *vanR, vanS, vanH, vanA, vanX, vanY* and *vanZ.* The genes found within the VanB phenotype are; $vanR_B$, $vanS_B$, $vanY_B$, $vanW_B$, $vanH_B$, *vanB* and finally $vanX_B$. In the case of the VanC phenotype the genes are; *vanC-1*, $vanXY_C$, *vanT*, *vanRC* and $vanS_C$. The genes for VanD include; $vanR_D$, $vanS_D$, $vanY_D$, $vanH_D$, *vanD* and $vanX_D$. In the VanE phenotype, the genes found present are; $vanT_F$, $vanR_F$, $vanS_F$, *vanE* and $vanXY_E$. The least commonly known VanF phenotype contains the $vanY_F$, $vanZ_F$, $vanH_F$, *vanF*, and $vanX_F$ genes. VanG has the following genes; $vanR_G$, $vanS_G$, $vanY_{G1}$, $vanW_G$, *vanG*, $vanY_{G2}$ and $vanT_G$ (Pootoolal *et al.*, 2002).

The resistance can be classified in three types, namely the VanA, VanB, and finally VanD, which is dependant upon the level of resistance to vancomycin and also the susceptibility or resistance to teicoplanin (Arthur *et al.*, 1996; Depardieu *et al.*, 2003). The resistance in enterococci results from the modified precursors, as mentioned above, ending with D-Ala-D-Lac corresponds with the phenotypes VanA, VanB and VanD or the modified precursors could also end with D-Ala-D-Ser and, therefore, will correspond with the VanC, VanE and the VanG phenotypes (refer to Table 1.1). The peptidoglycan terminus is shown relative to each phenotype as well as the resistance, which is calculated by the MIC values. It is also noticed that the two species that are the most prominent, in terms of causing infection, are the *E. faecium* and the *E. faecalis* (Pootoolal *et al.*, 2002).

Table 1.1. The six different phenotypes of glycopeptide antibiotic resistance in enterococci adapted from Pootoolal *et al.*, 2002.

Phenotype	Peptidoglycan terminus	Resistance (MIC in µg/ml)	Source	Induction	Organisms
VanA	D-Ala-D-Lac	Van (≥ 64) Tei (≥ 16)	Acquired e.g., *Tn 1546*	Inducible	*E. faecium* *E. faecalis*
VanB	D-Ala-D-Lac	Van (≥ 4)	Acquired e.g., *Tn 1547*	Inducible	*E. faecium* *E. faecalis*
VanC	D-Ala-D-Ser	Van (≥ 2)	Intrinsic	Constitutive and Inducible	*E. gallinarum* *E. casseliflavus*
VanD	D-Ala-D-Lac	Van (≥ 16) Tei (≥ 2)	Intrinsic	Constitutive	*E. faecium*
VanE	D-Ala-D-Ser	Van (16) Tei (0.5)	Acquired	Inducible	*E. faecalis*
VanG	D-Ala-D-Ser	Van (16)	Unknown	Unknown	*E. faecalis*

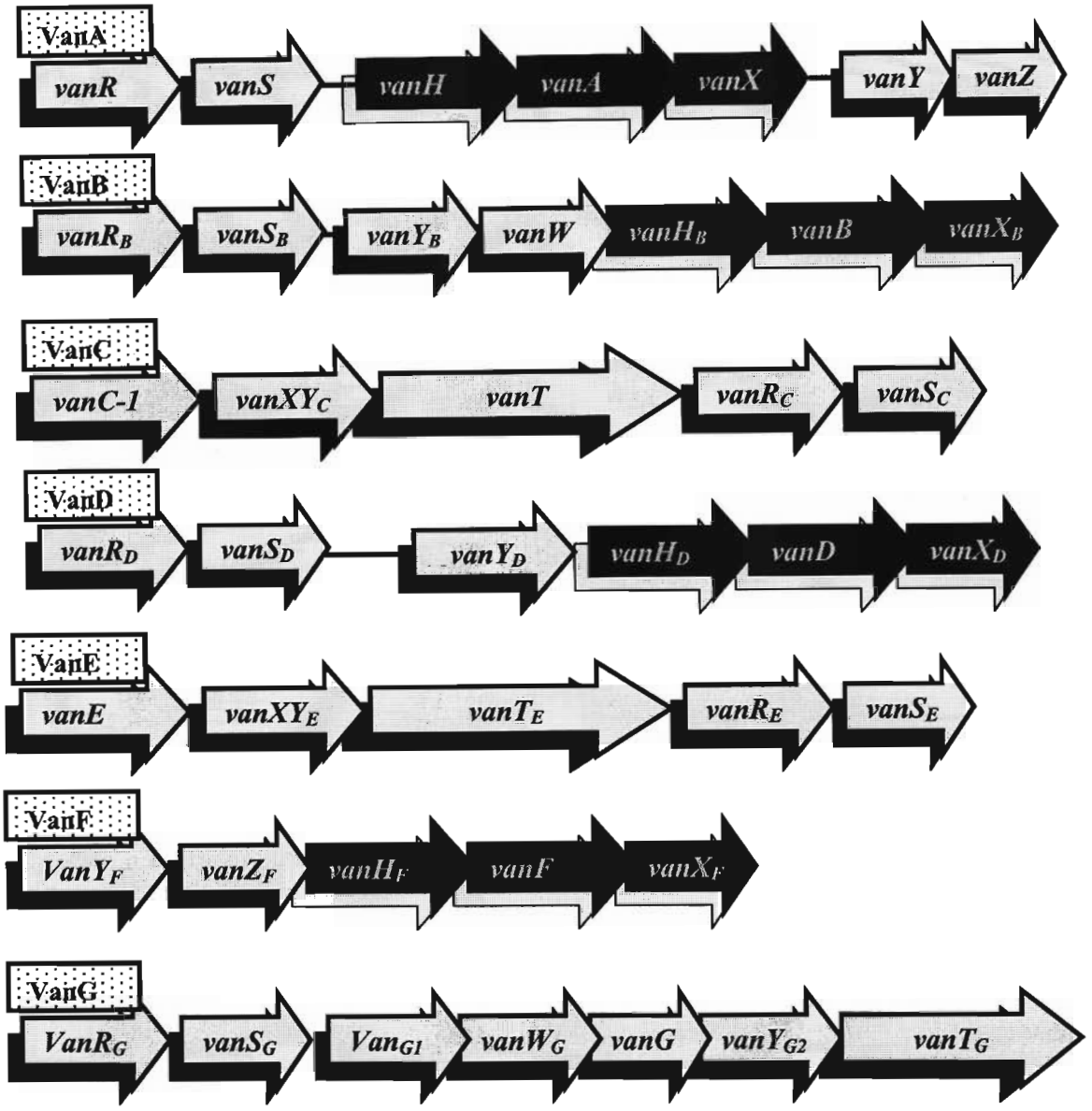


Figure 1.7. The different types of phenotypes and the genes that are within them (Pootoolal *et al.*, 2002).

1.2.2. VanA glycopeptide resistance

The details of vancomycin resistance have best been explained by the *vanA* gene cluster which is found on the transposons, or 'jumping gene,' Tn*1546*. The other genes associated with the regulation and the expression of vancomycin resistance is also found on this 10,851bp

transposon of *E. faecium*, which is often found on a plasmid. The genes that regulate and express vancomycin resistance are the *vanR, vanS, vanH, vanX,* and *vanZ* (Arthur *et al.*, 1993b). *VanA* type is defined by high-level resistance to both vancomycin and teicoplanin. It is stated by Evers & Courvalin that the *Tn1546* transposon codes for nine proteins that can be assigned to the following five groups (Evers & Courvalin, 1996). Firstly the transposon belongs to the Tn3 family of transposons and, therefore, carries with it two open reading frames (ORFs) namely the ORF1 (refer to Figure 1.8 for the structure), encoding for transposases, and ORF2 encoding for resolvases (Evers & Courvalin, 1996; Arthur & Courvalin, 1993a). Secondly, the production of the D-Ala-D-Lac requires the *vanA* gene which encodes the VanA ligase and is structurally similar to the D-Ala-D-Ala but has a broader specificity (Bugg *et al.*, 1991; Dukta-Malen *et al.*, 1990). The VanA catalyses ester bond creation between D-Ala and D-2hydroxy acids for the manufacture of D-Ala-D-Lac subsequently added to UDP-MurNAc-tripeptide. The result of this is the creation of the creation of the UDP-MurNAc-pentadepsipeptide. The VanH D-2-hydroxyacid dehydrogenase encoded by *vanH* reduces pyruvate to D-Lac, the substrate for VanA (Bugg *et al.*, 1991). Thirdly the *vanX* D, D-dipeptidase hydrolyzes the D-Ala-D-Ala precursor, which is produced by D-Ala-D-Ala ligase (Reynolds *et al.*, 1994), which then reducers the amount of D-Ala-D-Ala, so as to decrease the competition for the binding to the peptidoglycan precursors, with the D-Ala-D-Lac. The VanY D, D-dipeptidase encoded by the *vanY* gene is thought to contribute to the glycopeptide resistance at low concentrations of v*anA*, *vanH* and *vanX* by the method of hydolysis of UDP-MurNAc-pentapeptide (Arthur *et al.*, 1994). The fourth functional group is characterised by the *vanZ* gene, which gives low-level resistance MIC by a mechanism which is not clearly understood (Arthur *et al.*, 1995). Finally, the two genes *vanR* and *VanS* encode for the two component regulatory system those express glycopeptide resistance genes. The *vanR* is referred to as an activator, which is needed for the initiation of co-transcription of the *vanH, vanA,* and *vanX* genes. Phoshorylation of *vanR* by *vanS* changes its activity (Arthur *et al.*, 1992; Evers & Courvalin, 1996).

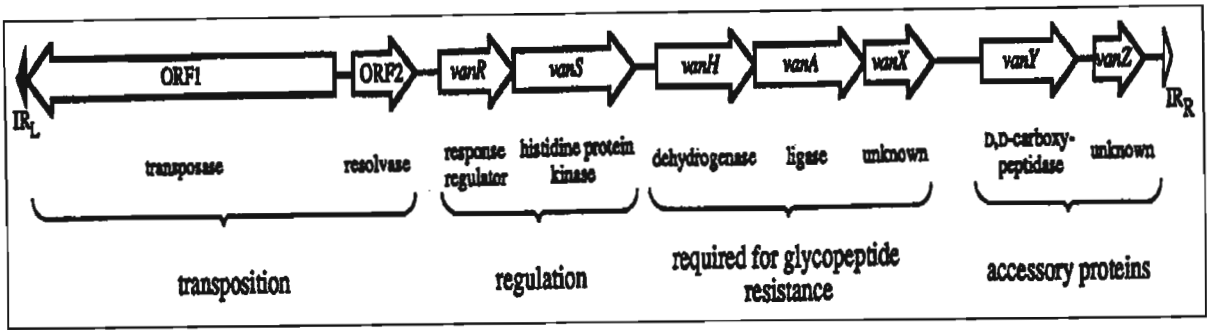


Figure 1.8. Map of the transposon Tn 1546 showing the coding sequences. The closed and open arrow heads labelled IR_L and IR_R indicating the left and right inverted repeats of the transposon. (Arthur & Courvalin, 1993)

It can be seen that the core proteins favouring synthesis of pentadepsipeptide terminating in D-Ala-D-Lac are the *vanA*, *vanH* and the *vanX*, with the *vanA* producing D-Ala-D-Lac in preference to D-Ala-D-Ala, the *vanH* creates a pool of D-lactate used for the reaction and finally the *vanX* protein decreases the number of D-Ala-D-Ala so as to decrease the competition for the binding to the peptidoglycan precursors. The resistance to the antibiotic is not achieved alone by the VanA protein. This is probably due to the D-hydroxy acids such as D-Lactate are not natural products occurring in the environment of the enterococci or neither is it produced by the *Enterococcus* genus (Leclercq & Courvalin, 1997; Cetinkaya *et al.*, 2000). Therefore, in order to synthesize the D-Lactate the VanA protein it must acquire the genes, like *vanH*, which is responsible for the production of D-Lactate (Cetinkaya *et al.*, 2000).

The members of the two component regulatory system, *vanR* and *vanS*, are responsible for regulating the transcription of the *vanHAX* gene cluster (Arthur *et al.*, 1992) (Refer to Figure 1.9). The VanS acts as a sensor to discover if vancomycin or teicoplanin is present and can also detect disturbance of cell wall precursors which are elicited by these drugs. When the *vanS* protein does detect the presence of vancomycin it then informs the *vanR,* which is the response regulator, and, thereafter, in turn responds by activating the transcription of the *vanHAX* gene cluster. The *vanY* and the *vanZ* genes are not entirely necessary for resistance. The *vanY* contributes moderately to resistance levels, while the *vanZ* moderately increases the

MIC values of the teicoplanin antibiotic and is, therefore, not essential for the expression of the VanA phenotype (Cetinkaya *et al.*, 2000).

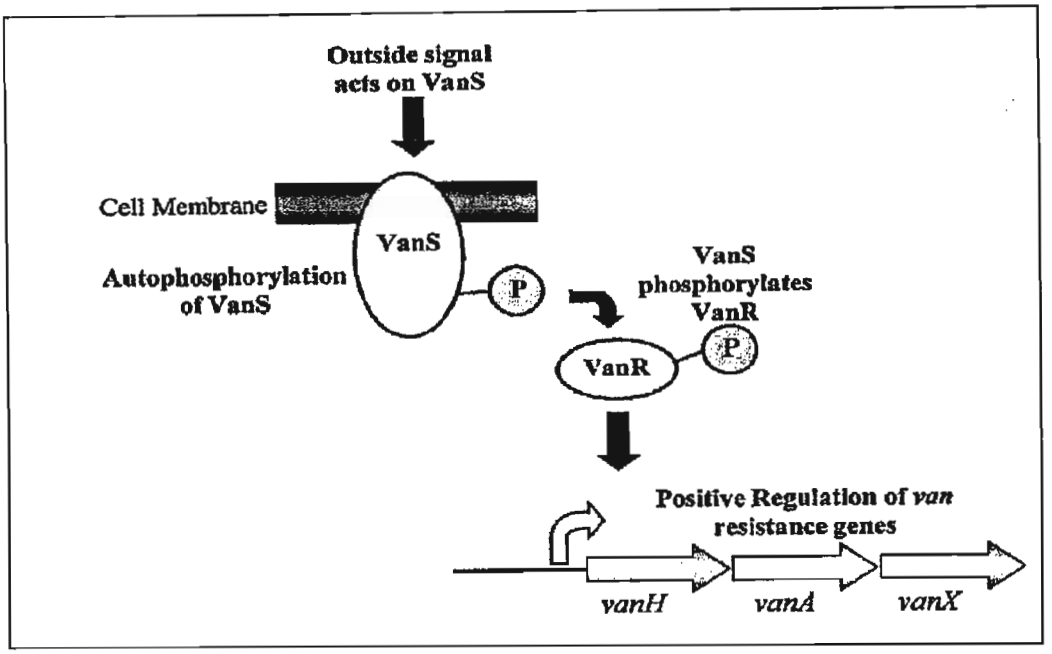


Figure 1.9. The regulation of the two component system and the events leading to the transcription of the VanA and VanB gene clusters (Arthur & Quintiliani, 2001).

1.2.3. VanB glycopeptide resistance

The VanB phenotype also produces the D-Ala-D-Lac terminating peptidoglycan like the VanA phenotype and, therefore also, requires the $vanH_B$, *vanB* and the $vanX_B$ enzymes with exact same functions as their VanA counterparts. The VanB glycopeptide resistance in enterococci is mediated by an abnormal ligase (*vanB*), which is structurally related to the *vanA* ligase (70% amino acid similarity) (Cetinkaya *et al.*, 2000). The *vanB* is located on large conjugative chromosomal elements (from 90 to 250 kb) (Quintiliani & Courvalin, 1994) or plasmids such as Tn*1547* which can be transferred from one bacterium to another by plasmid conjugation or alternatively with the use of the process, horizontal transmission (Lessard & Walsh, 1999). The two component regulatory system is also present in the VanB phenotype serving the same sort of function as it serves with the VanA phenotype. An important factor

that defines the VanB is that it is sensitive to teicoplanin but is resistant to vancomycin. It is not clearly understood why this occurs, because the VanA and VanB are similar in terms of the *vanHAX* and the *vanR* and *vanS*. Although there is a difference in these two genes, that is the presence of the *vanZ* gene in the VanA as well as the presence of $vanW_B$ in the VanB phenotype. Another difference between the VanA and VanB type resistance is that the VanA is found to be more widely distributed in the world (Cetinkaya *et al.*, 2000). It is also noticed that the *vanA* ligase gene is isolated from a wider range of enterococcal species, also being isolated from non-enterococcal species like *Corynebacterium* spp., *Arcanobacterium heamolyticum*, and *Lactococcus* spp., however the *vanB* was found to be isolated primarily from *E. faecium* and *E. faecalis* (Cetinkaya *et al.*, 2000). The possible reason for this could be due to the VanA gene cluster is often located on a transposon, which can be part of a conjugative (transferable) plasmid. While the VanB cluster is often located on a chromosome and was thought not to be transferable to other bacteria, this theory was incorrect as this gene cluster has been transferable as part of large mobile elements (Cetinkaya *et al.*, 2000). The VanB organisms can be resistant at an MIC of 32 – 62 μg/ml but can still be susceptible to teicoplanin (Lessard & Walsh, 1999).

In Figure 1.10, the positions of the genes mentioned above which are responsible for the correct functioning for vancomycin resistance are indicated. The $vanR_B$ and $vanS_B$ are found at the beginning of the gene cluster, followed by $vanY_B$ and *vanW*. Then in comparison to the VanA gene cluster, the $vanH_B$, *vanB* and $vanX_B$ are found toward the end of the gene cluster.

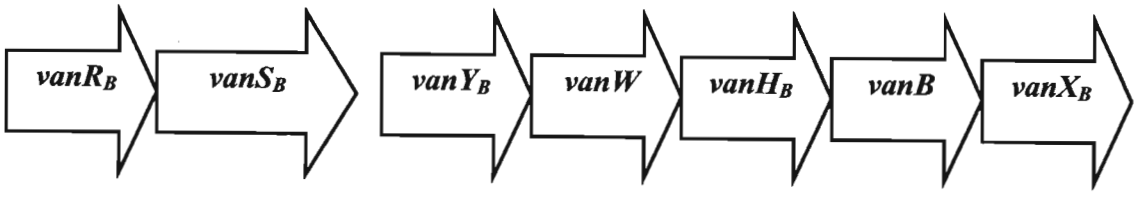


Figure 1.10. The schematic representation of the VanB gene cluster (Pootoolal *et al.*, 2002).

1.2.4. VanC glycopeptide resistance

The chromosomally located VanC gene cluster encodes five genes (refer to Figure 1.11). The VanC type resistance is known to have intrinsic, low-level resistance to vancomycin (MIC, 2 to 32 mg/l) and susceptibility to teicoplanin (MIC, 0.5 to 1.0 mg/l) which was first detected in *E. gallinarum* BM4174, subsequently detected in *E. casseliflavus* (VanC-2 type) and *E. flavescens* (VanC-3 type) (Reynolds & Courvalin, 2005). The VanC ligase of *E. gallinarum* favours the production of a pentapeptide terminating in D-Ala-D-Ser. The change of the D-Ala to D-Ser is assumed to weaken the binding of vancomycin to the novel pentapeptide (Cetinkaya *et al.*, 2000). Varying levels of D-Ala-D-Ala relative to D-Ala-D-Ser could account for the variable levels of vancomycin resistance observed among isolates of vancomycin resistant enterococci carrying the VanC phenotype (Cetinkaya *et al.*, 2000). The availability of D-Serine is due to the activity of *vanT*, which is a serine racemase (Arias *et al.*, 1999). The presence of the mutation of two amino acid residues at positions Phe250Tyr and Arg322Met, results in a 6000 fold switch of substrate specificity towards the production of D-Ala-D-Ala, which accounts for the loss of D-Ala-D-Ser synthetic activity (Reynolds & Courvalin, 2005; Healy *et al.*, 1998).

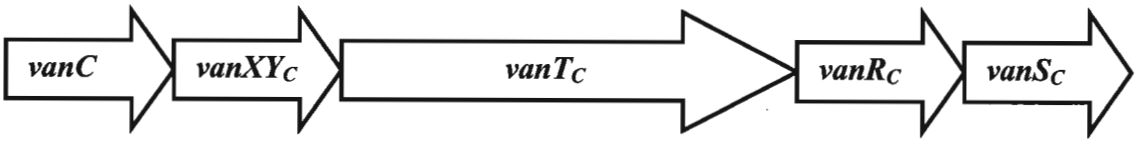


Figure 1.11. The schematic representation of the VanC gene cluster (Boyd *et al.*, 2002).

It is also noticed that the organization of the VanC operon is different compared to the VanA and VanB operons. The two component regulatory system is found at the end of the operon, also the presence of the *vanXY*$_C$ and *vanT* genes are noticed in any other gene clusters. The changed presence of these genes i.e. the *vanC* D-Ala-D-Ser ligase, the *vanXY*$_C$ D, D-carboxypeptidase and *vanT*$_C$ serine racemase, which is membrane-bound, is responsible for the resistance to the antibiotic. The presence of these genes results in a six fold decrease in

affinity for the antibiotic, due to the fact that the hydroxymethyl side chain of D-Serine is thought to sterically disrupt the binding of vancomycin to the normal D-Ala-D-Ala termini (Healy *et al.*, 2000).

1.2.5. VanD glycopeptide resistance

The VanD phenotype is thought to be located on the chromosome and is not transferable to other enterococci (Cetinkaya *et al.*, 2000). The VanD type resistance is known to have intrinsic, moderate-level resistance to vancomycin (MIC, 64 mg/l) (Pootoolal *et al.*, 2002). The VanD phenotype is grouped with the VanA and the VanB phenotype in the sense that they all produce D-Ala-D-Lac termini. Also the organizations of the genes are similar to those of VanA and VanB (refer to Figure 1.12). The VanD strains constitutively express D-Ala-D-Lac, and are not responsive to the $vanR_D$ and $vanS_D$ two component regulation system (Pootoolal *et al.*, 2002).

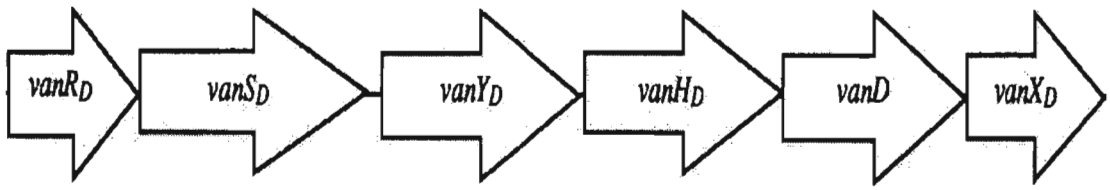


Figure 1.12. The schematic representation of the VanD gene cluster (Pootoolal *et al.*, 2002).

1.2.6. VanE glycopeptide resistance

VanE glycopeptide resistance is characterised by synthesis of peptidoglycan precursors ending in D-Ala-D-Ser, whereby low-level resistance is acquired. The genes that confer the resistance are chromosomal. The VanE type resistance is almost identical to the VanC type apart from the different relative activities of the enzymes (Reynolds & Courvalin, 2005). Similar to the VanC phenotype, the VanE type resistance is not transferable by conjugation,

and also contains genes necessary for resistance (refer to Figure 1.13). The VanE type is also resistant to low levels of vancomycin (MIC, 16 μg/ml) and is susceptible to teicoplanin (MIC, 0.5 μg/ml) (Cetinkaya *et al.*, 2000). The deduced amino acid sequence has a higher similarity to VanC (55%) than VanA (45%), VanB (43%), or VanD (44%) (Cetinkaya *et al.*, 2000). Hence, it is possible to say that the VanE-type resistance was acquired from a VanC-type strain; however it is unlikely to have occurred recently due to the overall low percentage similarities (Reynolds & Courvalin, 2005).

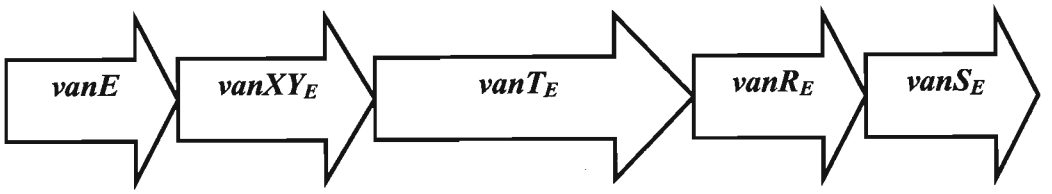


Figure 1.13. The schematic representation of the VanE gene cluster (Boyd *et al.*, 2002)

1.2.7. VanG glycopeptide resistance

Similar to the VanC and VanE types of resistance, the VanG type also synthesizes peptidoglycan precursors terminating in D-Ala-D-Ser. However, the VanG type resistance differs from the VanC and VanE isolates. For example, the VanG cluster is composed of seven genes (refer to Figure 1.14) as opposed to the five genes that are found in either VanC or VanE (Reynolds & Courvalin, 2005; McKessar *et al.*, 2000). The VanG operon seems to have been derived from the VanB, VanC and VanE operons. The transfer of VanG resistance to susceptible strains of *E. faecalis* seems to occur by the movement of genetic elements from one chromosome to the next chromosome (Reynolds & Courvalin, 2005).

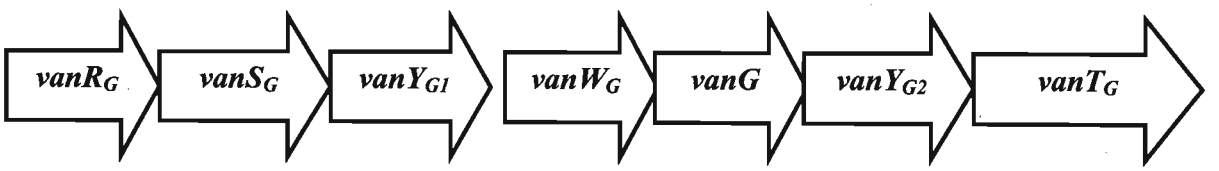


Figure 1.14. The schematic representation of the VanG gene cluster (McKessar *et al.*, 2000).

1.2.8. VanX

VanX is a zinc-dependent D-Alanyl-D-Alanine dipeptidase which is an important component in the system that provides vancomycin resistance to enterococci. The vanX is also a key drug target in circumventing clinical vancomycin resistance (Bussiere *et al.*, 1998). VanX has been detected in both Gram-positive and Gram-negative bacteria, where it appears to have adapted to at least three distinct physiological roles. In the first type, pathogenic vancomycin-resistant enterococci, vanX is joined by four other genes to form a five gene cluster which is responsible for the reprogramming of the cell-wall biosynthesis to produce peptidoglycan chain precursors terminating in D-Ala-D-Lac. The second type, in the glycopeptide antibiotic producers, *vanHAX* operon may have co-evolved with antibiotic biosynthesis genes to provide immunity by reprogramming of the cell-wall biosynthesis to produce peptidoglycan chain precursors terminating in D-Ala-D-Lac. Finally in the Gram-negative bacteria, which are not affected by the glycopeptide antibiotics, the reason being it is unable to penetrate the outer membrane permeability barrier (Lessard & Walsh, 1999).

1.3 Methicillin resistance in *Staphylococcus*

It was noticed in the 1950s, that the penicillin antibiotic was rapidly becoming useless against staphylococcal infections (Chambers, 1988). An outbreak of staphylococcal infections would therefore be calamitous for humankind. So alternate antibiotics are been explored to help overcome this problem. The penicillinase-resistant (later known as the β-lactamase-resistant) semi-synthetic penicillins were looked at, after a series of penicillin was developed (refer to Figure 1.2) (Rolinson, 1998), which was resistant to destruction by bacterial β-lactamases. The methicillin antibiotic was the one chosen and was introduced into clinical practice in 1959 (Hardy *et al.,* 2004). Shortly after the introduction of methicillin, (refer to Figure 1.15), methicillin resistant *Staphylococcus aureus* (MRSA) was discovered in 1961 (Ito *et al.,* 1999). The factors that increase the risk of infection include; advanced age, male gender, hospitalisation (both duration and previous hospitalisation), chronic medical illness, prior and prolonged antibiotic treatment, presence and size of wound, exposure to colonised or infected patient, and lastly the presence of invasive indwelling devices (Haddadin *et al.*, 2002). This nosocomial epidemic therefore soon became an issue of great concern.

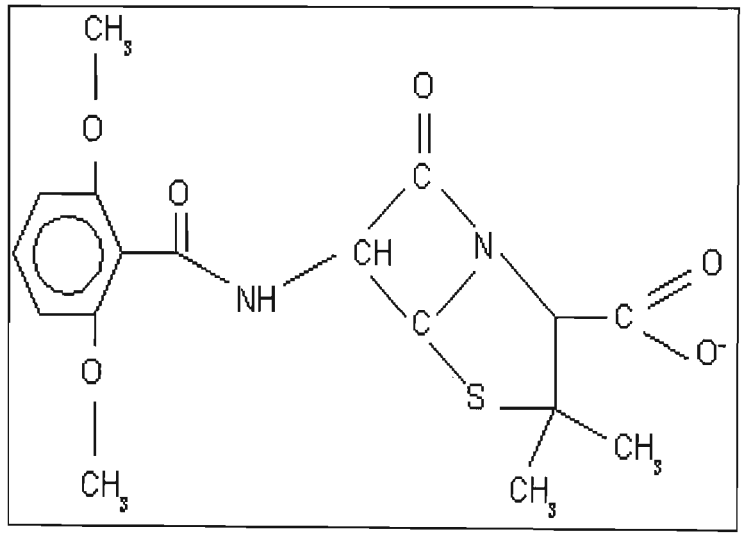


Figure 1.15. The structure of methicillin antibiotic, the β-lactam antibiotic responsible for keeping staphylococcal infections under control (Wikipedia, 2005).

The staphylococcal strains that express resistance to methicillin were studied and it was shown that the mechanism of resistance was not destruction of the drug and was labelled "intrinsic" resistance, which is a term commonly used with methicillin resistance (Chambers, 1988). As mentioned earlier the site for β-lactam antibiotics is the penicillin binding proteins (PBPs). The non-resistant staphylococcal strains produce five distinct PBPs. Namely the PBPs 1, 2, 3, and 4 are high-affinity binding proteins found in all strains. There is also an additional PBP, PBP 2a also known as PBP 2', which has two to three orders of magnitude lower affinity for most β-lactams, and is unique to methicillin-resistant strains (Chambers, 1989; Katayama *et al.*, 2003). PBPs 1 and 2 are essential, the rest of the PBPs are not required. However, when the bacterial cell is exposed to a β-lactam antibiotic, PBP 2a plays a very important role in resistance. By virtue of its low affinity for β-lactams, PBP 2a apparently can substitute for some functions of the other PBPs. PBP 2 also has a critical role in full expression of PBP 2a-mediated resistance (Katayama *et al.*, 2003; Pinho *et al.*, 2001; Pinho *et al.*, 2000).

The MRSA produces a 78 kDa protein of the PBP 2a, which is highly conserved. This additional low affinity PBP 2a is in contrast to the production of β-lactamase, which is responsible for the destruction of the non-stable β-lactams, as mentioned above (refer to section on β-lactamase). In susceptible strains of staphylococci the PBP 2a retains its transpeptidase activity and takes over the role of the normal PBPs in cell wall synthesis. The β-lactam resistance of methicillin-resistant *Staphylococcus* is determined by the function of penicillin binding protein 2a encoded by the methicillin resistance gene *mec*A (Matsuhashi *et al.*, 1986; Ito *et al.*, 2001).

3.1.1 *Mec* gene

The expression of the *mec*A gene can be constitutive or inducible. The presence of the *mec* gene is not sufficient for the *Staphylococcus* to be resistant to methicillin, it has been noticed that some strains of *S. aureus* (2%) have the *mec*A but remain susceptible to methicillin (Hiramatsu, 1995; Haddadin *et al.*, 2002). The *mec*A is widely distributed in both the coagulase positive and negative staphylococci, and is found on a transposon. This gene also appears to integrate into a single site in the staphylococcal chromosome with another 30 kb

DNA fragment, the *mec* locus. The regulatory locus, namely the *mecRI* and *mecI* is thought to have an insertion element, which might be an integration site for unrelated resistance determinants. The other important regulatory components that are involved in expression of the gene are the β-lactamase genes, namely the *blaI, blaRI*, and the *blaZ* gene. Due to the sequence similarities to the *mecRI* and *mecI* it can also down regulate *mec*A gene transcription (Haddadin *et al.*, 2002).

The MRSA is thought to have evolved from the methicillin sensitive *S. aureus* (MSSA) by acquiring a large genetic element known as the staphylococcal cassette chromosome *mec* (SCC*mec*). The *mec* gene complex along with various resistance genes, carried by the SCC*mec* (refer to Figure 1.16) (Foster, 2004), act against non β-lactam antibiotics (Katayama *et al.*, 2000; Hardy *et al.*, 2004). The induction of PBP2a is thought to be controlled by regulatory genes which are present on the plasmids encoding penicillinase production. The staphylococcal strains that are penicillinase negative with PBP 2a are probably induced by the chromosomal location of the penicillinase regulatory genes (Asheshov, 1966; Chambers, 1988).

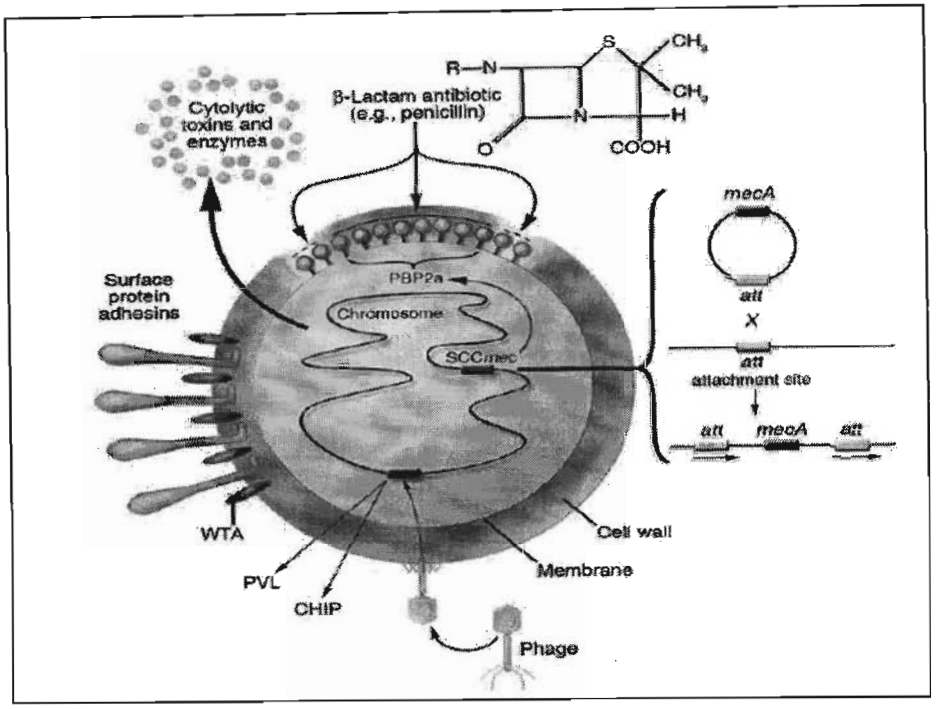


Figure 1.16. Schematic diagram showing the process whereby staphylococcus acquires resistance and its ability to express different virulence factors, the insertion of the horizontally transferred SCC*mec* (Foster, 2004).

SCC*mec* is a mobile genetic element which is characterised by the presence of terminal inverted and direct repeats, a set of site specific recombinase genes (*ccrA* and *ccr*B), and the *mec* gene complex (Ito *et al.*, 1999; Ito *et al.*, 2001). Excision from the chromosome N325 occurs precisely and this genetic element is integrates specifically into a *S. aureus* chromosome with the help of a unique set of recombinase genes, *ccrA* and *ccr*B (Ito *et al.*, 2001). The structures of the SCC*mec* (refer to Figure 1.17) show that the recombinase genes, *ccrA* and *ccr*B are found upstream from the *mec* gene complex. There are also different types of the *ccr* complex (e.g. type 1, type 2, type 3, etc.). There are two classes of the *mec* gene complex, namely the class A *mec* complex, which has the complete structure of *mec* genes (*mecI-mecRI-mecA*-IS*431*), and the class B *mec* complex, which has a deletion and integration of an insertion sequence (IS*1272*-Δ*mecRI-mecA*-IS*431*) (Ito *et al.*, 2001).

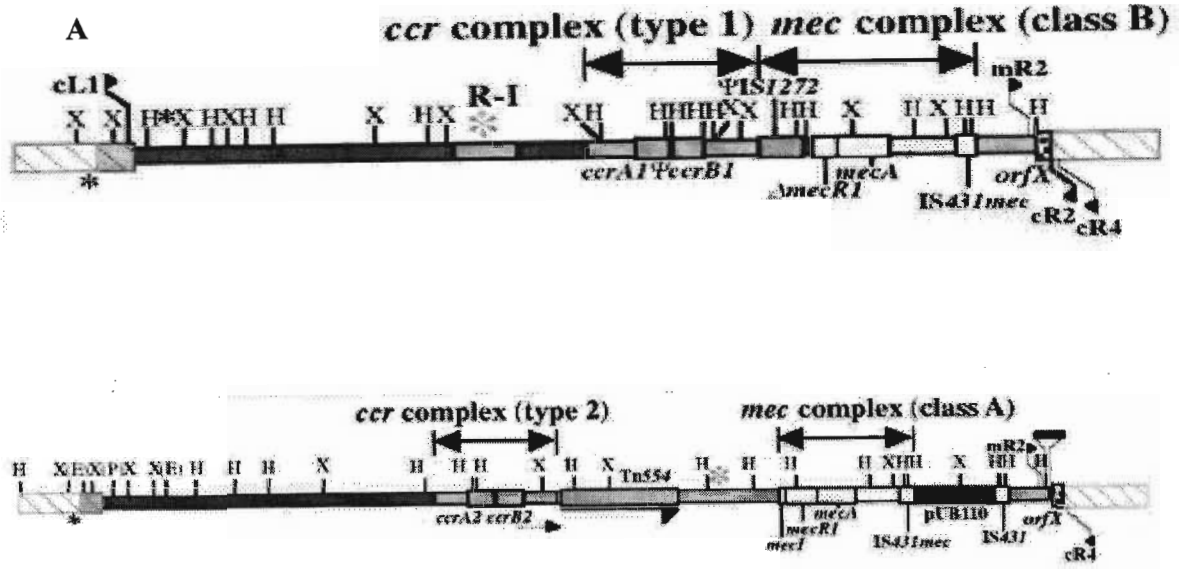


Figure 1.17. The structure of the SCC*mec* showing the *ccr* complex with the *mec* complex. The H and X refer to the *Hind* and *XbaI* restriction sites, the blocks in colour refer to the open reading frames (ORF). (A) Indicates the *ccr* type 1 complex and the mec complex class B. (B) Indicates the *ccr* type 2 complex and the *mec* complex class A (Ito *et al.*, 2001)

3.1.2. Factors essential for methicillin resistance

The expression of resistance also depends on another gene known as the factor essential for the expression of methicillin resistance or *fem* gene. The *fem* factors were first noticed in 1983, and defined the *fem* genes via a reduction of methicillin resistance acquired by transposon mediated inactivation of a gene even though the expression of *mec*A gene was not influenced (Berger-Bachi, 1983; Labischinski *et al.*, 1998). At least six *fem* genes have been described, as well as ten equivalents, but distinctly named, auxiliary (*aux*) genes (Berger-Bachi, 1994; de Lencastre & Tomasz, 1994; Labischinski *et al.*, 1998). Each of these genes affects the different steps in the synthesis of peptidoglycan. These *aux* genes are part of the cellular peptidoglycan metabolism and can regulate the degree of resistance without changing the levels of PBP 2a (Haddadin *et al.*, 2002).

It was shown that the *fem* genes belong to the family of housekeeping genes, with the majority of the genes playing a role in cell wall biosynthesis and some of genes being common to other bacteria. An example of this is FemF, which has been shown to catalyse the addition of the di-amino acid common to all peptidoglycan stem peptides (Labischinski *et al.*, 1998). The mutation of the *femAB* operon affects the glycine content of the cell wall. The FemA adds the glycines 2 and 3 of the pentaglycine interpeptide and the FemB adds the glycines 4 and 5. The mutant *femAB* lacks the resistance to the methicillin and therefore become susceptible to many unrelated antibiotics (Ling & Berger-Bachi, 1998). The *femAB* mutants are pseudo-multicellular with a thicker cell wall, have abnormal septa as a result of the damaged cell wall, and are resistant to the glycyl-glycine endopeptidase lysostaphin (Rohrer & Berger-Bachi, 2003). An additional factor termed the *fem* factor or *femX* is thought to be responsible for the addition for the glycine residue. So there are three genes that are required to move from one step to the next they are the *femA, femB*, and the *femX*, even though they have similar gene products they cannot complement each other (Ehlert *et al.*, 1997; Labischinski *et al.*, 1998).

A significant part of the strength of an antibacterial target is its spread between clinically vital organisms. The important *femA* and *femB* and the analogy *femX* all occur in *S. aureus* strains and, as may be expected from the relatively identical peptidoglycan interpeptide bridge, also occur in all coagulase-negative staphylococci observed so far. It was recently noticed that *femAB*-like genes accountable for amalgamation of serine instead of glycine into the interpeptide bridge of staphylococci shows that further *femAB*-like genes do exist. *FemABX*-like proteins implicated in cell wall interpeptide bridge formation appear belong to a multi-target protein family, which, because of their structural and functional similarity, might be reachable to inhibition by one and the same type of inhibitor (Labischinski *et al.*, 1998).

1.4 Bioinformatics Approach

1.4.1. Sequence Analysis

The cross-discipline of biological and computer science, which can be called the science of biological information used to study the biological insights. Bioinformatics is the changing of biology, traditionally an entirely lab-based science, to a more information-based science. Bioinformatics includes searching the biological databases, comparing sequences, and looking at protein structures and also just asking the biological questions with a computer (Claverie & Notredame, 2003). Phylogenetics is also a combination the biological and computer science. Phylogenetic trees makes it easy for you to organise your thinking about a protein of interest in terms of its relationship to other proteins, and may also allow you to show important results about its biological functions that would not otherwise be noticeable (Hall, 2001).

Usually the starting point of an analysis in phylogenetics or bioinformatics is the process of obtaining the sequences, whether it is obtained from sequencing the gene of interest or downloading it from the Internet. The latter option involves obtaining the sequences from a website (sequence databases), which hosts billions of sequences from many different species; the required sequence is obtained by doing a search using the keywords of the required gene. Sequence databases make it easier to analyse biological questions by analysing sequences that may have been determined as many as 20 years ago (Claverie & Notredame, 2003). The required sequence and the sequences similar to it are downloaded in the appropriate format to be used for the analysis.

The type of format that the sequences are downloaded in depends on which program is used to analyse the sequences. In this study the sequences were downloaded in the FASTA format so they could be manipulated in the Clustal program (Higgins & Sharp, 1988). There are two forms of this program, namely ClustalW (Thompson *et al.*, 1994) and ClustalX (Thompson *et al.*, 1997). Both these programs are identical in terms of the alignment; however the ClustalW program is a simple text-based interface, while the ClustalX is suitable for high-throughput

tasks or a graphical interface (Salemi & Vandamme, 2003). The latter program was used for all the alignments in this study.

The ClustalX program creates a multiple alignment in three stages. Firstly, the program independently aligns each sequence to each other sequence in a succession of pairwise alignments. Thereafter, the program uses the set of pairwise alignments created to produce a guide tree. Thirdly, this guide tree is then used to help create the multiple alignments. There are also alignment and gap penalty parameters that need to be changed in order to obtain the most optimum alignment (Hall, 2001). The optimum alignment is achieved by attempting to decrease the number as well as the size of the gaps while trying to increase the number as well as the size of the conserved blocks. The conserved block is a region in the alignment where similar or identical residues occur across all or the majority of the sequences (Hall, 2001).

Once the most optimum alignment is obtained then the next step is generally to perform a phylogenetic analysis. There are many different methods that are used to produce phylogenies from both the nucleic acid and the protein sequence alignments. The different methods are firstly, distance methods, the favoured implementation of this method is the Neighbour Joining (NJ). The second method is the Maximum Parsimony (MP). The next method that is used to create a phylogeny is the Maximum Likelihood (ML). The final method is the Bayesian (BAY). There is no better method; each method is used based on what you want as well as each method has is advantages and disadvantages (Hall, 2001).

The NJ method is an algorithmic method; this approach uses an algorithm to construct trees from the data that is provided. The algorithmic method has two important advantages, namely, they are fast and it only produces one tree from the given dataset. The other algorithmic method is the UPGMA (Unweighted Pair-group Method with Arithmetic Mean). The NJ method has almost totally replaced the UPGMA method, due to the NJ method being a faster and effective method. Both the NJ and UPGMA methods are distance methods (Hall, 2001). The distance method changes the aligned sequences into a distance matrix and reduces it in size at each step which consists of pairwise difference (distances) connecting the sequences thereafter reconstructs the tree from that series of matrices. Using the original

matrix the algorithm calculates, for each taxon, its net divergence from all the other taxa as the sum of the individual distances from the taxon. Thereafter it uses the created net divergence to calculate a corrected distance matrix. The NJ algorithm finds the two taxa with the lowest corrected distance and then calculates the distance from each of those taxa to the node that joins them. Finally a new matrix is then created whereby the new node is substituted for those two taxa (Hall, 2001; Salemi & Vandamme, 2003).

The method of maximum parsimony is based the assumption that the almost certainly or most likely tree is the one that needs the least number of changes to describe the data in the alignment. The idea behind parsimony is that for those taxa that share common characteristics do so because they have inherited those characteristics from a common ancestor. However, when there is disagreement with that assumption the reversal, convergence, or parallelism are used to explain. These explanations grouped collectively under the term homoplasy. The reversal refers to the characteristic changed but then reverted back to its original state. While the convergence refers to the dissimilar taxa evolved the same characteristic separately. When different taxa have related embryological mechanisms that influence a characteristic, to develop in a certain way, is termed the parallelism. The tree or trees that minimize the number of evolutionary steps required to explain the data is selected as the most parsimonious tree. Parsimony, or alternatively termed minimum change, is criterion for choosing the best tree. The algorithm is used to calculate the smallest number of steps essential for any given tree to be consistent with data; this smallest number is referred to as the score for the tree. The most parsimonious tree or trees is also obtained from the tree with the lowest score (Hall, 2001).

The maximum likelihood method attempts to deduce an evolutionary tree by finding that tree which maximises the probability of observing the data. The ML method is similar to the MP method the reason for this is it observes all rational tree topologies and estimates the support for each by examining each sequence position. The algorithm computes the possibility of expecting each possible nucleotide or amino acid in the ancestral or internal nodes and deduces the likelihood of the tree structure from these possibilities. The likelihood of all rational tree topologies is explored in this way, and the most likely tree is selected as the best tree (Salemi & Vandamme, 2003; Hall, 2001).

The final method described here is the Bayesian analysis, in this method the idea of posterior probabilities, the probability based o a model from previous expectations, after learning something about the data. The posterior probability is a better estimate over observing the probability that is estimated without prior knowledge (Hall, 2001).

It is important to get a statistical estimate of the reliability of some groupings, and the technique that helps with this is termed a Bootstrap Analysis. The analysis is widely used for determining the statistical error in situations in which the underlying sampling distribution is either unidentified or is not easy to obtain analytically (Efron & Gong, 1983; Salemi & Vandamme, 2003). This method was first used for the estimation of confidence intervals for phylogenies inferred from sequence data (Felsenstein, 1985). This process includes bootstrapping the sequence data; this is done by obtaining a new alignment from the original data by randomly choosing columns from it. Each of these columns in the alignment can be selected more than once or could not be selected at all until a new set of sequences; this is named the bootstrap replicate, which is the same size as the original dataset. It is noticed that some characters are randomly not chosen while some other characters are randomly chosen more than once, perhaps even twice, thrice or maybe more (Salemi & Vandamme, 2003). Also for each dataset reproduced a tree is constructed and the proportion of each clade among all the bootstrap replicates is computed, the proportion is known as the statistical confidence supporting the monophyly of the subset (Salemi & Vandamme, 2003). A strict, 50% majority rule or semi-strict consensus is then performed on the many trees produced to get the best tree (Salemi & Vandamme, 2003; Hall, 2001).

1.4.2. Protein Structure

Proteins may have four levels of structure, namely the primary, secondary, tertiary and quaternary structures. The primary structure is determined by the sequence of specific amino acids that codes for the protein, which is performed with the aid of the mRNA. The secondary structure consists of the accurate folding of the polypeptide chain. The secondary structure can be broken down in to sections of the protein that contain alpha helices (as the name suggests it has a helical shape) and beta sheet (which are flat). There might also be turns,

bends and coils present between the helices and sheets. The interaction of the protein with all the helices, sheets, bends, turns and coils forms the tertiary structure, which forms the exclusive three-dimensional (3D) structure. The quaternary structure, consists of the interactions between the different subunits (Claverie & Notredame, 2003).

Determining the 3D structure involves the use of a technique called X-ray crystallography. The theory behind this technique is based on scattering X-rays by electrons in the crystal's atoms. The 3D map is then constructed by measuring the diffraction pattern that emerges when the X-rays strike. Using this technique, many proteins structures have been determined and are freely available in public databases (Claverie & Notredame, 2003).

Some proteins are, however, much more difficult to crystallise, and, therefore, a new technique needs to be implemented to analyse these proteins. Nuclear Magnetic Resonance (NMR) is used, and is based on the principle that the nuclei of some elements resonate when placed in a powerful magnetic field. NMR, therefore, measures the chemical shifts of the atoms nuclei in the protein. The NMR creates a set of distances between specific pairs of atoms, and thereafter generates models of many possible structures as opposed to producing a single structure. Smaller proteins 3D structures can, therefore, very accurately be determined by the NMR technique (Salemi & Vandamme, 2003; Hall, 2001).

The folding of the protein allows the amino acids that are found in different parts of the protein to interact with each other. In enzymes such as the PBPs, active site are formed which involves the interactions of amino acids to form a site in the structure that catalyses the enzymatic reaction. This site also binds specifically to the substrate molecule termed the ligand. Changes in the amino acid sequence leads to changes in the overall structure of the protein and therefore also effects the functioning. So a change in the active site will result in a malfunctioning or inactive enzyme (Salemi & Vandamme, 2003; Hall, 2001).

The use of computer software has become increasingly used for the discovery of new drugs to inactivate proteins involved in diseases. The computer programs can be used to determine the 3D structure and its active site and thereafter determine ways of inactivating the protein;

therefore, the use of computer software has made major advancement in science (Salemi & Vandamme, 2003; Hall, 2001).

1.5. Aim of the study

The main aim of this study was to characterise the three different genera, namely *Streptococcus*, *Enterococcus* and *Staphylococcus,* using the genes that confer cell wall antibiotic resistance in a phylogenetic analysis. The strains or sequences used were compared according to their resistance or susceptibility, which were determined by the MIC values. Another aim of this study was to determine the important nucleotides or amino acids responsible for changes between susceptibility and resistance. Furthermore, analysis was conducted to determine the conformational 3D structure of the protein responsible for resistance.

CHAPTER TWO – MATERIALS AND METHODS

2.1. *Streptococcus* Analysis

DNA sequences were downloaded from the NCBI database, www.ncbi.nlm.nih.gov with the keyword search "penicillin binding protein in *Streptococcus*". All sequences of relevant importance were downloaded in the GenBank format. Sequences were separated using the BioEdit program (Figure 2.1). First, the partial (i.e. only a part of the PBP gene) and then the complete PBP sequence were separated. The complete sequences were separated via two parts: (i) according to the species, i.e. the *S. pneumonia, mitis, mutans* etc. and (ii) according to the PBPs, i.e. PBP 1a, PBP 1b, PBP 2a etc. Next was to perform an analysis on each group of data. Comparisons were made between the different species, *S. pneumonia, mitis, mutans* etc. Comparisons were also made between and within the PBP groups. The comparisons were done via an alignment using the ClustalX program. The initial alignment was done using the default settings, only if there were large gaps or improperly aligned regions the gaps were reweighed and the sequences were realigned. The chosen output options of the alignment were the Phylip and Nexus.

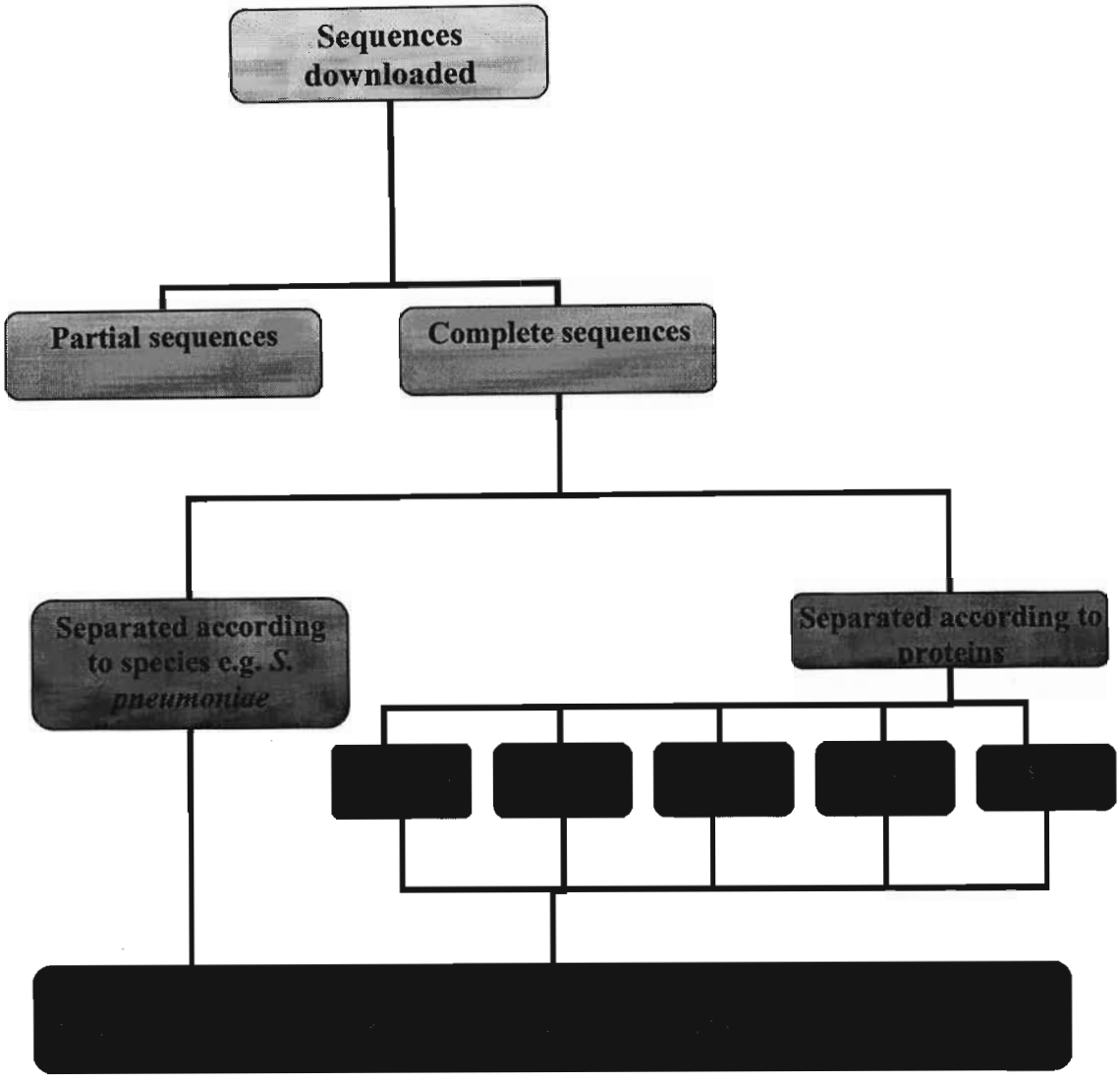


Figure 2.1. Flow diagram showing steps taken to separate the data in order to perform the analysis on the sequences.

The results of the alignment were exported in the Nexus format to be used by the PAUP* program (Swofford 2000) for the construction of the distance matrix. The phylip format, out file, created by the ClustalX multiple alignment was subsequently used for a Bootstrap analysis (refer to Figure 2.2) which was performed using the SEQBOOT program in PHYLIP. A 1000 replicates were chosen for the data with the random seed number (which must be an odd number) of 101, was chosen for all the analysis using Bootstrap.

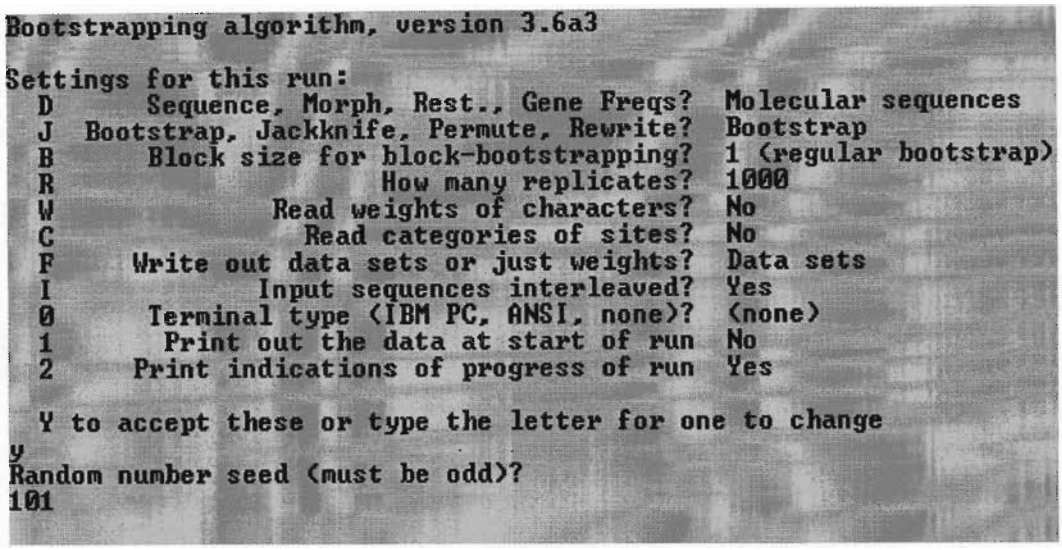


Figure 2.2. The Bootstrap options that were chosen for the Bootstrapping algorithm.

Using the output file created by Bootstrap, a parsimony analysis was performed using the DNAPARS program available in PHYLIP (Figure 2.3). Multiple datasets were chosen with the number of replicates created by the Bootstrapping algorithm being 1000. The random seed number chosen was also 101, and the number of times to jumble was 10. The other default options were not altered.

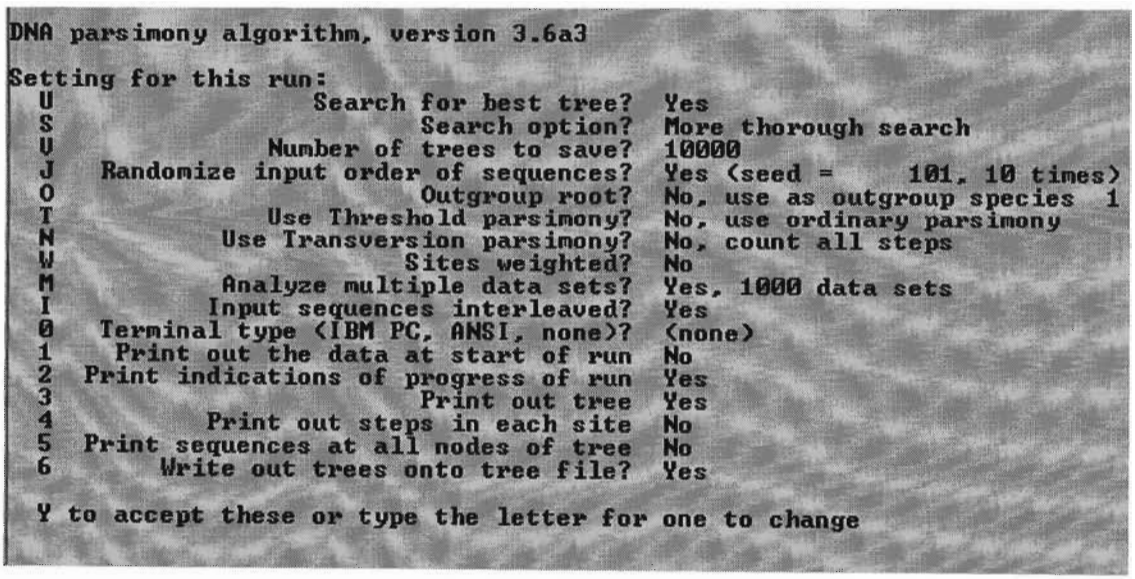


Figure 2.3. The DNA parsimony options that are chosen for the algorithm.

The output of the DNA parsimony algorithm was an out file and an out tree. The out tree file, which contains multiple trees, was used for the next step. A consensus tree was subsequently obtained from the number of trees produced using the CONSENSE program available in PHYLIP (the options chosen are shown in Figure 2.4). All the default options were unaltered for the consensus analysis.

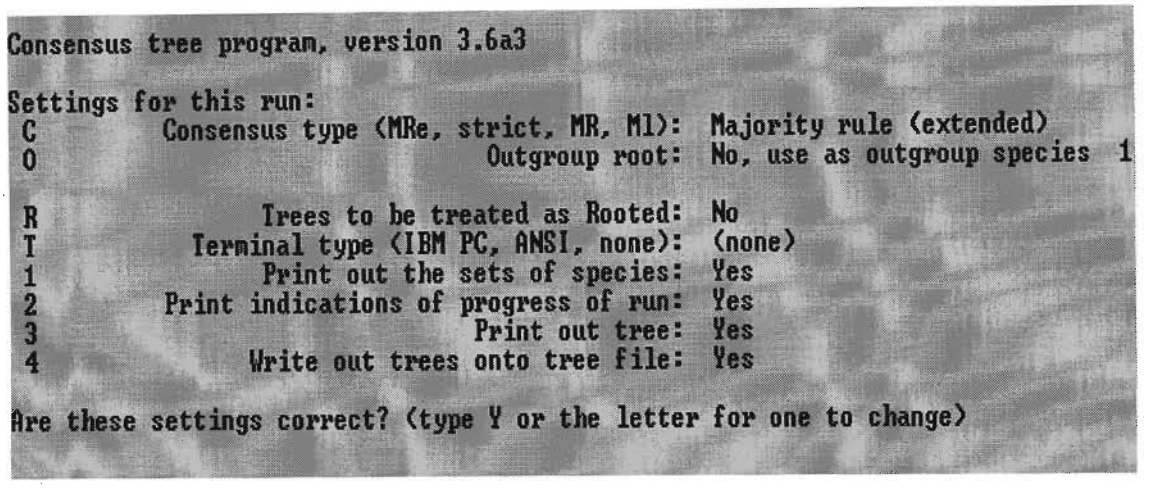


Figure 2.4. The Consensus analysis options that were chosen for this Consensus tree algorithm.

Protein analysis involved the translation of DNA sequences to protein sequences, using the tools on the Expasy website. Also more protein sequences were downloaded from the Expasy website with the keyword search "penicillin binding protein in *Streptococcus*". Thereafter the relevant protein sequences were downloaded in FASTA format. The downloaded protein sequences were separated in a similar format to the DNA sequences (refer to Figure 2.1). Using the ClustalX program the various sequences were aligned. However, in comparison to the DNA sequence alignment some changes were made with respect to the gap opening penalty and the gap extension penalty. The default values for the gap opening penalty (10.00) and the gap extension penalty (0.10) was used for the DNA sequences. The gap opening penalty (35.00) and the gap extension penalty (0.75) was used for the protein sequences in order to give better alignments (Hall, 2001). The comparison with protein data was conducted in a similar fashion using the same programs with the exception of the PHYLIP program PROTPARS. PROTPARS was used instead of the DNAPARS program since the data that

was analyzed was protein sequences. All other programs and options chosen remained the same, i.e. in Bootstrap the number of replications was 1000. And the random seed number chosen was 101. The PROTPARS program had similar changes made to the algorithm in comparison to DNA sequences, i.e. multiple datasets was chosen with the number being 1000, and the number of times to jumble was 10. The other default options were not altered in the PROTPARS program. The CONSENSE programs default options remained the same. The distance matrix was obtained from the PAUP* program as mentioned above.

The protein modelling part of the project involved the use of the Swiss-PDB program (Koop & Schede, 2004) which was downloaded from SwissPDB website. The 3D models were obtained by submitting the protein sequence to the Expasy database. Also, some of the 3D structures were downloaded from the database using the keyword search "penicillin binding proteins'. The active sites of the PBPs were obtained from literature and comparisons were made between the resistant and the susceptible strains.

2.2. *Enterococcus* Analysis

The *Enterococcus* sequences were obtained from the NCBI database with the keyword searches "Vancomycin resistance in Enterococcus" and "Van genes in Enterococcus". The sequences was subsequently separated according to their phenotypes i.e., the VanA, VanB, VanC, etc was grouped together, with the genes that are found within them being separated. This was followed by a comparative analysis using all the phenotypes (the entire VanA, VanB, VanC, etc cluster). This was done via the same process previously mentioned, by constructing a consensus tree using the PHYLIP programs, and the distance matrix using the PAUP program. The same options were chosen for this analysis (refer to Figures 2.2, 2.3 and 2.4). Where possible, the individual vancomycin genes were used for comparative analysis. The protein analysis was also conducted using similar options as mentioned for the proteins above.

The 3D models of the proteins were obtained from the Expasy database as indicated before using the keyword searches "VanA", "VanB", "VanC", "VanD" "VanE" and "vanX". These protein models were subsequently downloaded and analyzed by inspecting the active sites and the way that the antibiotic interacts with it.

2.3. *Staphylococcus* Analysis

The *Staphylococcus* sequences were obtained from the NCBI database with the keyword searches "Methicillin resistance in *Staphylococcus*" and "Mec *Staphylococcus* genes" and "fem *Staphylococcus* genes". The sequences were separated according to their phenotypes *mecA*, *mecI*, *mecR*, and *fem*. Analysis was performed using both the DNA and protein sequences with the respective options stipulated above using the PHYLIP program to create the consensus trees, and the distance matrices using the PAUP program.

CHAPTER THREE – RESULTS AND DISCUSSION

3.1. Penicillin resistance in *Streptococcus*

3.1.1. Sequence Analysis - DNA Sequences

PBP nucleotide sequences were downloaded from the GenBank database, characterized from species with MIC values ranging from <0.0075 μg/ml to 8 μg/ml. In this part of the study, nucleotide sequences associated with penicillin resistance were tested. There are a total of 57 different PBP 1a *S. pneumoniae* sequences used in this study, 41 different PBP 3 *S. pneumoniae* sequences, 40 different PBP 2b *S. pneumoniae* sequences, and 59 different PBP 2x *S. pneumoniae* sequences. Analysis was performed on these different nucleotide sequences. Comparisons were then made using the MIC values associated with each strain.

The tree shown in Figure 3.1 is the consensus tree of *S. pneumonia* penicillin binding protein 1a. When we compare Figure 3.1 and Table 3.1, the MIC values indicate that the susceptible strains (<0.1 μg/ml) are found towards the top and bottom of the tree. Furthermore, the resistant strains are found in the middle of the tree. They are found within the inner group leading to a common ancestor, strain SP00065 with the accession number AB119767. This strain is susceptible to penicillin due to its MIC value of 0.063 μg/ml. It could, therefore, be speculated that the resistant phenotypes evolved from this susceptible phenotype, since all the resistant types are found in one group and have this strain as a common ancestor. They could have gained a number of mutations and, therefore, would have become resistant to penicillin. When the tree in Figure 3.1 is compared with the Distance Matrix 1 of the *S. pneumonia* PBP 1a (refer to Appendix A) it is noticed that the strain SP00065, when compared with all the sequences within the resistant group (highlighted in the table) which is thought to be their common ancestor, has distance values grater than 0.07315. This implies that the sequences that are being compared are not considerably similarly to each other. This result seems to be valid as we are comparing the susceptible, strain SP00065, with all the resistant and intermediate strains that are found within the group. Alignment 1 (refer to Appendix B, Alignment 1 of the *S. pneumonia* PBP 1a) shows that there are a number of mutations found in

the resistant strains in comparison with the susceptible strains. It is also seen that the higher the MIC values the greater the number of mutations are found in the alignment. In other words, the more resistant a strain is, the more mutations are found throughout the length of the PBP when it is compared with the susceptible strains to penicillin. When we compare, for example, a highly resistant strain, AB119791 strain SP00091, with a highly susceptible strain, AB119754 strain SP00052, it is noticed that there is a far greater number of mutations present in the resistant strain. This, therefore, means the greater the number of mutations, the more the protein will change in structure and, therefore, will not be able to bind specifically to the antibiotic and, thus confer resistance.

The susceptible strains, which are found toward the ends of the tree forming the roots, are examined using the Distance Matrix 1, of the *S. pneumonia* PBP 1a (refer to Appendix A). It can be seen that for the susceptible associated sequences found toward the end of the tree the distance values does not exceed the value of 0.00463. This, therefore, shows that these sequences are greatly similar to each other, with very low level of changes noticed between the different strains. Furthermore, when the alignment is looked at (refer to Appendix B Alignment 1) it can be seen that there are very few mutations present between the sequences. This overall result could mean that the resistant strains may have evolved from the susceptible strains. PBP 1a, is one of the proteins implicated in resistance to penicillin based antibiotic, therefore, this result is significant in terms of the origin of the resistant strains.

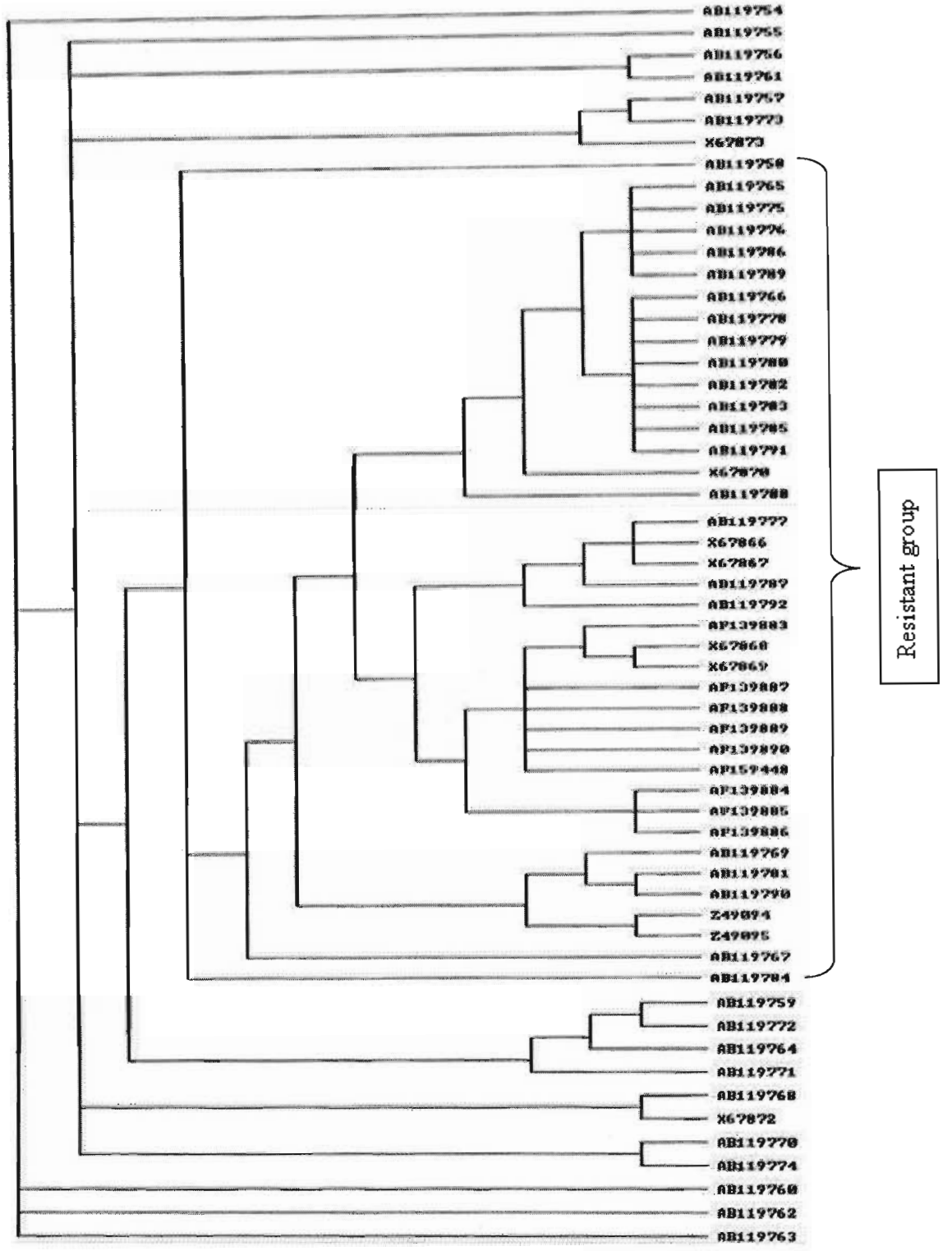


Figure 3.1. Consensus tree produced using the nucleotide sequences from *S. pneumonia* penicillin binding protein 1a. Resistance is based on the MIC values of each strain.

Table 3.1. The accession numbers corresponding to the strains used, including the affinity for penicillin with the MIC (μg/ml) values for penicillin binding protein 1a.

Accession Numbers	*S. pneumoniae* PBP 1a Strains	Affinity for penicillin (MIC μg/ml)[a]	Reference
AB119753	SP00051	S - 0.063	Sanbongi *et al.*, 2004
AB119754	SP00052	S - 0.008	Sanbongi *et al.*, 2004
AB119755	SP00053	S - 0.063	Sanbongi *et al.*, 2004
AB119756	SP00054	S - 0.063	Sanbongi *et al.*, 2004
AB119757	SP00055	I - 0.125	Sanbongi *et al.*, 2004
AB119758	SP00056	I - 0.5	Sanbongi *et al.*, 2004
AB119759	SP00057	I - 0.25	Sanbongi *et al.*, 2004
AB119760	SP00058	S - 0.063	Sanbongi *et al.*, 2004
AB119761	SP00059	S - 0.063	Sanbongi *et al.*, 2004
AB119762	SP00060	S - 0.063	Sanbongi *et al.*, 2004
AB119763	SP00061	S - 0.063	Sanbongi *et al.*, 2004
AB119764	SP00062	I - 0.25	Sanbongi *et al.*, 2004
AB119765	SP00063	I - 0.5	Sanbongi *et al.*, 2004
AB119766	SP00064	I - 0.25	Sanbongi *et al.*, 2004
AB119767	SP00065	S - 0.063	Sanbongi *et al.*, 2004
AB119768	SP00067	I - 0.125	Sanbongi *et al.*, 2004
AB119769	SP00068	I - 0.125	Sanbongi *et al.*, 2004
AB119770	SP00069	I - 0.25	Sanbongi *et al.*, 2004
AB119771	SP00070	I - 0.25	Sanbongi *et al.*, 2004
AB119772	SP00072	S - 0.031	Sanbongi *et al.*, 2004
AB119773	SP00073	I - 0.125	Sanbongi *et al.*, 2004
AB119774	SP00074	I - 0.25	Sanbongi *et al.*, 2004
AB119775	SP00075	R – 2	Sanbongi *et al.*, 2004
AB119776	SP00076	R – 2	Sanbongi *et al.*, 2004
AB119777	SP00077	I - 1	Sanbongi *et al.*, 2004
AB119778	SP00078	R – 2	Sanbongi *et al.*, 2004
AB119779	SP00079	R – 2	Sanbongi *et al.*, 2004
AB119780	SP00080	R – 4	Sanbongi *et al.*, 2004
AB119781	SP00081	I - 0.5	Sanbongi *et al.*, 2004
AB119782	SP00082	R - 2	Sanbongi *et al.*, 2004
AB119783	SP00083	R - 2	Sanbongi *et al.*, 2004
AB119784	SP00084	R – 4	Sanbongi *et al.*, 2004
AB119785	SP00085	I – 1	Sanbongi *et al.*, 2004
AB119786	SP00086	R - 2	Sanbongi *et al.*, 2004
AB119787	SP00087	R – 2	Sanbongi *et al.*, 2004
AB119788	SP00088	I – 1	Sanbongi *et al.*, 2004
AB119789	SP00089	R – 2	Sanbongi *et al.*, 2004
AB119790	SP00090	R – 4	Sanbongi *et al.*, 2004
AB119791	SP00091	R - 4	Sanbongi *et al.*, 2004

Table 3.1. continued...

Accession Numbers	*S. pneu* PBP 1a Strains	Affinity for penicillin (MIC µg/ml)[a]	Reference
AB119792	SP00092	R - 2	Sanbongi *et al.*, 2004
AF139883	SP665	R - 0.5-2	Coffey *et al.*, 1999
AF139884	PO273	R - 0.5-2	Coffey *et al.*, 1999
AF139885	PO341	R - 0.5-2	Coffey *et al.*, 1999
AF139886	PO342	R - 0.5-2	Coffey *et al.*, 1999
AF139887	M134	R - 0.5-2	Coffey *et al.*, 1999
AF139888	URU135	R - 0.5-2	Coffey *et al.*, 1999
AF139889	URU157	R - 0.5-2	Coffey *et al.*, 1999
AF139890	URU159	R - 0.5-2	Coffey *et al.*, 1999
AF159448	URU206	R - 0.5-2	Coffey *et al.*, 1999
X67866	681	I - 1	Martin *et al.*, 1992
X67867	670	R - 2	Martin *et al.*, 1992
X67868	456	R - 2	Martin *et al.*, 1992
X67869	56742	R - 4	Martin *et al.*, 1992
X67870	2039	R - 2	Martin *et al.*, 1992
X67872	63915	S - 0.015	Martin *et al.*, 1992
X67873	45607	S - 0.12	Martin *et al.*, 1992
Z49094	CS109	R	Coffey *et al.*, 1995
Z49095	CS111	S	Coffey *et al.*, 1995

[a] S - Susceptible, I - Intermediate, R – Resistant.

The PBP 3 produces a very interesting result. In Figure 3.2 with reference to Table 3.2, it can be noticed that the resistant sequences are spread throughout the tree, unlike Figure 3.1, and are not found in groups. The susceptible strains are also found spread throughout the tree in-between the resistant and the intermediate phenotypes. This result is significant due to the fact that the PBP 3 is not one of the essential PBPs to confer resistance. Therefore, we can speculate that the resistance noticed for these strains may be due to other genes involved. The tree in Figure 3.2 shows that in one group found towards the middle of the phylogram containing the sequences AB119990, AB119955 and AB119964 with the corresponding strains SP00090, SP00053 and SP00062 respectively, that this group has three different phenotypes, resistant, intermediate and susceptible (highlighted in the figure). An interesting factor of this group is that no differences are noticed between the sequences (refer to the Appendix B – Alignment 2 for the *S. pneumonia* PBP 3). When the Distance Matrix 2 (refer

to Appendix A – Distance Matrix 2 for *S. pneumonia* PBP 3) is studied, this result is confirmed showing values of zero for these three different strains. Therefore, based on this information, it can be said that this gene, the PBP 3, does not play a significant role in resistance.

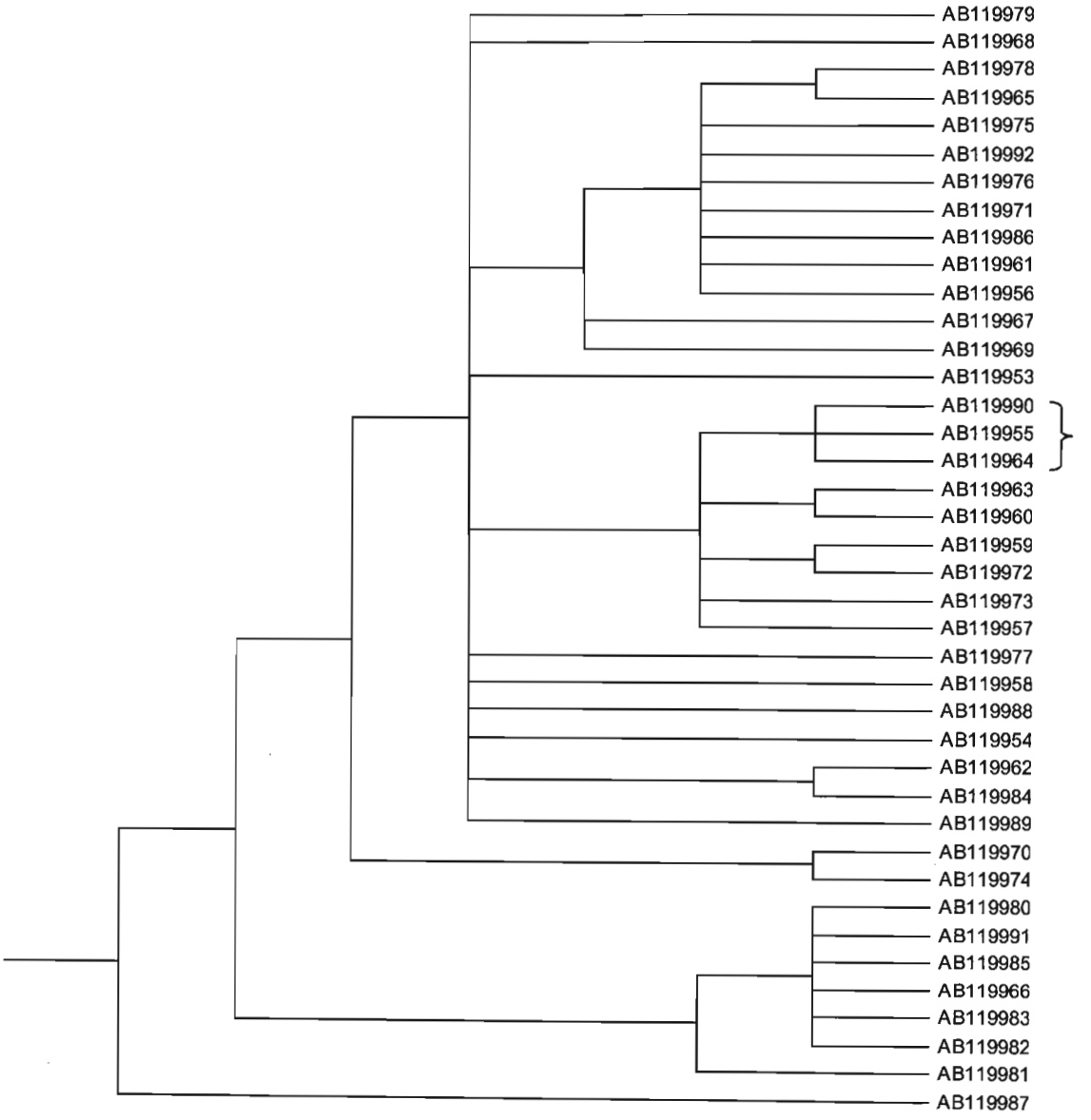


Figure 3.2. Consensus tree of *S. pneumonia* penicillin binding protein 3.

Table 3.2. Accession numbers corresponding to the strains used, and the affinity for penicillin with the MIC (μg/ml) values for penicillin binding protein 3.

Accession Numbers	*S. pneumonia* PBP 3 Strains	Affinity for penicillin[a] (MIC μg/ml)
AB119992	SP00092	R – 2
AB119991	SP00091	R – 4
AB119990	SP00090	R – 4
AB119989	SP00089	R – 2
AB119988	SP00088	I – 1
AB119987	SP00087	R – 2
AB119986	SP00086	R – 2
AB119985	SP00085	I – 1
AB119984	SP00084	R – 4
AB119983	SP00083	R – 2
AB119982	SP00082	R – 2
AB119981	SP00081	I - 0.5
AB119980	SP00080	R – 4
AB119979	SP00079	R – 2
AB119978	SP00078	R – 2
AB119977	SP00077	I – 1
AB119976	SP00076	R – 2
AB119975	SP00075	R – 2
AB119974	SP00074	I - 0.25
AB119973	SP00073	I - 0.125
AB119972	SP00072	S - 0.031
AB119971	SP00070	I - 0.25
AB119970	SP00069	I - 0.25
AB119969	SP00068	I - 0.125
AB119968	SP00067	I - 0.125
AB119967	SP00065	S - 0.063
AB119966	SP00064	I - 0.25
AB119965	SP00063	I - 0.5
AB119964	SP00062	I - 0.25
AB119963	SP00061	S - 0.063
AB119962	SP00060	S - 0.063
AB119961	SP00059	I - 0.25
AB119960	SP00058	S - 0.063
AB119959	SP00057	I - 0.25
AB119958	SP00056	I - 0.5
AB119957	SP00055	I - 0.125
AB119956	SP00054	S - 0.008
AB119955	SP00053	S - 0.063
AB119954	SP00052	S - 0.008
AB119953	SP00051	S - 0.063

[a] All strains were obtained from the reference Sanbongi *et al.*, 2004. S - Susceptible, I - Intermediate, R – Resistant.

Figure 3.3 shows the consensus tree produced with the nucleotide sequences from *S. pneumoniae* PBP 2b. This figure in combination with the Table 3.3 shows the relatedness of the PBP 2b sequences. It can be noticed that the resistant strains, with the exception of the strain SP00090, accession number AB119910, which is found in the middle of a group containing only intermediate strains, are found toward the bottom of the tree in a single group, and this group of resistant strains (highlighted in Figure 3.3) are linked to the group of susceptible and intermediate strains, which is found in the upper section of the tree. The susceptible strains are found grouped together on the top of the tree, with some susceptible strains being found in-between the intermediate strains. This result, therefore, indicates to us that the resistant strains are different in comparison to the susceptible strains. This is based on their PBP 2b nucleotide sequences, due to the fact that they are found on separate ends of the tree. It can also be seen in the tree that the strain SP00078, accession number AB119898, forms the out-group or the root of the tree. It is not clearly understood why this strain forms the out-group, but it is speculated that due to its number of mutations it is unable to group with the rest of the strains.

The Distance Matrix three (refer to Appendix A, Distance Matrix 3 of *S. pneumonia* PBP 2b using the DNA sequences) confirms the result obtained in the tree shown in Figure 3.3. It can be noticed that the values obtained in the distance matrix are high when the susceptible and resistant strains are compared, with the lowest values of 0.04276 obtained. When the susceptible strains are compared to each other the highest value obtained is 0.00534, showing that these strains are very closely related to each other. If these different values, i.e. the values for the resistant and susceptible phenotypes, are compared to each other, it can be seen that based on the nucleotide sequences, there is a clear difference between the susceptible and the resistant phenotypes.

The result of Alignment three (refer to Appendix B, Alignment 3 of *S. pneumonia* PBP 2b using the DNA sequences) shows that there is a clear difference between the resistant and susceptible strains, in terms of their PBP 2b nucleotide sequences. The regions of 180 – 200 bp, 980 – 1200 bp and 1300 - 1400, show a number of changes between the resistant and susceptible strains. There are also a number of point mutations found throughout the

sequences. Therefore, from the result obtained from this section we can say that the sequences for the resistant and susceptible strains are clearly different from each other. This is very important due to the fact that this protein, PBP 2b, is one of the PBPs implicated in resistance.

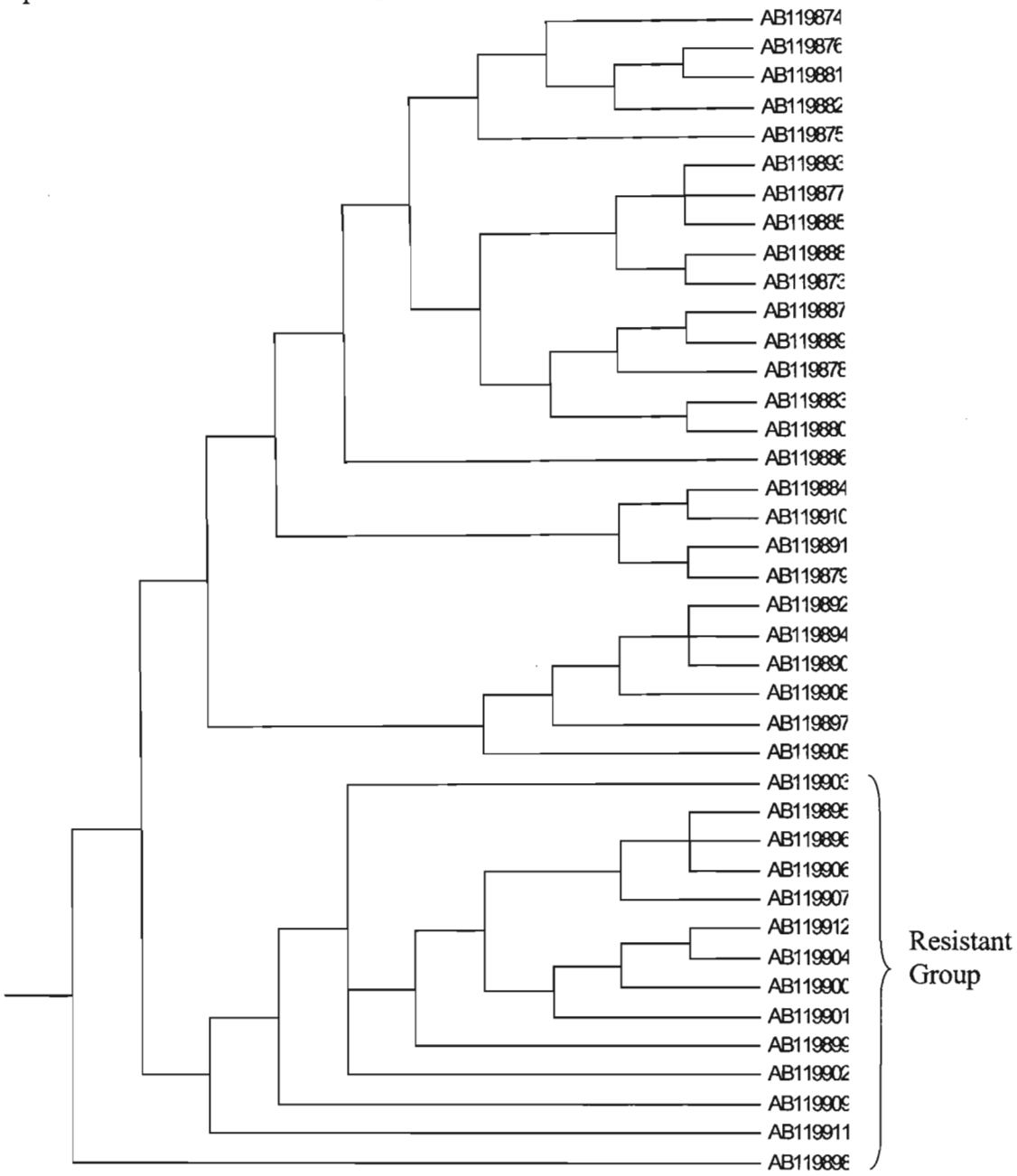


Figure 3.3. Consensus tree of *S. pneumonia* penicillin binding protein 2b, shown with the accession numbers. Also shown on the tree is the resistant group, i.e. the group that contains strains that are resistant to penicillin.

Table 3.3. The accession numbers corresponding to the strains used, and the affinity for penicillin with the MIC (μg/ml) values for penicillin binding protein 2b

Accession Numbers	*S. pneumonia* PBP 2b Strains	Affinity for penicillin (MIC μg/ml)[a]
AB119912	SP00092	R – 2
AB119911	SP00091	R – 4
AB119910	SP00090	R – 4
AB119909	SP00089	R – 2
AB119908	SP00088	I – 1
AB119907	SP00087	R – 2
AB119906	SP00086	R – 2
AB119905	SP00085	I – 1
AB119904	SP00084	R – 4
AB119903	SP00083	R – 2
AB119902	SP00082	R – 2
AB119901	SP00081	I - 0.5
AB119900	SP00080	R – 4
AB119899	SP00079	R – 2
AB119898	SP00078	R – 2
AB119897	SP00077	I – 1
AB119896	SP00076	R – 2
AB119895	SP00075	R – 2
AB119894	SP00074	I - 0.25
AB119893	SP00073	I - 0.125
AB119892	SP00072	S - 0.031
AB119891	SP00070	I - 0.25
AB119890	SP00069	I - 0.25
AB119889	SP00068	I - 0.125
AB119888	SP00067	I - 0.125
AB119887	SP00065	S - 0.063
AB119886	SP00064	I - 0.25
AB119885	SP00063	I - 0.5
AB119884	SP00062	I - 0.25
AB119883	SP00061	S - 0.063
AB119882	SP00060	S - 0.063
AB119881	SP00059	S - 0.063
AB119880	SP00058	S - 0.063
AB119879	SP00057	I - 0.25
AB119878	SP00056	I - 0.5
AB119877	SP00055	I - 0.125
AB119876	SP00054	S - 0.063
AB119875	SP00053	S – 0.063
AB119874	SP00052	S – 0.008
AB119873	SP00051	S - 0.063

[a] All strains obtained from Sanbongi *et al.*, 2004. S-Susceptible, I-Intermediate, R–Resistant.

In Figure 3.4 with reference to Table 3.4, showing the PBP 2x, the resistant strains is separated towards the ends of the tree while the susceptible strains are found toward the middle of the tree. The susceptible strains are split into two groups by the intermediate strains and this is very unusual. Another very unusual observation is the presence of the susceptible strains SP00058 and SP00064, accession numbers AB119920 and AB119926 respectively with a MIC value of 0.063 μg/ml for both strains, which is found in the middle of the resistant group. A possible explanation for this could be that there are a number of mutations found throughout the gene, but these mutations are not critical for the resistance to the antibiotic. If we look at the alignment (refer to Appendix B, Alignment 4 of *S. pneumonia* PBP 2x using the nucleotide sequences) it is clearly visible that the susceptible strains, SP00058 and SP00064, have several mutations when compared to the other susceptible strains. However, when these strains are compared to the resistant strains it can be observed that there are some regions of homology between the sequences but there are also more mutations found in the resistant strains, which could be the mutations responsible for some level of resistance.

When we compare the two groups of resistant strains found towards the ends of the tree (refer to Appendix B, Alignment 4 of *S. pneumonia* PBP 2x using the nucleotide sequences) it can be noticed that there are several changes between these two groups. The bottom group of resistant strains has more mutations in comparison to the top resistant group. This shows how the two different groups have diverged from each other although they both give resistance. The additional mutations that are found in the bottom resistance group are probably not important for the resistance, therefore the MIC values are about the same for both the top and bottom groups.

The Distance Matrix 4 (refer to refer to Appendix A, Distance Matrix 4 of *S. pneumonia* PBP 2x using the nucleotide sequences) shows that the susceptible strains, which were found in the middle of the resistant group, have very high distance values when compared with the other susceptible strains. The lowest values for the susceptible strains SP00058 and SP00064, when compared to the other susceptible strains, are 0.17976 and 0.06880 respectively, which are very high values for susceptible strains to have. Another important observation is that the strain SP00064 has a matrix value of zero when compared with the other resistant strains

SP00082 and SP00083 with MIC values of 2 μg/ml. A possible explanation for this might be that the MIC values might have been miscalculated and this strain SP00064 is perhaps a resistant one, however this is just speculation.

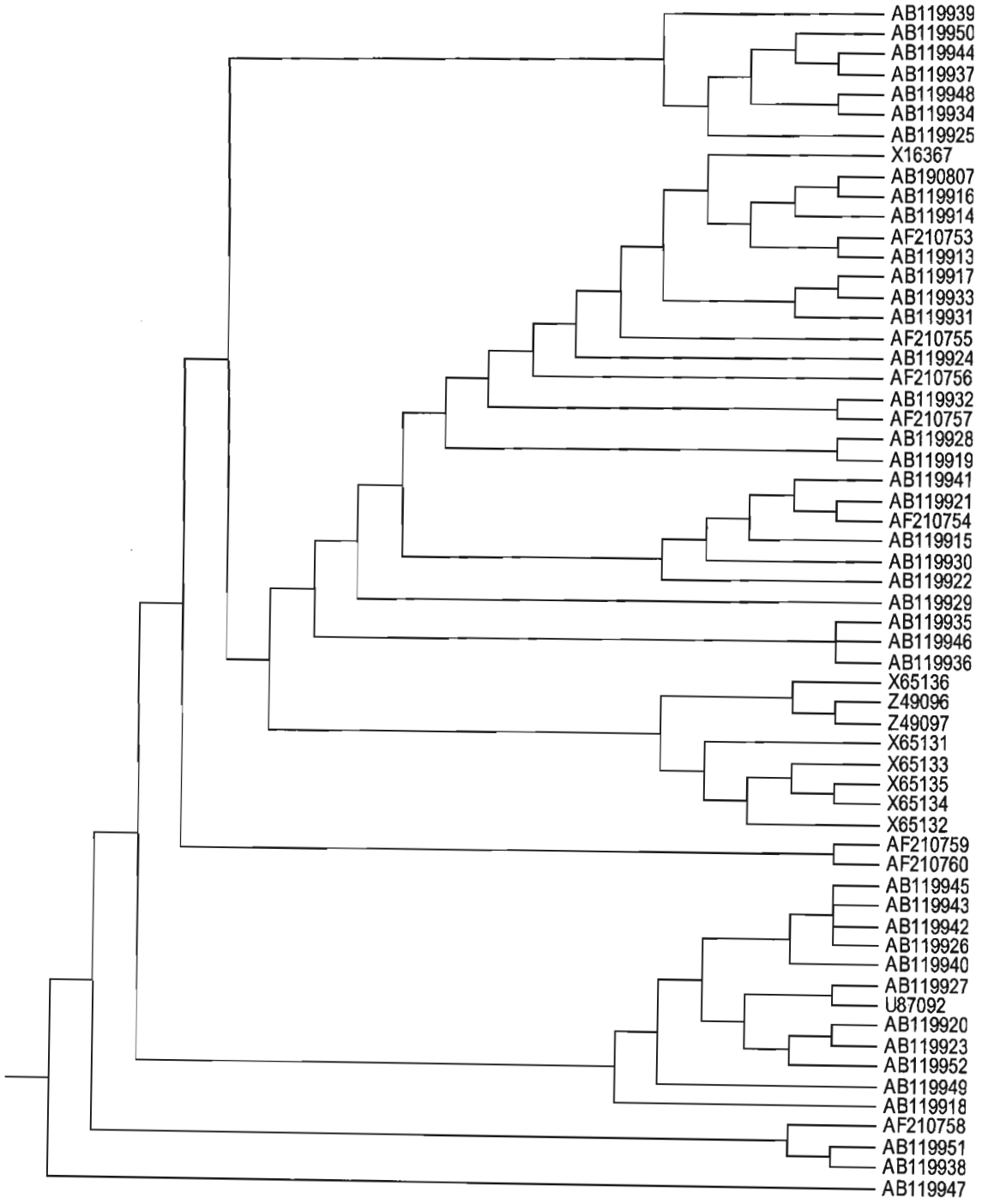


Figure 3.4. The consensus tree of *S. pneumonia* PBP 2x, based on the nucleotide sequences

Table 3.4. The accession numbers corresponding to the strains used, also contains the affinity for penicillin with the MIC (μg/ml) values for PBP 2x

Accession Numbers	*S. pneumonia* PBP 2x Strains	Affinity for penicillin (MIC μg/ml)	Reference
AB119913	SP00051	S - 0.063	Sanbongi *et al.*, 2004
AB119914	SP00052	S - 0.008	Sanbongi *et al.*, 2004
AB119915	SP00053	S - 0.063	Sanbongi *et al.*, 2004
AB119916	SP00054	I - 0.125	Sanbongi *et al.*, 2004
AB119917	SP00055	I - 0.5	Sanbongi *et al.*, 2004
AB119918	SP00056	I - 0.25	Sanbongi *et al.*, 2004
AB119919	SP00057	S - 0.063	Sanbongi *et al.*, 2004
AB119920	SP00058	S - 0.063	Sanbongi *et al.*, 2004
AB119921	SP00059	S - 0.063	Sanbongi *et al.*, 2004
AB119922	SP00060	S - 0.063	Sanbongi *et al.*, 2004
AB119923	SP00061	I - 0.25	Sanbongi *et al.*, 2004
AB119924	SP00062	I - 0.5	Sanbongi *et al.*, 2004
AB119925	SP00063	I - 0.25	Sanbongi *et al.*, 2004
AB119926	SP00064	S - 0.063	Sanbongi *et al.*, 2004
AB119927	SP00065	I - 0.125	Sanbongi *et al.*, 2004
AB119928	SP00067	I - 0.125	Sanbongi *et al.*, 2004
AB119929	SP00068	I - 0.25	Sanbongi *et al.*, 2004
AB119930	SP00069	I - 0.25	Sanbongi *et al.*, 2004
AB119931	SP00070	S - 0.031	Sanbongi *et al.*, 2004
AB119932	SP00072	I - 0.125	Sanbongi *et al.*, 2004
AB119933	SP00073	I - 0.25	Sanbongi *et al.*, 2004
AB119934	SP00074	R – 2	Sanbongi *et al.*, 2004
AB119935	SP00075	R – 2	Sanbongi *et al.*, 2004
AB119936	SP00076	I – 1	Sanbongi *et al.*, 2004
AB119937	SP00077	R – 2	Sanbongi *et al.*, 2004
AB119938	SP00078	R – 2	Sanbongi *et al.*, 2004
AB119939	SP00079	R – 4	Sanbongi *et al.*, 2004
AB119940	SP00080	I - 0.5	Sanbongi *et al.*, 2004
AB119941	SP00081	S - 0.063	Sanbongi *et al.*, 2004
AB119942	SP00082	R – 2	Sanbongi *et al.*, 2004
AB119943	SP00083	R – 2	Sanbongi *et al.*, 2004
AB119944	SP00084	R – 4	Sanbongi *et al.*, 2004
AB119945	SP00085	I – 1	Sanbongi *et al.*, 2004
AB119946	SP00086	R – 2	Sanbongi *et al.*, 2004
AB119947	SP00087	R – 2	Sanbongi *et al.*, 2004
AB119948	SP00088	I – 1	Sanbongi *et al.*, 2004
AB119949	SP00089	R – 2	Sanbongi *et al.*, 2004
AB119950	SP00090	R – 4	Sanbongi *et al.*, 2004

Table 3.4. Continued...

Accession Numbers	*S. pneu* PBP 2x Strains	Affinity for penicillin (MIC µg/ml)	Reference
AB119951	SP00091	R – 4	Sanbongi *et al.*, 2004
AB119952	SP00092	R – 2	Sanbongi *et al.*, 2004
AB190807	KK97	S - 0.016	Maeda *et al.*, 2004
AF210753	SP1261	S - 0.008	Ferroni & Berche, 2001
AF210754	SP1513	S - 0.006	Ferroni & Berche, 2001
AF210755	SP1465	I - 0.12	Ferroni & Berche, 2001
AF210756	SP1258	I - 0.25	Ferroni & Berche, 2001
AF210757	1053	I – 0.5	Ferroni & Berche, 2001
AF210758	BM 4200	I – 0.5	Ferroni & Berche, 2001
AF210759	1470	R – 2	Ferroni & Berche, 2001
AF210760	22861	R – 8	Ferroni & Berche, 2001
U87092	328	R – 4	Mouz *et al.*, 1998
X16367	6/C506	Unknown[a]	Laible *et al.*, 1989
X65131	29044	Unknown[a]	Laible *et al.*, 1991
X65132	8249	Unknown[a]	Laible *et al.*, 1991
X65133	669	Unknown[a]	Laible *et al.*, 1991
X65134	53139/72	Unknown[a]	Laible *et al.*, 1991
X65135	110K/70	Unknown[a]	Laible *et al.*, 1991
X65136	577	Unknown[a]	Laible *et al.*, 1991
Z49096	CS109	Unknown[a]	Coffey *et al.*, 1995
Z49097	CS111	Unknown[a]	Coffey *et al.*, 1995

[a] MIC values were unobtainable,

S – Susceptible, I – Intermediate and R – Resistant.

3.1.2 Sequence Analysis - Protein Sequences

The next part of this project included the use of the Streptococcal protein sequences. The protein sequences were obtained for all the Streptococcal species that were available. In this part of the study there were eight different species used, namely, *S. pneumoniae*, *S. lactis*, *S. agalactiae*, *S. faecalis*, *S. mutans*, *S. pyogenes*, *S. gordonii* and *S. thermophilus*. For each of the PBPs there were sequences from several different strains downloaded. PBP 1a has a total of 36 sequences; PBP 1b has a total of 23 sequences; PBP 2a contains 31 different sequences, PBP 2b has 30 sequences, PBP 2x has 49 sequences. The levels of resistance varied from susceptible to highly resistant. A comparative analysis was performed on the respective groups of data, based on their resistance levels.

Figure 3.5 shows the consensus tree of the various Streptococcal species using the PBP 1a amino acid sequences. When the figure is analysed, with respect to the information shown in Table 3.5, it can be noticed that *S. pneumoniae* species form a group in the middle of the tree, while the other species are found on either ends of the tree. The *S. pyogenes* species is found at the base or the root of the tree. The *S. lactis* and *S. faecalis* are found at the top of the tree with *faecalis* species forming an out-group when compared to the *pneumoniae* group. Within the *pneumoniae* cluster it can be noticed that there is a group of susceptible strains found at the top. Also within the *pneumoniae* cluster the resistant strains are grouped together, while some intermediate strains are found in-between. The fact that the resistant and the susceptible types are found on two separate groups implies that they are different.

The MIC values for some strains could not be obtained. Also, for some sequences with accession numbers Q75YL8, Q75YM1, Q75YM2 and Q75YM5, there was more than one strain associated with the protein with each of the strains having a different MIC. An example of this is for the accession number Q75YL8 where there are eight different strains given to the one protein sequence. These eight strains include SP00078 (2 μg/ml), SP00064 (0.5 μg/ml), SP00079 (2 μg/ml), SP00080 (4 μg/ml), SP00082 (2 μg/ml), SP00083 (2 μg/ml), SP00085 (0.1 μg/ml), and SP00091 (4 μg/ml). MIC values are indicated in brackets. This shows that there are different MIC values for the strains and yet they correspond to one protein therefore

it could not be determined for sure what the exact MIC values for the strains are, for the protein in question.

When the Distance Matrix 5 (refer to Appendix A, Distance Matrix 5 of various *Streptococcus* species using the PBP 1a protein sequences) is examined it can be seen that the total character differences between the various species has the lowest value of 184, which is shown for a *pyogenes* strain and an *agalactiae* strain. When the distance matrix is studied for the *pneumoniae* group, the susceptible types have a highest value of 4 total character differences among the susceptible types. However, when the resistant and susceptible phenotypes are compared, it can be noticed that the minimum number of total character differences is 35, which is noticed for the resistant strain SP00084, accession number Q75YL2 (MIC value of 4 μg/ml), when compared with the susceptible strain 1261, accession number Q9REU0 (MIC value of <0.0075 μg/ml). This therefore suggests that there are 35 mutations between the PBP 1a amino acid sequences of the susceptible and resistant strains.

Considering Alignment 5 (refer to Appendix B, Alignment 5 of various *Streptococcus* species using the PBP 1a protein sequences), it can be noted that there are several mutations found throughout the sequences when the species are compared. In the *pneumoniae* group it can be noticed that the susceptible strains, when compared to each other, show few changes, which is consistent with the result obtained from the Distance Matrix 5. When we compare the resistant and susceptible strains it can be noticed that there are much more mutations found throughout the sequence. If we look at the sequences we observed that the active site STMK at position 397 in the alignment plays a role in resistance. This is due to the observation that the resistant and intermediate strains all have an altered active site to either SAMK or SAFK which is found at position 397, while the susceptible phenotypes have the STMK active site. The second active site SRN found at position 455 in the alignment remains the same in all the strains. Also, the third active site KTG at position 589 in the alignment is also found to be the same throughout the strains. Therefore, the important active site would be the STMK, and a change in it could lead to resistance.

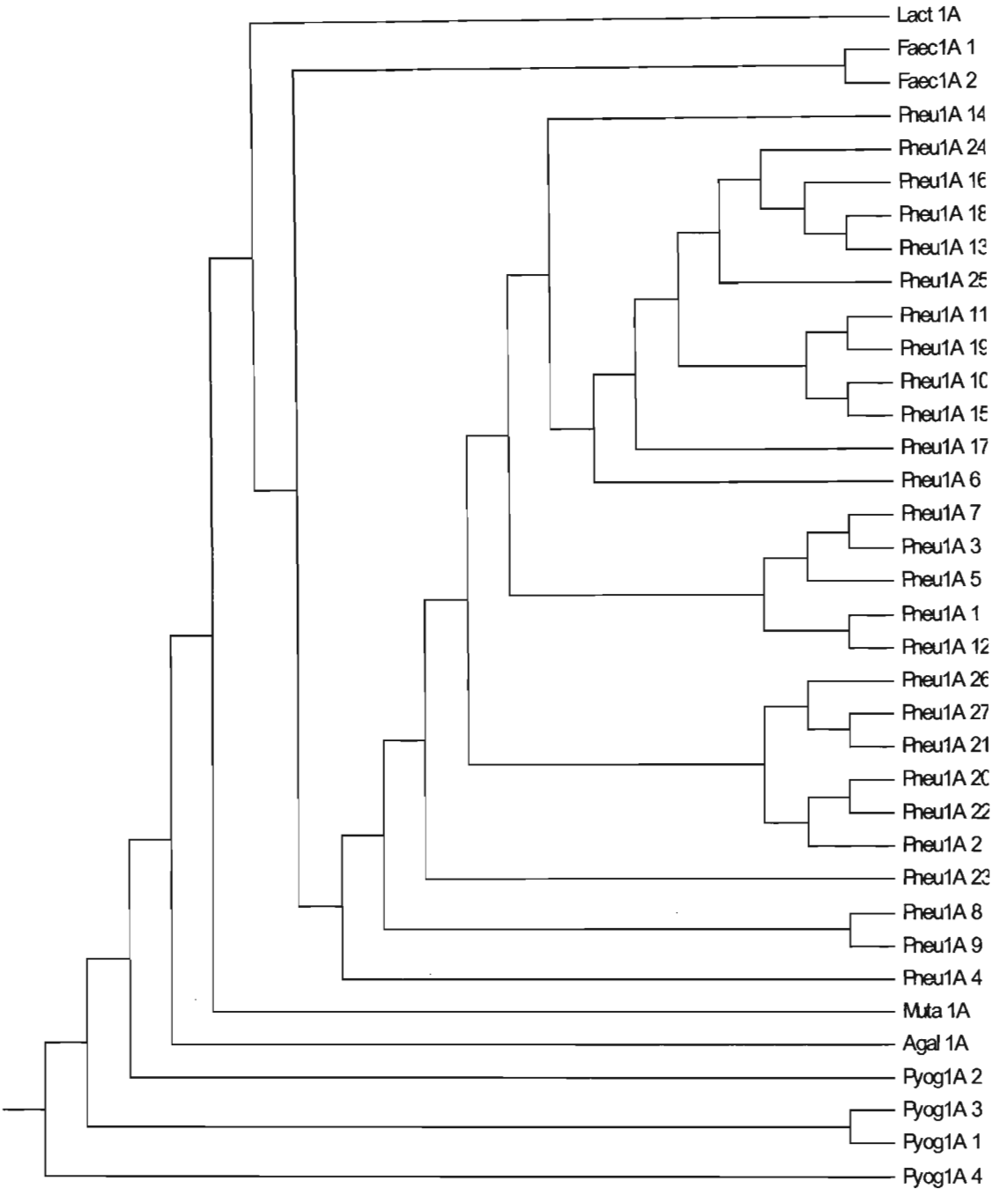


Figure 3.5. Consensus tree of the PBP 1a protein sequences from the various streptococcal species used.

Table 3.5. The names and the description for the proteins from various Streptococcal species for PBP 1a as well as the affinity for penicillin and the MIC (μg/ml) values.

Names	Description and Strain	Affinity for penicillin (MIC μg/ml)	Reference
Agalac_1A	Q8E1Q5 PBP 1a – *S. agalactiae* Strain = 2603 V/R / ATCC BAA-611 / Serotype V	Unknown[a]	Tettelin *et al.*, 2002
Faecal1A_1	Q9EXN1 PBP 1a - *S. faecalis.* Strain = JH2-2	Intermediate	Hallut & Coyette, 2001
Faecal1A_2	Q836G4 PBP 1a *S. faecalis.* Strain = V583 / ATCC 700802	Unknown[a]	Paulsen *et al.*, 2003
Lactis_1A	Q9CI23 PBP 1a – *S. lactis* Strain = IL1403	Unknown[a]	Bolotin *et al.*, 2001
Mutans_1A	Q8DVL4 PBP 1a - *S. mutans.* Strain = UA159 / ATCC 700610 / Serotype c	Unknown[a]	Ajdic *et al.*, 2002
Pneu1A_1	Q57114 PBP 1a – *S. pneumonia.* Strain = CS111	Resistant	Coffey *et al.*, 1995
Pneu1A_2	Q75YK4 PBP 1a – *S. pneumonia.* Strain = SP00092	R - 2	Sanbongi *et al.*, 2004
Pneu1A_3	Q75YK6 PBP 1a – *S. pneumonia.* Strain = SP00090	R - 4	Sanbongi *et al.*, 2004
Pneu1A_4	Q75YK8 PBP 1a – *S. pneumonia.* Strain = SP00088	I - 1	Sanbongi *et al.*, 2004
Pneu1A_5	Q75YK9 PBP 1a – *S. pneumonia.* Strain = SP00087	R - 2	Sanbongi *et al.*, 2004
Pneu1A_6	Q75YL2 PBP 1a – *S. pneumonia.* Strain = SP00084	R - 4	Sanbongi *et al.*, 2004
Pneu1A_7	Q75YL5 PBP 1a – *S. pneumonia.* Strain = SP00081	I - 0.5	Sanbongi *et al.*, 2004
Pneu1A_8	Q75YL8 PBP 1a – *S. pneumonia.* Strain = SP00078, SP00064, SP00079, SP00080, SP00082, SP00083, SP00085, and SP00091	Unknown[b]	Sanbongi *et al.*, 2004
Pneu1A_9	Q75YM1 PBP 1a – *S. pneumonia.* Strain = SP00075, SP00063, SP00076, SP00086, and SP00089	Unknown[b]	Sanbongi *et al.*, 2004
Pneu1A_10	Q75YM2 PBP 1a – *S. pneumonia.* Strain = SP00072, and SP00057	Unknown[b]	Sanbongi *et al.*, 2004
Pneu1A_11	Q75YM3 PBP 1a – *S. pneumonia.* Strain = SP00070	S - 0.031	Sanbongi *et al.*, 2004
Pneu1A_12	Q75YM4 PBP 1a – *S. pneumonia.* Strain = SP00068	I - 0.25	Sanbongi *et al.*, 2004

Table 3.5. Continued...

Names	Description and Strain	Affinity for penicillin (MIC µg/ml)	Reference
Pneu1A_13	Q75YM5 PBP 1a – *S. pneumonia.* Strain = SP00067, and SP00051	Unknown[b]	Sanbongi *et al.*, 2004
Pneu1A_14	Q75YM6 PBP 1a – *S. pneumonia.* Strain = SP00065	I - 0.125)	Sanbongi *et al.*, 2004
Pneu1A_15	Q75YM9 PBP 1a – *S. pneumonia.* Strain = SP00062	I - 0.5	Sanbongi *et al.*, 2004
Pneu1A_16	Q75YN0 PBP 1a – *S. pneumonia.* Strain = SP00060	S - 0.063	Sanbongi *et al.*, 2004
Pneu1A_17	Q75YN2 PBP 1a – S *pneumonia.* Strain = SP00056	I - 0.25	Sanbongi *et al.*, 2004
Pneu1A_18	Q75YN3 PBP 1A – *S. pneumonia.* Strain = SP00053	S - 0.063	Sanbongi *et al.*, 2004
Pneu1A_19	Q9RET3 PBP 1A – *S. pneumonia.* Strain = 22861	Unknown[a]	Ferroni & Berche, 2001
Pneu1A_20	Q9RET4 PBP 1A – *S. pneumonia.* Strain = 1470	R - 2	Ferroni & Berche, 2001
Pneu1A_21	Q9RET5 PBP 1A – *S. pneumonia.* Strain BM 4200	I - 0.5	Ferroni & Berche, 2001
Pneu1A_22	Q9RET6 PBP 1A – *S. pneumonia.* Strain = 1053	I - 0.5	Ferroni & Berche, 2001
Pneu1A_23	Q9RET8 PBP 1A – *S. pneumonia.* Strain = 1465	I - 0.12	Ferroni & Berche, 2001
Pneu1A_24	Q9RET9 PBP 1A – *S. pneumonia.* Strain = 1513	S - 0.06	Ferroni & Berche, 2001
Pneu1A_25	Q9REU0 PBP 1A – *S. pneumonia.* Strain = 1261	S - <0.0075	Ferroni & Berche, 2001
Pneu1A_26	Q9WVW0 PBP 1a – *S. pneumonia.* Strain = PO-342, PO-273, and PO-341	Resistant	Coffey *et al.*, 1999
Pneu1A_27	Q9WW11 PBP 1a – *S. pneumonia.* Strain = URU-E135, and URU-E157	Resistant	Coffey *et al.*, 1999
Pyog1A_1	Q7CMY6 PBP 1a – *S. pyogenes* (serotype M18). Strain = MGAS8232 / Serotype M18	Unknown[a]	Smoot *et al.*, 2002
Pyog1A_2	Q879F4 PBP 1a – *S. pyogenes* Strain = SSI-1 / Serotype M3	Unknown[a]	Nakagawa *et al.*, 2003
Pyog1A_3	Q99YL1 PBP 1a – *S. pyogenes.* Strain = SF370 / ATCC 700294 / Serotype M1	Unknown[a]	Ferretti *et al.*, 2001
Pyog1A_4	Q8K6D6 PBP 1a – *S. pyogenes* Strain = MGAS315 / Serotype M3	Unknown[a]	Beres *et al.*, 2002

[a] Refers to the MIC values that could not be obtained.
[b] Refers to the MIC values that were obtained from different strains with the same MIC value.

The consensus tree produced from PBP 1b using different Streptococcal species (Figure 3.6) shows that the non-*pneumoniae* species are found towards the root of the tree forming their own group, while the *pneumoniae* group are found at the upper portion of the tree. The MIC values could not be obtained for most of the sequences (refer to Table 3.6), therefore, correct evaluation of the different strains could not be performed. Using the MIC values that were obtained it can be noticed that the resistant, intermediate and susceptible strains are spread throughout the *pneumoniae* cluster. To make more accurate observations all the MIC values need to be obtained.

When Alignment 6 is examined (refer to Appendix B, Alignment 6 of various *Streptococcus* species using the PBP 1b protein sequences) it is noted that the *pneumoniae* strains have a similar alignment to each other. However, when the non-*pneumoniae* group is analysed it is seen that there are numerous mutations found throughout the gene. The Distance Matrix 6 (refer to Appendix A, Distance Matrix 6 of various *Streptococcus* species using the PBP 1b protein sequences) confirms this result as the matrix values are over 300 total character differences. When the *pneumoniae* group is compared it is seen that there is a high of seven total character differences between some of the strains. This indicates that the proteins are very similar to each other and that; even the resistant and susceptible strains have values that are very low. Therefore, it could be speculated that this gene does not play a major role in resistance.

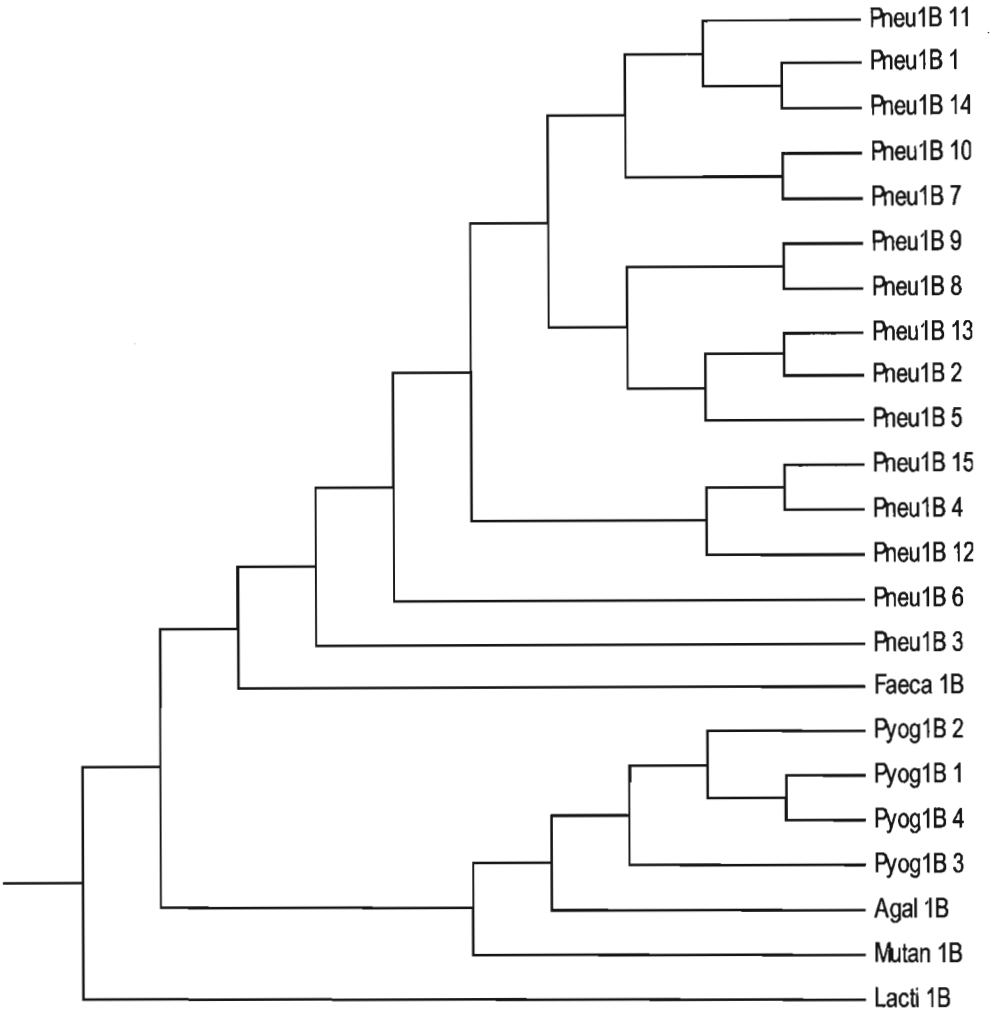


Figure 3.6. Consensus tree of the PBP 1b protein sequences using different streptococcal species.

Table 3.6. The accession numbers corresponding to the strains that was used, also the affinity for penicillin with the MIC (μg/ml) values for penicillin binding protein 1b.

Names	Description	Affinity for penicillin (MIC μg/ml)	Reference
Agal_1B	Q8E240 - PBP1b- *S. agalactiae* Strain = 2603 V/R / ATCC BAA-611 / Serotype V	Unknown[a]	Tettelin *et al.*, 2002
Faeca_1B	Q834C6 - PBP1b- *S. faecalis*. Strain = V583 / ATCC 700802	Unknown[a]	Paulsen *et al.*, 2003
Lacti_1B	Q9CIH4 - PBP1b- *S. lactis*. Strain = IL1403	Unknown[a]	Bolotin *et al.*, 2001
Mutan_1B	Q8DS45 - PBP1b- *S. mutans*. Strain = UA159 / ATCC 700610 / Serotype c	Unknown[a]	Ajdic *et al.*, 2002
Pneu1B_1	O70038 - PBP1b- *S. pneumonia*. Strain R6	R - 2	Hakenbeck *et al.*, 1998
Pneu1B_2	Q75YG9 - PBP1b- *S. pneumonia*. Strain SP00087, SP00089, and SP00057	Unknown[b]	Sanbongi *et al.*, 2004
Pneu1B_3	Q75YH2 - PBP1b- *S. pneumonia*. Strain SP00084	R – 4	Sanbongi *et al.*, 2004
Pneu1B_4	Q75YH5 - PBP1b- *S. pneumonia*. Strain SP00081	I - 0.5	Sanbongi *et al.*, 2004
Pneu1B_5	Q75YH8 - PBP1b- *S. pneumonia*. Strain SP00078, SP00080, SP00082, SP00083, SP00085, SP00091, and SP00064	Unknown[b]	Sanbongi *et al.*, 2004
Pneu1B_6	Q75YI4 - PBP1b- *S. pneumonia*. Strain SP00072, SP00074, and SP00069	Unknown[b]	Sanbongi *et al.*, 2004
Pneu1B_7	Q75YI8 - PBP1b- *S. pneumonia*. Strain SP00067, and SP00051	Unknown[b]	Sanbongi *et al.*, 2004
Pneu1B_8	Q75YI9 - PBP1b- *S. pneumonia*. Strain SP00065	I - 0.125	Sanbongi *et al.*, 2004
Pneu1B_9	Q75YJ1 - PBP1b- *S. pneumonia*. Strain SP00063	I - 0.25	Sanbongi *et al.*, 2004
Pneu1B_10	Q75YJ6 - PBP1b- *S. pneumonia*. Strain SP00058, SP00061, SP00070, SP00073, and SP00055	Unknown[b]	Sanbongi *et al.*, 2004

Table 3.6. Continued...

Names	Description	Affinity for penicillin (MIC µg/ml)	Reference
Pneu1B_11	Q75YJ8 – PBP1b – *S. pneumonia.* Strain SP00056	I - 0.25	Sanbongi *et al.*, 2004
Pneu1B_12	Q75YK0 - PBP1b – *S. pneumonia.* Strain SP00054, SP00059, SP00060, SP00062, SP00068, SP00075, SP00076, SP00086, SP00088, SP00090, SP00092, and SP00053;	Unknown[b]	Sanbongi *et al.*, 2004
Pneu1B_13	Q75YK2 - PBP1b – *S. pneumonia.* Strain SP00052	S - 0.008	Sanbongi *et al.*, 2004
Pneu1B_14	Q7CRA4 - PBP 1b – *S. pneumonia* (ATCC BAA-255 / R6).	Unknown[a]	Hoskins *et al.*, 2001
Pneu1B_15	Q97NE4 - PBP1b – *S. pneumonia.* Strain ATCC BAA-334 / TIGR4	Unknown[a]	Tettelin *et al.*, 2001
Pyog1B_1	Q879Q7 - PBP1b – *S. pyogenes* Strain SSI-1 / Serotype M3	Unknown[a]	Nakagawa *et al.*, 2003
Pyog1B_2	Q8K8W4 - PBP1b – *S. pyogenes* Strain MGAS315 / Serotype M3	Unknown[a]	Beres *et al.*, 2002
Pyog1B_3	Q8P2Y4 - PBP1b – *S. pyogenes* Strain MGAS8232 / Serotype M18	Unknown[a]	Smoot *et al.*, 2002
Pyog1B_4	Q9A1U2 - PBP1b – *S. pyogenes.* Strain SF370 / ATCC 700294 / Serotype M1	Unknown[a]	Ferretti *et al.*, 2001

[a] Refers to the MIC values that could not be obtained.

[b] Refers to the MIC values that were obtained from different strains with the same MIC value.

The consensus tree produced in Figure 3.7 using the protein sequences PBP 2a of the various Streptococcal species, shows that the *S. pneumoniae* cluster are found toward the upper portion of the tree while the other species are in the lower portion forming the root of the tree. The MIC values (refer to Table 3.7) could also not be obtained for the majority of these sequences, but from the information that is present it can be seen that the resistant, intermediate and susceptible sequences are found to be scattered throughout the tree. The Distance Matrix 7 (refer to Appendix A, Distance Matrix 7 of various *Streptococcus* species using the PBP 2a protein sequences) shows that the highest value obtained for the total number of character differences for the *pneumoniae* group is 26. This value is only noticed for one sequence given the name Pneu2A_2 which forms the root of the *pneumoniae* group, all

the other *pneumoniae* strains when compared to each other gives the highest value of seven, which is low, therefore, implying that there is very little differences between the resistant and susceptible phenotypes. In the Distance Matrix 7 it can also be seen that the total number of differences between the different species is very high with values exceeding 300. The alignment shows (refer to Appendix B, Alignment 7 of various *Streptococcus* species using the PBP 2a protein sequences) that there are several changes present when the different species are compared to each other. Within the pneumococcus group it can be noticed that there are very few mutations present, i.e. the resistant, intermediate and susceptible strains have almost the same sequence to each other, therefore, showing that the PBP 2a protein is not important for resistance to penicillin.

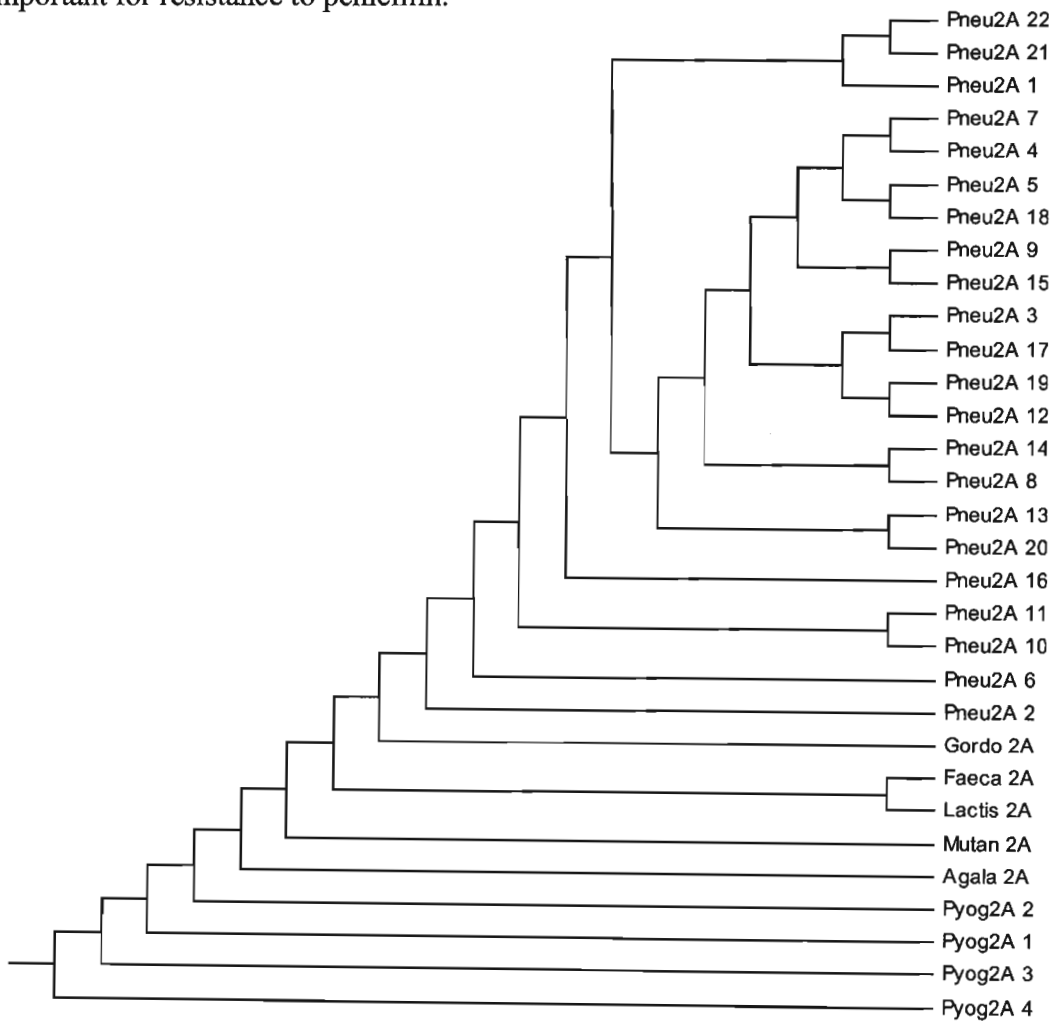


Figure 3.7. Consensus tree of the PBP 2a protein sequences from different streptococcal species.

Table 3.7. The accession numbers corresponding to the streptococcal strains, also shown is the affinity for penicillin with the MIC (μg/ml) values for penicillin binding protein 2a.

Names	Description	Affinity for penicillin (MIC μg/ml)	Reference
Agala_2A	Q8DWZ3 *S. agalactiae* Strain 2603 V/R / ATCC BAA-611 / Serotype V	Unknown[a]	Tettelin *et al.*, 2001
Faeca_2A	Q837Z4 *S. faecalis.* Strain V583 / ATCC 700802	Unknown[a]	Paulsen *et al.*, 2003
Gordo_2A	Q83YQ8 *S. gordonii.* Strain 38	Unknown[a]	Xu *et al.*, 2003
Lactis_2A	Q9CDV3 *S. lactis.* Strain IL1403	Unknown[a]	Bolotin *et al.*, 2001
Mutan_2A	Q8DS80 *S. mutans.* Strain UA159 / ATCC 700610 / Serotype c	Unknown[a]	Ajdic *et al.*, 2002
Pneu2A_1	O70039 *S. pneumonia* Strain R6	R - 2	Hakenbeck *et al.*, 1998
Pneu2A_2	Q6S9W1 *S. pneumonia.*	Unknown[a]	Smith *et al.*, 2005
Pneu2A_3	Q75YC6 *S. pneumonia.* Strain SP00090, and SP00062	Unknown[b]	Sanbongi *et al.*, 2004
Pneu2A_4	Q75YC9 *S. pneumonia.* Strain SP00087	R - 2	Sanbongi *et al.*, 2004
Pneu2A_5	Q75YD1 *S. pneumonia.* Strain SP00085	I - 1	Sanbongi *et al.*, 2004
Pneu2A_6	Q75YD2 *S. pneumonia.* Strain SP00084	R - 4	Sanbongi *et al.*, 2004
Pneu2A_7	Q75YD8 *S. pneumonia.* Strain SP00078, SP00080, SP00081, SP00082, SP00083, SP00088, SP00089, SP00091, and SP00064	Unknown[b]	Sanbongi *et al.*, 2004
Pneu2A_8	Q75YD9 *S. pneumonia.* Strain SP00077, SP00079, and SP00056	Unknown[b]	Sanbongi *et al.*, 2004
Pneu2A_9	Q75YE3 *S. pneumonia.* Strain SP00073, and SP00055	Unknown[b]	Sanbongi *et al.*, 2004
Pneu2A_10	Q75YE4 *S. pneumonia.* Strain SP00072, SP00074, and SP00070	Unknown[b]	Sanbongi *et al.*, 2004
Pneu2A_11	Q75YE6 *S. pneumonia.* Strain SP00069	I - 0.25	Sanbongi *et al.*, 2004
Pneu2A_12	Q75YE7 *S. pneumonia.* Strain SP00068	I - 0.25	Sanbongi *et al.*, 2004

Table 3.7. Continued...

Names	Description	Affinity for penicillin (MIC µg/ml)	Reference
Pneu2A_13	Q75YE8 *S. pneumonia.* Strain SP00067	I - 0.125	Sanbongi *et al.*, 2004
Pneu2A_14	Q75YF1 *S. pneumonia.* Strain SP00063	I - 0.5	Sanbongi *et al.*, 2004
Pneu2A_15	Q75YF4 *S. pneumonia.* Strain SP00060	S - 0.063	Sanbongi *et al.*, 2004
Pneu2A_16	Q75YF5 *S. pneumonia.* Strain SP00059, SP00075, SP00076, SP00086, SP00092, and SP00054	Unknown[b]	Sanbongi *et al.*, 2004
Pneu2A_17	Q75YF6 *S. pneumonia.* Strain SP00058, SP00061, SP00065, and SP00057	Unknown[b]	Sanbongi *et al.*, 2004
Pneu2A_18	Q75YG1 *S. pneumonia.* Strain SP00053	S - 0.063	Sanbongi *et al.*, 2004
Pneu2A_19	Q75YG2 *S. pneumonia.* Strain SP00052	S - 0.008	Sanbongi *et al.*, 2004
Pneu2A_20	Q97NL3 *S. pneumonia.* Strain ATCC BAA-334 / TIGR4	Unknown[a]	Tettelin, H. *et al.*, 2001
Pneu2A_21	Q9RQJ1 *S. pneumonia.* Strain SP00051	S - 0.063	Sanbongi *et al.*, 2004
Pneu2A_22	Q8DNB6 *S. pneumonia* strain ATCC BAA-255 / R6	Unknown[a]	Hoskins *et al.*, 2001
Pyog2A_1	Q877X5 *S. pyogenes* Strain SSI-1 / Serotype M3	Unknown[a]	Nakagawa *et al.*, 2003
Pyog2A_2	Q8K5N0 *S. pyogenes* Strain MGAS315 / Serotype M3	Unknown[a]	Beres *et al.*, 2002
Pyog2A_3	Q8NZ61 *S. pyogenes* Strain MGAS8232 / Serotype M18	Unknown[a]	Smoot *et al.*, 2002
Pyog2A_4	Q99XS5 *S. pyogenes*. Stain SF370 / ATCC 700294 / Serotype M1	Unknown[a]	Ferretti *et al.*, 2001

[a] Refers to the MIC values that could not be obtained.

[b] Refers to the MIC values that were obtained from different strains with the same MIC value.

The result obtained from Figure 3.8, with reference to Table 3.8 showing the proteins sequences of PBP 2b, is very unusual in that the *S. faecalis*, *lactis*, *agalactiae* and *mutans* species are found in the middle of the *S. pneumoniae* cluster. The two strains, namely Pneu2B 22 and Pneu2B 20, are separated from the other *pneumoniae* strains by the other Streptococcal

species. When we look at the rest of the tree it can be noticed that the intermediate strains are clustered toward the bottom of the tree while the resistant strains are found grouped together toward the middle of the tree just below the group of non-streptococcal species. One of the susceptible strains is found in the middle of the tree in between the intermediates.

When we examine the Distance Matrix 8 (refer to Appendix A, Distance Matrix 8 of various *Streptococcus* species using the PBP 2b protein sequences), it can be noticed for the two sequences found at the top of the tree that there are about the same number of total character differences present within most of the pneumococcal strains. Furthermore, when the *S pneumoniae* group is examined, it is noticed that the highest value obtained is 50. When the interspecies groups are compared it can be noticed that there are large differences between the different species with values of greater than 188.

Alignment 8 (refer to Appendix B, Alignment 8 of various *Streptococcus* species using the PBP 2b protein sequences) shows that there are several differences between the species, while within the pneumococcus species it is noticed that there are few changes, even the active site SXXK at position 416 in the alignment is completely homologous. The second active site SXN at position 476 also does not have any changes present. The third active site KTG at position 656 also shows no mutations (Nagai *et al.*, 2002).

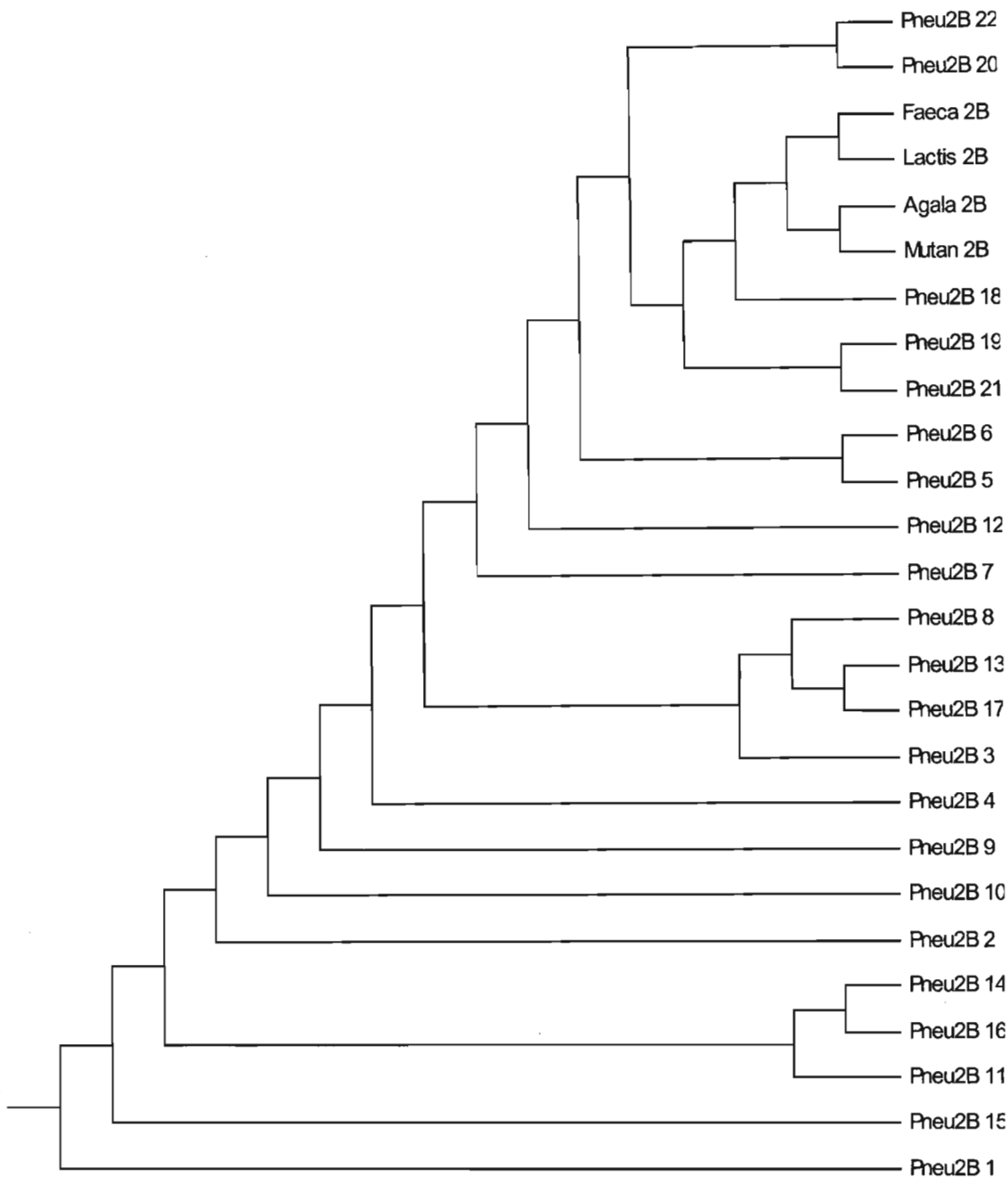


Figure 3.8. Consensus tree of the PBP 2b protein sequences from various streptococcal species.

Table 3.8. The accession numbers corresponding to the strains used, including the affinity for penicillin with the MIC (μg/ml) values for penicillin binding protein 2b.

Names	Accession number and Description	Affinity for penicillin (MIC μg/ml)	Reference
Agala_2B	Q8E0G8 *S. agalactiae* Strain 2603 V/R / ATCC BAA-611 / Serotype V	Unknown[a]	Tettelin *et al.*, 2002
Faeca_2B	Q830D1 *S. faecalis.* Strain V583 / ATCC 700802	Unknown[a]	Paulsen *et al.*, 2003
Lacti_2B	Q9CIL7 *S. lactis.* Strain IL1403	Unknown[a]	Bolotin *et al.*, 2001
Mutan_2B	Q8DVA0 *S. mutans.* Strain UA159 / ATCC 700610 / Serotype c	Unknown[a]	Ajdic *et al.*, 2002
Pneu2B_1	Q54616 *S. pneumonia.* Strain 27222	I - 0.25	Smith & Klugman, 1995
Pneu2B_2	Q54617 *S. pneumonia.* Strain 8859	I - 1	Smith & Klugman, 1995
Pneu2B_3	Q54618 *S. pneumonia.* Strain 22012	S - 0.06	Smith & Klugman, 1995
Pneu2B_4	Q54619 *S. pneumonia.* Strain 17230	I - 0.25	Smith & Klugman, 1995
Pneu2B_5	Q54620 *S. pneumonia.* Strain 52328	R - 4	Smith & Klugman, 1995
Pneu2B_6	Q54621 *S. pneumonia.* Strain M11	R - 4	Smith & Klugman, 1995
Pneu2B_7	Q54622 *S. pneumonia.* Strain E957	I - 0.25	Smith & Klugman, 1995
Pneu2B_8	Q54623 *S. pneumonia.* Strain 48478	R - 4	Smith & Klugman, 1995
Pneu2B_9	Q54624 *S. pneumonia.* Strain 52075	I - 0.5	Smith & Klugman, 1995
Pneu2B_10	Q54625 *S. pneumonia.* Strain 56739	I - 1	Smith & Klugman, 1995
Pneu2B_11	Q54626 *S. pneumonia.* Strain 39030	I - 0.25	Smith & Klugman, 1995
Pneu2B_12	Q54627 *S. pneumonia.* Strain 56762	R - 4	Smith & Klugman, 1995
Pneu2B_13	Q54628 *S. pneumonia.* Strain 43	I - 0.25	Smith & Klugman, 1995
Pneu2B_14	Q54629 *S. pneumonia.* Strain 53135	I - 0.125	Smith & Klugman, 1995

Table 3.8. Continued...

Names	Accession number and Description	Affinity for penicillin (MIC µg/ml)	Reference
Pneu2B_15	Q54630 *S. pneumonia*. Strain 23884	I - 0.25	Smith & Klugman, 1995
Pneu2B_16	Q54631 *S. pneumonia*. Strain 21241	I - 0.125	Smith & Klugman, 1995
Pneu2B_17	Q57504 *S. pneumonia*. Strain 65654	I - 0.25	Smith & Klugman, 1995
Pneu2B_18	Q75Y84 *S. pneumonia* Strain SP00092	R - 2	Sanbongi *et al.*, 2004
Pneu2B_19	Q75Y85 *S. pneumonia*. Strain SP00091	R - 4	Sanbongi *et al.*, 2004
Pneu2B_20	Q75Y86 *S. pneumonia*. Strain SP00090, and SP00062	Unknown[b]	Sanbongi *et al.*, 2004
Pneu2B_21	Q75Y87 *S. pneumonia*. Strain SP00089	R - 2	Sanbongi *et al.*, 2004
Pneu2B_22	Q75Y88 *S. pneumonia*. Strain SP00088	I - 1	Sanbongi *et al.*, 2004

[a] Refers to the MIC values that could not be obtained.

[b] Refers to the MIC values that were obtained from different strains with the same MIC value.

The consensus tree, Figure 3.9, of the different species using the PBP 2x protein sequences, shows that the *S. pyogenes*, *lactis*, *agalactiae* and *mutans* species are found toward the bottom of the tree forming the root. It is also noticed, in conjunction with Table 3.9, that in the *S. pneumoniae* group the susceptible strains are found toward the top end of the tree and the resistant strains are found in the middle of the tree. The tree also shows that some intermediate strains are found in-between the susceptible strains. A very interesting observation to make is that there are two susceptible strains found in the resistant group, these sequences are Q75Y69 and Q75Y73. With the exception of these two sequences the tree shows that there is a clear difference between the resistant and susceptible phenotypes, based on their MIC values for the PBP 2x protein sequences.

The Distance Matrix 9 (refer to Appendix A, Distance Matrix 9 of various *Streptococcus* species using the PBP 2x protein sequences) shows that there is a clear difference between the

different species used for this section with the matrix values noticeably greater than 200 for the total number of character differences. If the two susceptible sequences that are found in the middle of the resistant group are looked at, it is noticed that the Q75Y69 sequences has very high values when compared to other susceptible strains. Minimum values of 79 total character differences are noted. When this sequence is compared to the resistant strains it is noticed that the total character difference values are as low as 13. The result is unexpected due to the resistant and susceptible strains just have 13 mutations found throughout the PBP 2x. A possible explanation could be that the MIC values are incorrect and that these susceptible strains are in fact resistant strains, however this is just speculation, and more work is necessary to find out if this is true. When we compare the susceptible strains with the resistant strains it is noticed that they have total character difference values of greater than 50 and mean character difference values of greater than 0.06800, which implies that their sequences are not similar to each other. Therefore, from the result obtained, we can say that the sequences for the resistant and susceptible strains are clearly different from each other. This is very important due to the fact that, PBP 2x, is one of the PBPs implicated in resistance.

If Alignment 9, (refer to Appendix B, Alignment 9 of various *Streptococcus* species using the PBP 2x protein sequences) is examined it can be noticed that there are numerous differences present when the different species are compared to each other. Also, when the *S. pneumoniae* resistant group is compared to the susceptible group it can be noticed that there are a number of changes found throughout the length of the protein. It is also observed that the first active site, SXXK (Nagai *et al.*, 2002), found at position 350 in the alignment, shows that the resistant and intermediate strains have a change of SAMK or SAFK while the susceptible sequences have the STMK active site. This therefore indicates the importance of this site. At the second active site, SXN (Nagai *et al.*, 2002) found at position 349, there is no mutation found in this site for all the sequences examined. At the third active site LKSG (Nagai *et al.*, 2002), found at position 560, it is noticed that the resistant and intermediate phenotypes have altered sites (VKSG), while the susceptible sequences have the LKSG active site. This also indicates that this site is also important for resistance to the antibiotic.

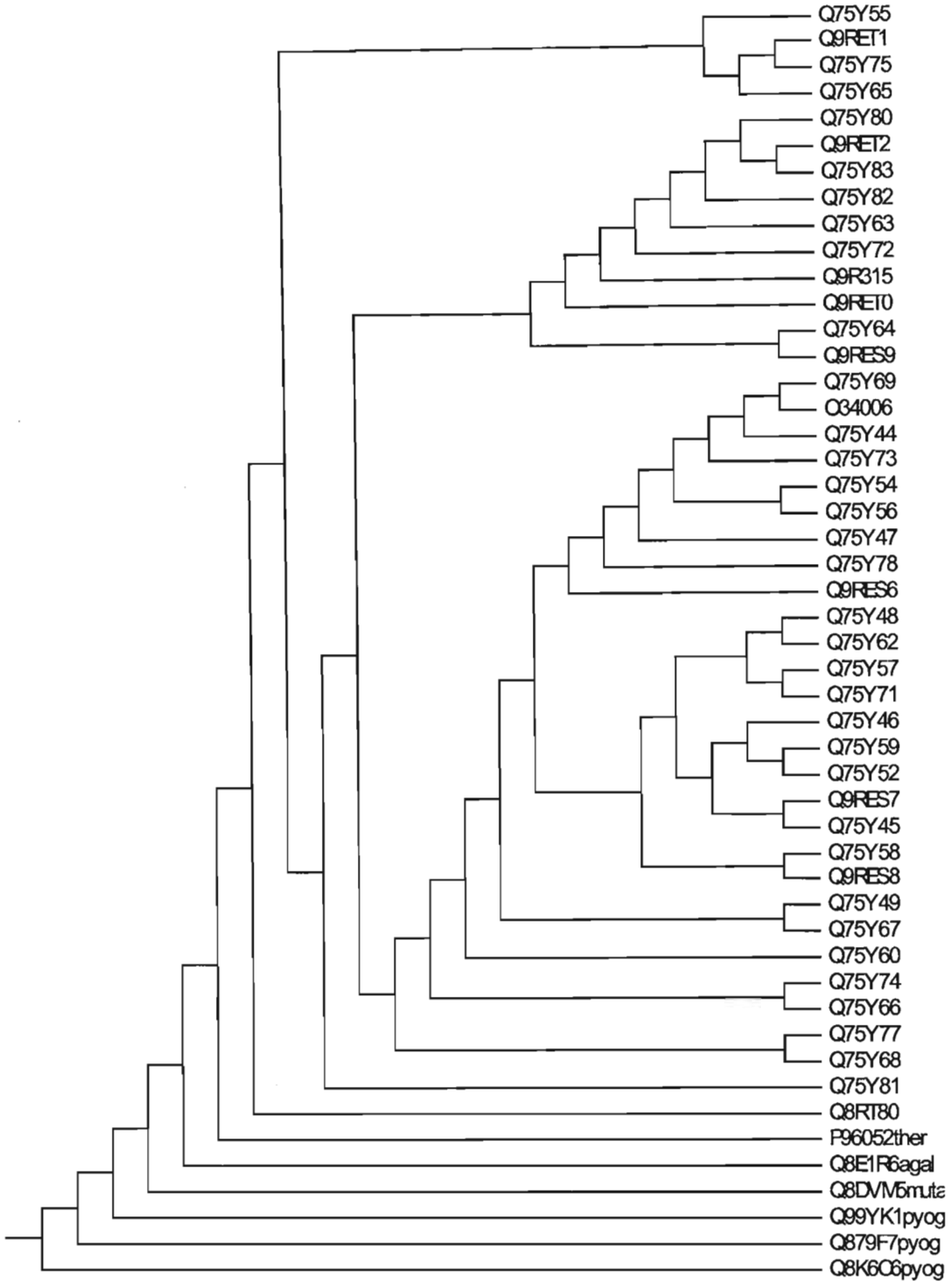


Figure 3.9. The consensus tree of five different streptococcal species using the PBP 2x protein sequences.

Table 3.9. The accession numbers corresponding to the strains, including the affinity for penicillin with the MIC (μg/ml) values for penicillin binding protein 2x.

Accession number	Description	Affinity for penicillin (MIC μg/ml)	Reference
O34006	*S. pneumoniae* Strain 328	R- 4	Mouz *et al.*, 1998
Q75Y44	*S. pneumoniae* Strain SP00092	R - 2	Sanbongi *et al.*, 2004
Q75Y45	*S. pneumoniae* Strain SP00091	R - 4	Sanbongi *et al.*, 2004
Q75Y46	*S. pneumoniae* Strain SP00090	R - 4	Sanbongi *et al.*, 2004
Q75Y47	*S. pneumoniae* Strain SP00089	R - 2	Sanbongi *et al.*, 2004
Q75Y48	*S. pneumoniae* Strain SP00088	I – 1	Sanbongi *et al.*, 2004
Q75Y49	*S. pneumoniae* Strain SP00087	R - 2	Sanbongi *et al.*, 2004
Q75Y52	*S. pneumoniae* Strain SP00084	R - 4	Sanbongi *et al.*, 2004
Q75Y54	*S. pneumoniae* Strain SP00082, SP00083, SP00085, and SP00064	Unknown[a]	Sanbongi *et al.*, 2004
Q75Y55	*S. pneumoniae* Strain SP00081	I - 0.5	Sanbongi *et al.*, 2004
Q75Y56	*S. pneumoniae* Strain SP00080	R - 4	Sanbongi *et al.*, 2004
Q75Y57	*S. pneumoniae* Strain SP00079	R - 2	Sanbongi *et al.*, 2004
Q75Y58	*S. pneumoniae* Strain SP00078	R - 2	Sanbongi *et al.*, 2004
Q75Y59	*S. pneumoniae* Strain SP00077	I - 1	Sanbongi *et al.*, 2004
Q75Y60	*S. pneumoniae* Strain SP00076, SP00086, and SP00075	R - 2[b]	Sanbongi *et al.*, 2004
Q75Y62	*S. pneumoniae* Strain SP00074	I - 0.25	Sanbongi *et al.*, 2004
Q75Y63	*S. pneumoniae* Strain SP00073, and SP00055	I - 0.125[b]	Sanbongi *et al.*, 2004
Q75Y64	*S. pneumoniae* Strain SP00072	S - 0.031	Sanbongi *et al.*, 2004
Q75Y65	*S. pneumoniae* Strain SP00070	I - 0.25	Sanbongi *et al.*, 2004

Table 3.9. Continued …

Accession number	Description	Affinity for penicillin (MIC µg/ml)	Reference
Q75Y66	*S. pneumoniae* Strain SP00069	I - 0.25	Sanbongi *et al.*, 2004
Q75Y67	*S. pneumoniae* Strain SP00068	I - 0.5	Sanbongi *et al.*, 2004
Q75Y68	*S. pneumoniae* Strain SP00067	I - 0.125	Sanbongi *et al.*, 2004
Q75Y69	*S. pneumoniae* Strain SP00065	S - 0.063	Sanbongi *et al.*, 2004
Q75Y71	*S. pneumoniae* Strain SP00063	I - 0.5	Sanbongi *et al.*, 2004
Q75Y72	*S. pneumoniae* Strain SP00062	I - 0.25	Sanbongi *et al.*, 2004
Q75Y73	*S. pneumoniae* Strain SP00061, and SP00058	S - 0.063[b]	Sanbongi *et al.*, 2004
Q75Y74	*S. pneumoniae* Strain SP00060	S - 0.063	Sanbongi *et al.*, 2004
Q75Y75	*S. pneumoniae* Strain SP00059	S - 0.063	Sanbongi *et al.*, 2004
Q75Y77	*S. pneumoniae* Strain SP00057	I - 0.25	Sanbongi *et al.*, 2004
Q75Y78	*S. pneumoniae* Strain SP00056	I - 0.5	Sanbongi *et al.*, 2004
Q75Y80	*S. pneumoniae* Strain SP00054	S - 0.063	Sanbongi *et al.*, 2004
Q75Y81	*S. pneumoniae* Strain SP00053	S - 0.063	Sanbongi *et al.*, 2004
Q75Y82	*S. pneumoniae* Strain SP00052	S - 0.008	Sanbongi *et al.*, 2004
Q75Y83	*S. pneumoniae* Strain SP00051	S - 0.063	Sanbongi *et al.*, 2004
Q8RT80	*S. pneumoniae* Isolate 3032	Unknown[a]	Peimbert & Segovia 2002
Q9R315	*S. pneumoniae* Strain G54	Unknown[a]	Massidda *et al.*, 1998
Q9RES6	*S. pneumoniae* Strain 22861	R - 8	Ferroni & Berche, 2001
Q9RES7	*S. pneumoniae* Strain 1470	R - 2	Ferroni & Berche, 2001
Q9RES8	*S. pneumoniae* Strain BM 4200	I - 0.5	Ferroni & Berche, 2001
Q9RES9	*S. pneumoniae* Strain 1053	I - 0.5	Ferroni & Berche, 2001

Table 3.9. Continued ...

Accession number	Description	Affinity for penicillin (MIC µg/ml)	Reference
Q9RET0	*S. pneumoniae* Strain 1258	I - 0.25	Ferroni & Berche, 2001
Q9RET1	*S. pneumoniae* Strain 1513	S - 0.06	Ferroni & Berche, 2001
Q9RET2	*S. pneumoniae* Strain 1261	S - 0.008	Ferroni & Berche, 2001
Q8E1R6	*S. agalactiae* Strain 2603 V/R / ATCC BAA-611 / Serotype V	Unknown[a]	Tettelin *et al.*, 2002
Q8DVM5	*S. mutans* Strain UA159 / ATCC 700610 / Serotype c	Unknown[a]	Ajdic *et al.*, 2002
Q879F7	*S. pyogenes* Strain SSI-1 / Serotype M3	Unknown[a]	Nakagawa *et al.*, 2003
Q8K6C6	*S. pyogenes* Strain MGAS315 / Serotype M3	Unknown[a]	Beres *et al.*, 2002
Q99YK1	*S. pyogenes* Strain SF370 / ATCC 700294 / Serotype M1	Unknown[a]	Ferretti *et al.*, 2001
P96052	*S. thermophilus* Strain Sfi6	Unknown[a]	Stingele & Mollet 1996

[a] Refers to the MIC values that could not be obtained.

[b] Refers to the MIC values that were obtained from different strains with the same MIC value.

3.1.3. Protein modelling

Protein models were obtained to determine the structural changes between the resistant and susceptible PBPs, and the effect that an amino acid change has on the structure and the function of the protein. The active site was also closely inspected to determine which changes occur between those implicated in resistance and those implicated susceptibility ones. Figures 3.10, 3.11, 3.12, 3.13 and 3.14 show the 3D structures of five penicillin-binding proteins. Their actives sites are shown in color and each binding motif is shown in a different color.

In Figure 3.10 it can be seen that the strain of *S. pneumoniae,* sp328 (PDB code: 1K25A) with the accession number O34006 is highly resistant (Dessen *et al.*, 2001). The active site SAMK (red) found at position 337, SSN (green) can be seen at position 395 and finally the third active site VKSG (blue) is at position 546. When the two active sites, SAMK (337) and VKSG (546) are examined it can be noticed that this strain has substitutions in its active site. In the SAMK (337) active site when compared with the other proteins, which are susceptible, it can be seen that they have an active site of STMK (as noticed from the results above). This, therefore, shows that a change from a T to an A, affects the structure of the proteins' active sites. Alanine is a non-polar and hydrophobic amino acid as opposed to the polar and hydrophilic threonine. The enlarged sections, of the active sites, of Figures 3.10, 3.11, 3.12, 3.13 and 3.14 show the difference when there is an amino acid substitution in the active site. A change will therefore result in the penicillin being unable to bind in the pockets due to the hydrophobic and hydrophilic nature. In addition the number of changes found throughout the protein determines how resistant the proteins are.

The other active site is the VKSG (546), when compared to other proteins, which are susceptible to penicillin; it can be observed that their active site is LKSG (546). The amino acid substitution of V to L follows the same non-polar and hydrophobic group of amino acids, but their structures are very different from each other and this could contribute to resistance. When Figure 3.10 is compared to Figure 3.12, the 1K25A and the 1QMEA proteins respectively, it can be seen that the latter protein has the STMK (337), HSSN (395) and LKSG

(546) active sites, it can be noticed that these two active sites are very different from each other, showing the difference between the resistant and possibly susceptible strain.

Figure 3.11, which contains the 3D structure of the protein with PDB ID: 1pyyA and the accession number Q9R3H6 (Chesnel *et al.*, 2003), shows that the active site contain SAFK (red) at position 337, the second active site SSN (green) seen at position 395 and finally the third active site LKSG (blue) seen at position 546. It can be noticed that there are two changes at the first site, when compared to the STMK site. The presence of an F (phenylalanine) as opposed to the M (methionine) causes a considerable change in the binding pocket, seen in the enlarged section of the active site, in Figure 3.11. This, therefore, would mean that penicillin would not be able to bind in the binding pocket and, therefore, contribute to resistance.

In Figure 3.12, PDB code: 1qmeA with the accession number Q9Z9X3 (Gordon *et al.*, 2000), it can be noticed that the active site contains the STMK (red) sequence found at position 337, it also has the SSN (green) active site located at position 395 and the LKSG (blue) active site found at position 546. This strain, therefore, has the active sites of a potential susceptible strain, in accordance with the results obtained above where susceptible strains have the active sites of STMK, SSN and LKSG. Thus, when we compare the resistant strain (Figure 3.10) to the susceptible strain Figure 3.12 it can be noticed that these structures are considerably different.

Figure 3.13, the protein with the PDB code 1rp5B and accession number Q9EW70 (Pernot *et al.*, 2004), the active site contains STMK (red) which is in position 337, SSN (green) position 395 and LKSG (blue) 546. This protein is very similar to the protein in Figure 3.12, with the PDB code: 1qmeA. Therefore, it has a sequence similar to that of susceptible strains. This figure can also be used for the comparison to the resistant strains shown above.

Figure 3.14 shows the PBP 1b 3D structure, PDB code of 2bg1A with the accession number Q75YG9 (Kopp & Schwede, 2004). Also indicated is the transpeptidase (TP) domain, which harbors the three conserved structural motifs SXXK (Ser-460-Thr-461-Thr-462-463), this active site contains the nucleophilic Ser-460 located towards the N-terminal helix α2. The

next highly conserved motif is the SXN (Ser-516-Trp-517-Asn-518), which forms the turn between helices α4 and α5 on the left side of the cavity. The final active site is KTG (Lys-651-Thr-652-Gly-653), which lines strand β3 (Macheboueff *et al.*, 2004). The PBP 1b enzyme is unique and is unambiguously unavailable for ligand binding. The interactions between the C terminus of β3 (Thr-654) and the N-terminal region of β3 are interrupted by movement of the intervening loop into the active site region. Due to the stability of this form, the C terminus of β3 is "pulled" away from the β4, which disrupts the local anti-parallel nature of the two strands (Macheboueff *et al.*, 2004).

The low β-lactam affinity PBPs; PBP 1a, PBP 2b and PBP 2x are the primary resistance determinants, the PBP 1b has not been linked to the development of resistance. The structure is shown here in order to compare it with the PBP 2x. It can be used as a framework for understanding how the streptococci can develop resistance through mutations. Macheboueff *et al,* noticed a very interesting characteristic of PBP 1b. It was observed that an activation phenomenon reflected in the existence of the active site cavity in closed and open conformations. The open conformation was noticed to exist only in the presence of ligands. Macheboueff *et al.* proposed that the PBPs could exist in non-dividing cells in their inactive or closed states, and when their need becomes prominent in the cell-division cycle, they would become active by one of two methods. Either by recognizing a substrate or by interacting with other members of a potential cell division or peptidoglycan elongation macromolecular complex. It is also thought that the PBPs might exist in both the conformations in the cell and is switch to whichever conformation is needed in the cell cycle, depending on the substrate availability (Macheboueff *et al.*, 2004).

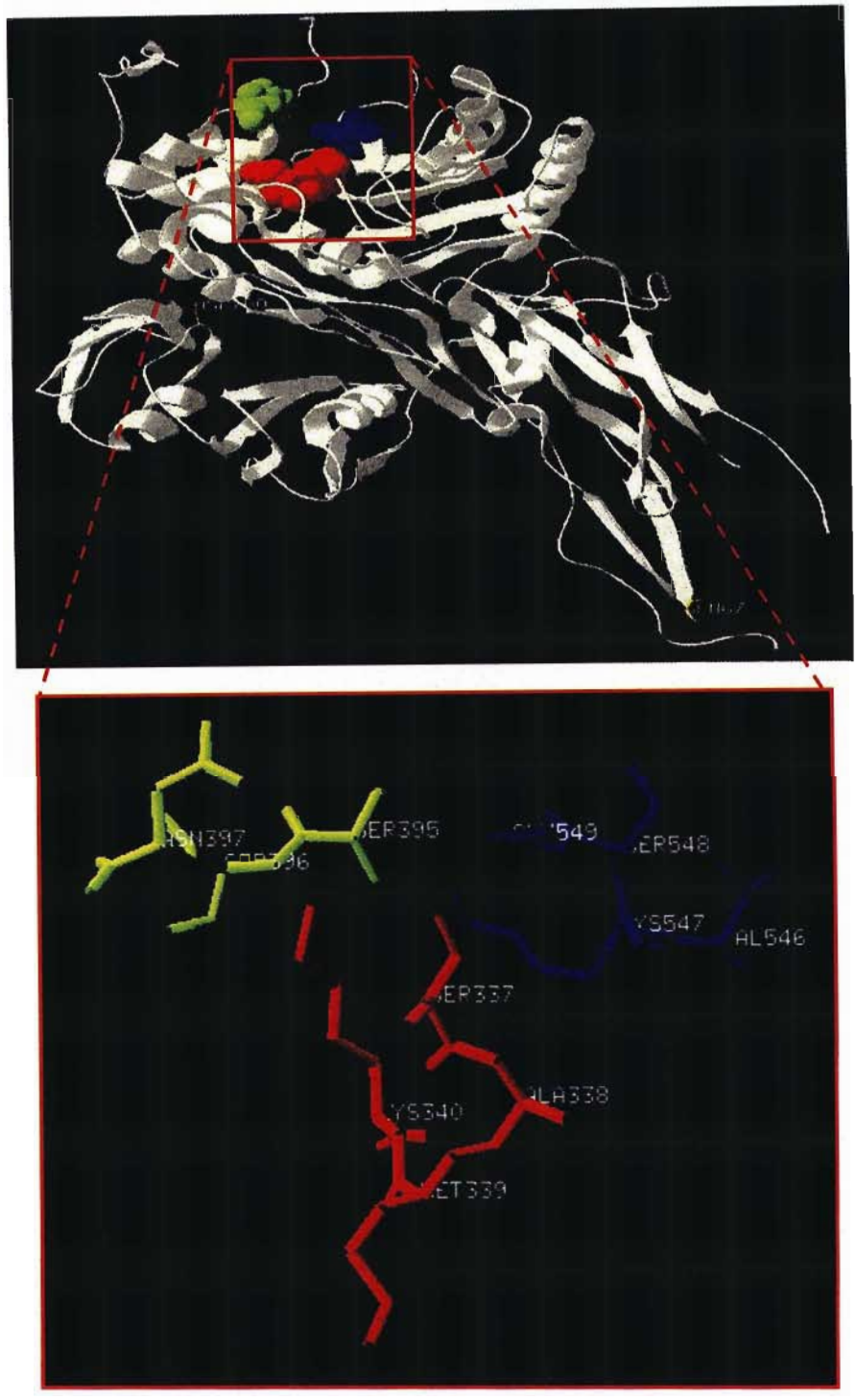


Figure 3.10. The 3D structure, position 67 (GLN67 in figure) to 750 (ASP750 in figure), of penicillin binding protein 2x from *S. pneumonia,* sp328 (PDB code: 1K25A) the active site contains SAMK (red) position 337 - 340, SSN (green) position 395 – 397 and VKSG (blue) position 546 – 549 (Dessen *et al.*, 2001).

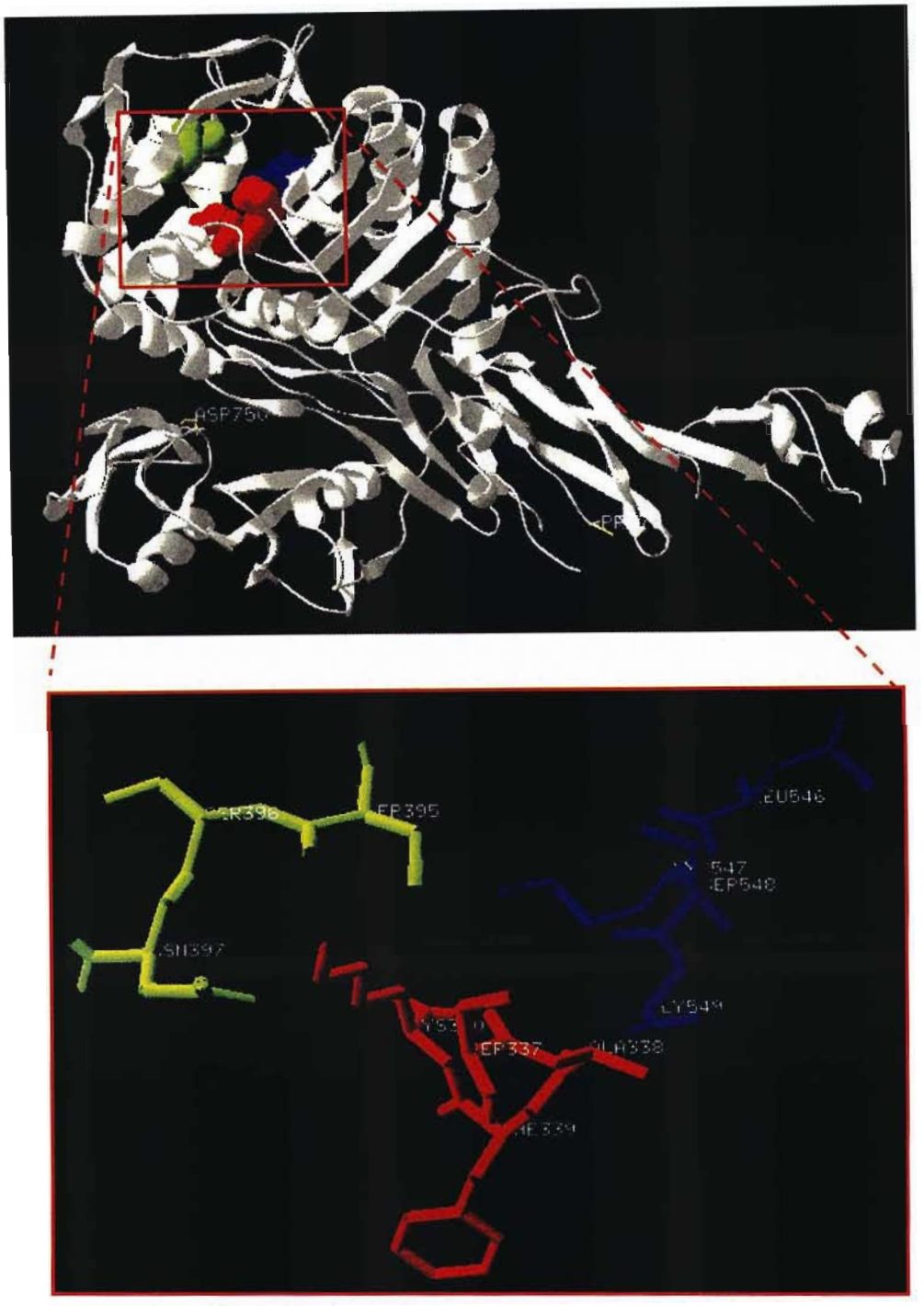


Figure 3.11. The 3D structure of penicillin binding protein 2x for *S. pneumonia* Strain R6, from position 73 (PRO73 in figure) to position 749 (GLY749 in figure), (PDB code: 1pyyA) the active site contains SAFK (red) position SER337 – LYS340, SSN (green) SER395 – ASN397 and LKSG (blue) LEU546 – GLY549 (Chesnel *et al.*, 2003).

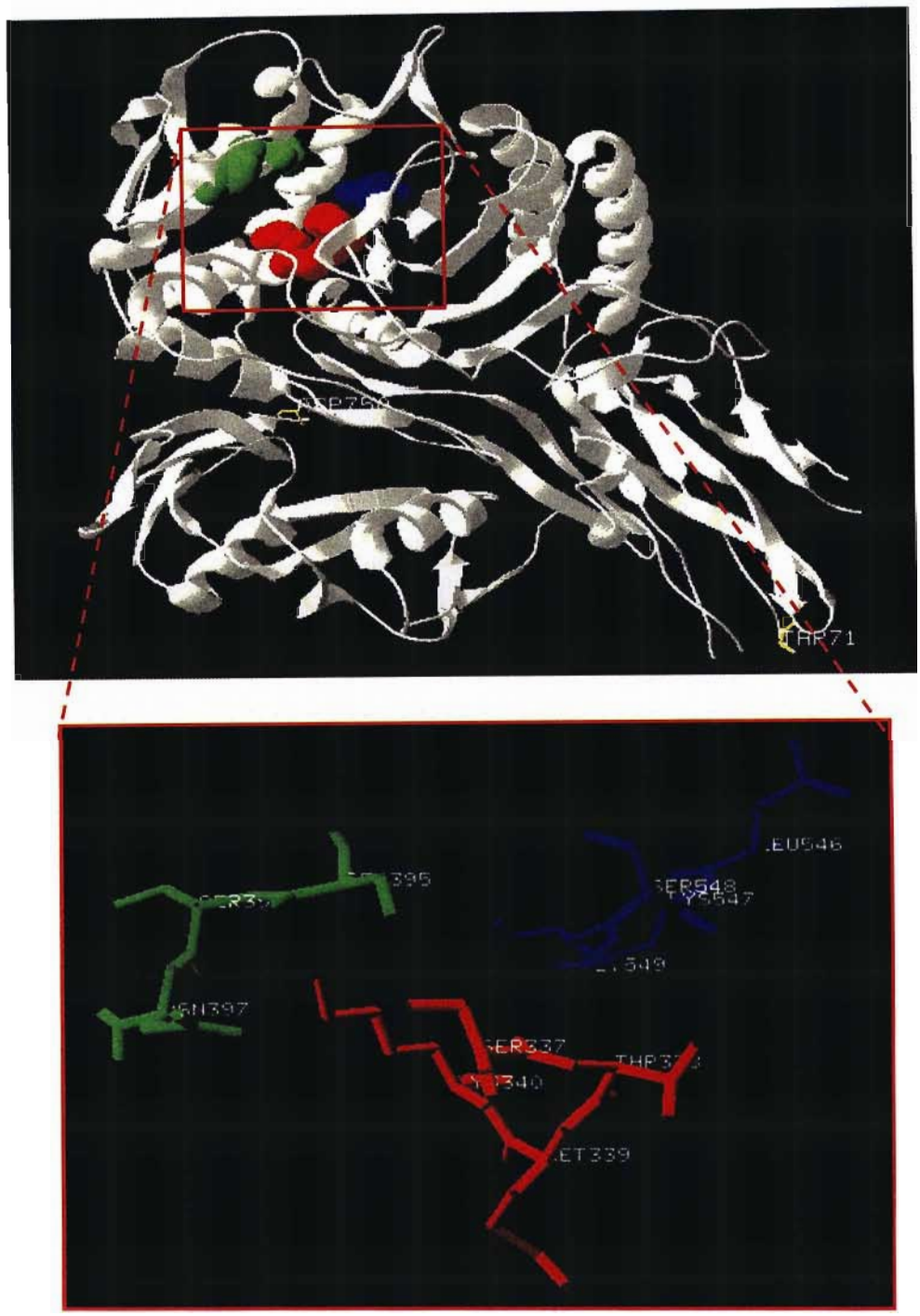


Figure 3.12. The 3D structure of penicillin binding protein 2x for *S. pneumonia* Strain MC1061, from position 71 (THR71 in the figure) to position 750 (ASP750 in the figure), (PDB code: 1qmeA) the active site contains STMK (red) position SER337 – LYS340, SSN (green) position SER395 – ASN397 and LKSG (blue) LEU546 – GLY549 (Gordon *et al.*, 2000).

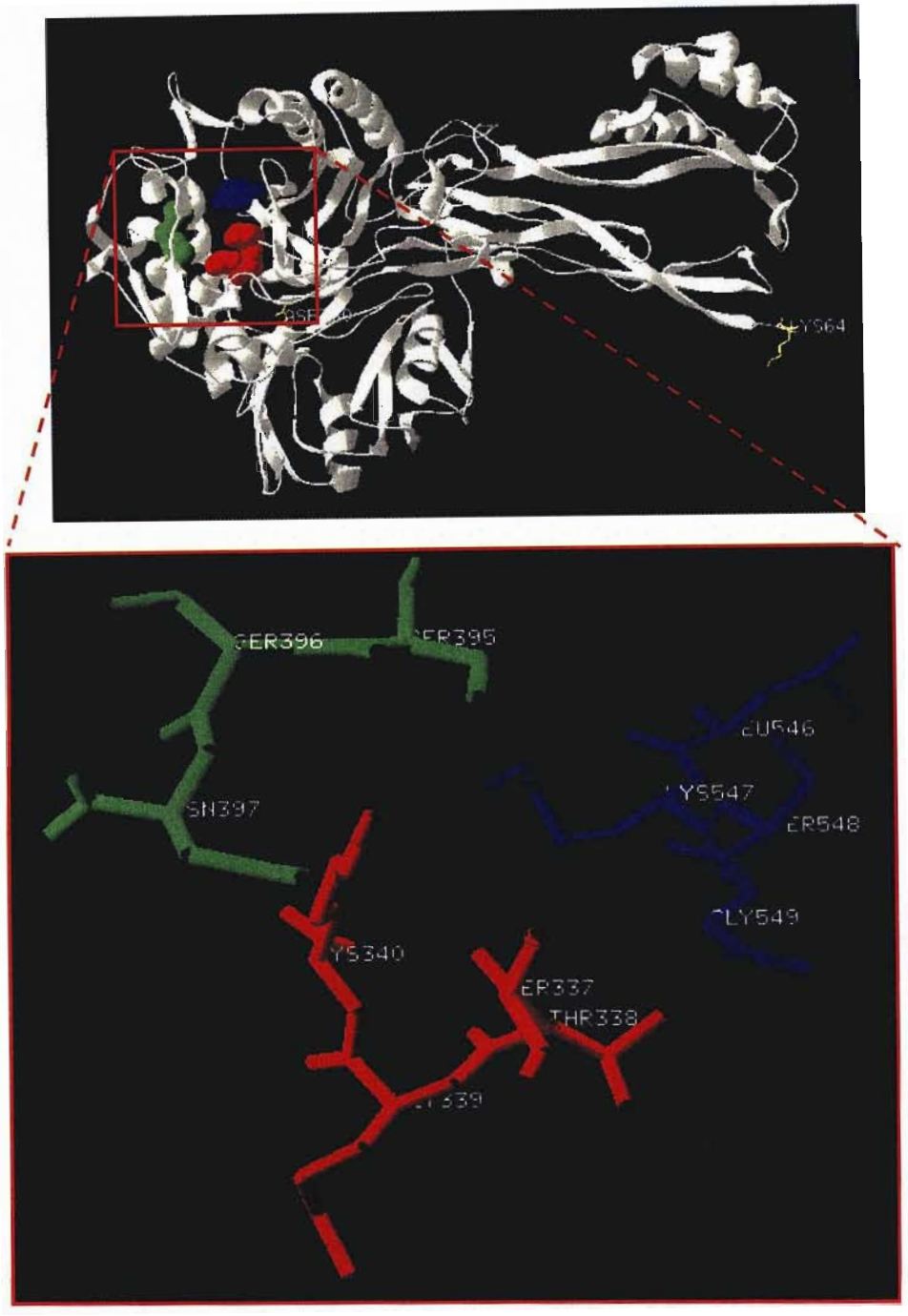


Figure 3.13. The 3D structure of penicillin binding protein 2x for *S. pneumonia* Strain 5259 (PDB code: 1rp5B), from position 64 (LYS64 in the figure) to position 750 (ASP750 in the figure) the active site contains STMK (red) position SER337 – LYS340, SSN (green) position SER395 – ASN397 and LKSG (blue) LEU546 – GLY549 (Pernot *et al.*, 2004).

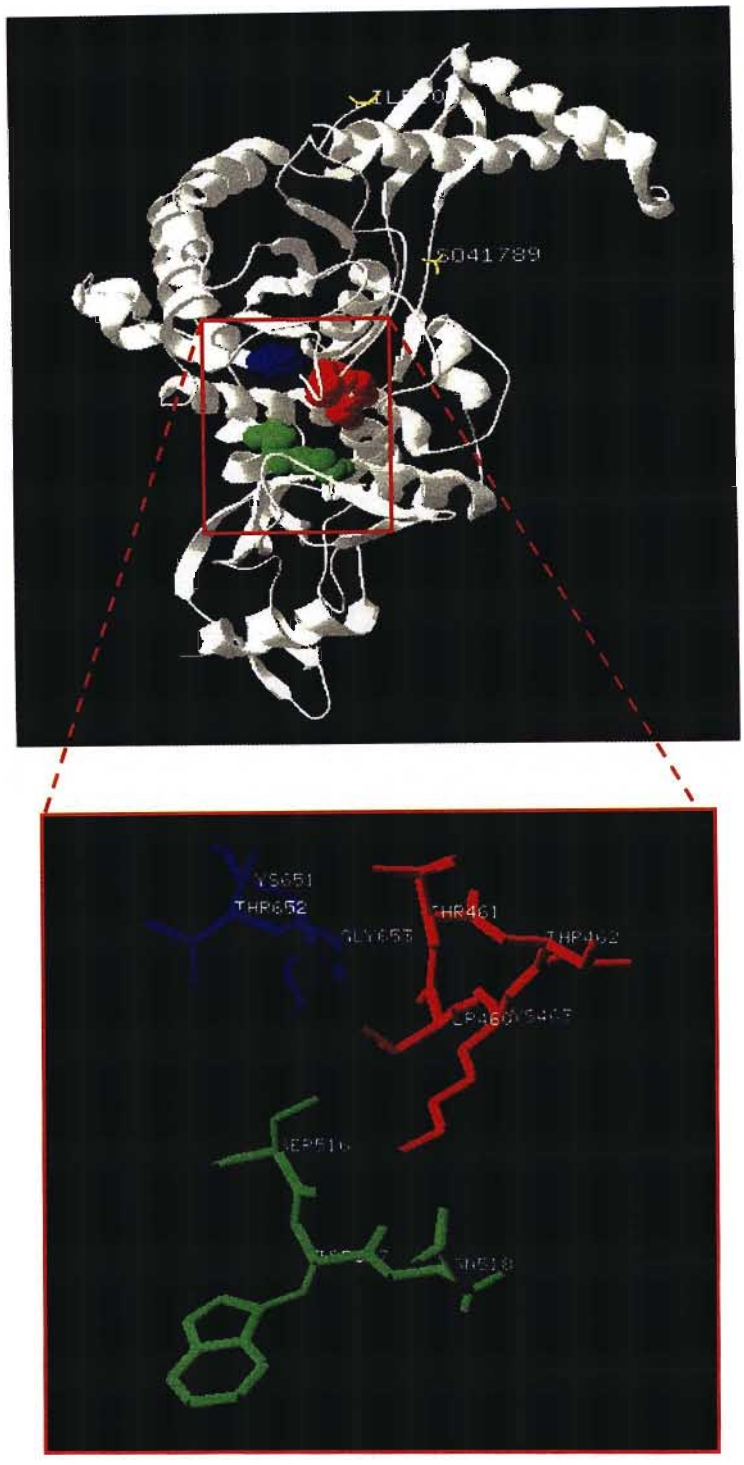


Figure 3.14. The 3D structure of penicillin binding protein 1b for *S. pneumonia* with the PDB code of 2bg1A, from position 108 to 789. The active site is noticed in the figure as STTK found at position 460-463, the second active site is SWN at position 516-518, and the final active site is KTG 651-653 (Kopp & Schwede 2004).

Table 3.10. Accession number, description, proteins affinity for penicillin and the references of strains used in the 3D structures of this study.

Accession number	Description	Reference
O34006	*S. pneumoniae* PBP 2x strain Sp 328 PDB code: 1K25A	Dessen *et al.*, 2001
Q9EW70	*S. pneumonia* PBP 2x Strain 5259 PDB code: 1rp5B	Pernot *et al.*, 2004
Q9Z9X3	*S. pneumonia* PBP 2x Strain MC1061 PDB code: 1qmeA	Gordon *et al.*, 2000
Q9R3H6	*S. pneumonia* PBP 2x Strain R6 PDB code: 1pyyA	Chesnel *et al.*, 2003
Q75YG9	*S. pneumoniae* PBP 1b PDB code: 2bg1A	Kopp & Schwede, 2004

3.2. Vancomycin resistance in *Enterococcus*

3.2.1. Sequence Analysis

The genes that contribute to resistance to vancomycin in Enterococci were downloaded. These sequences represent strains with resistance levels ranging from susceptible to extremely resistant, with MIC values ranging from a susceptible 1 μg/ml to resistant values up to 64 000 μg/ml. This part of the study included the use of different Enterococci species, among these are *E. faecium, faecalis, gallinarum, flavescens, casseliflavus* and *raffinosus*. The different phenotypes of the vancomycin resistant genes were also studied, the VanA, VanB, VanC, VanD and VanE phenotypes. The analysis was based on the levels of resistance to vancomycin determined by their respective MIC values of each strain and phenotype.

In Figure 3.15 with reference to Table 3.11, the entire Van clusters, of the 5 different phenotype, are compared to each other. The same phenotypes group together. The two VanA strains are found together towards the bottom of the tree and while the VanB strains are found to be grouped as an out-group. The VanB is also seen at the root of the tree. The VanD group is found towards the top of the tree, it can be noted that strain *E. faecium* N03-0072 of the VanD group, has a MIC value of 64 000 μg/ml. The VanC phenotype is found in the middle of the tree, while the VanE phenotype is found to be grouped with the *E. gallinarum* BM4174A VanC strain with an MIC value of 16 μg/ml, while the *E. faecalis* N00-410 VanE strain has the MIC value of 24 μg/ml. As mentioned previously, the VanE type resistance is almost identical to the VanC type apart from the different relative activity of enzymes (Reynolds & Courvalin, 2005). The result obtained here confirms those findings.

Distance Matrix 10 (refer to Appendix A) shows that when the different species are compared the distances obtained are relatively high, with the values ranging from 0.47248 to a high of 0.70641. If the VanE gene cluster is compared to the VanC gene cluster it is noticed that the distance values obtained are 0.45265, 0.45711 and 0.48333 for the strains *E. flavescens* CCM 439, *E. casseliflavus* ATCC 25788 and *E. gallinarum* BM4174 respectively. However, when the VanE gene cluster is compared to the other phenotypes the distance is higher than 0.67084.

Therefore, this result confirms once more that the VanE type resistance is similar to the VanC type.

Alignment 10 (refer to Appendix B, Alignment 10 showing five different phenotypes using various Enterococci species) shows that the sequences for the different phenotypes are fairly different from each other, with a number of changes being noticed throughout the gene clusters. When the VanE phenotype is compared to the VanC phenotype it can be noticed that the alignment has regions of similarity when compared to the other phenotypes. In addition when the similar phenotypes are compared to each other it can be noticed that there are regions of homology found throughout the alignment. It could be speculated that VanE could have evolved from VanC.

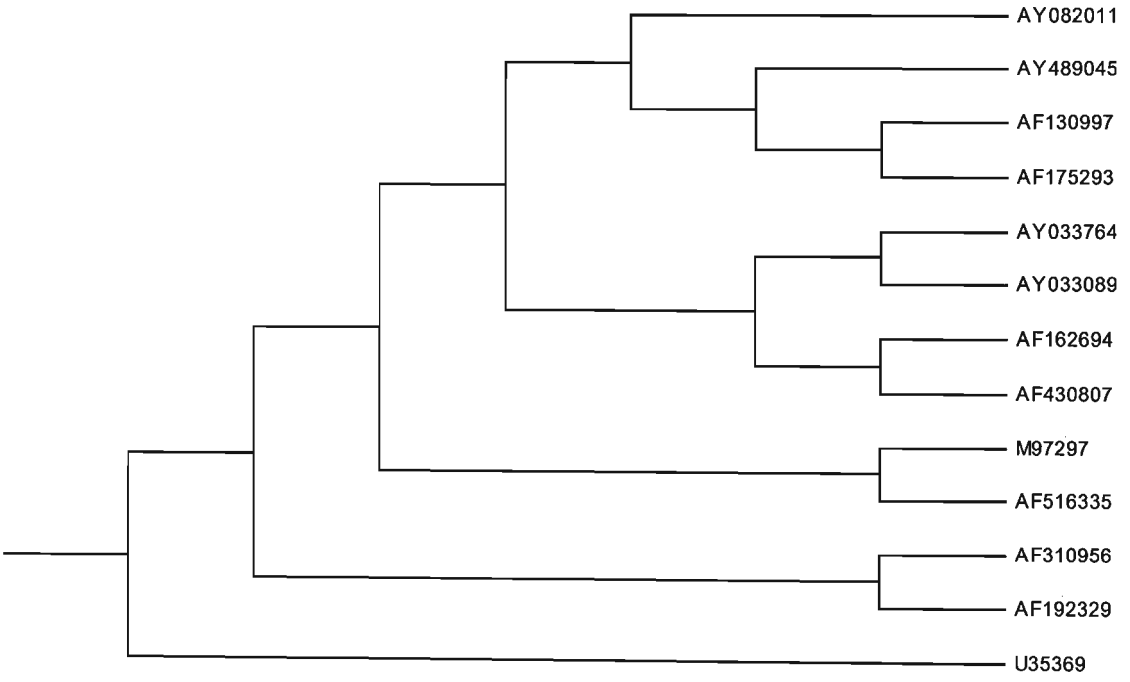


Figure 3.15. The consensus tree obtained using the Van gene clusters of VanA, VanB, VanC, VanD and VanE phenotypes from various enterococcal species.

Table 3.11. The Accession numbers and the description of the Van gene clusters also showing the level of resistance.

Accession Number	Description	Resistance MIC (µg/ml)	Reference
M97297	*E. faecium* BM4147 - VanA	Unknown[a]	Arthur *et al.*, 1993b
AF516335	*E. faecium* UW786 - VanA	Unknown[a]	Werner *et al.*, 2002
AF192329	*E. faecalis* BM4382 - VanB	Unknown[a]	Garnier *et al.*, 2000
AF310956	*E. faecium* TSGH1 – VanB2	>256	Lu *et al.*, 2001
U35369	*E. faecalis* V583 – VanB1	96	Evers & Courvalin, 1996
AF162694	*E. gallinarum* BM4174 – VanC	16	Dukta-Malen *et al.*, 1992
AY033764	*E. flavescens* CCM 439 – VanC-3	1	Dutta & Reynolds, 2003
AY033089	*E. casseliflavus* ATCC 25788 – VanC	8	Dutta & Reynolds, 2002
AY489045	*E. faecium* N03-0072 – VanD	64000	Boyd *et al.*, 2004
AY082011	*E. faecium* 10/96A – VanD	256	Depardieu *et al.*, 2003
AF175293	*E. faecium* N97-330 – VanD	>256	Boyd *et al.*, 2000
AF130997	*E. faecium* BM4339 – VanD	64	Casadewall & Courvalin, 1999
AF430807	*E. faecalis* N00-410 – VanE	24	Boyd *et al.*, 2002

[a] Refers to the MIC values that could not be obtained.

When Figure 3.16 is examined in conjunction with Table 3.12 it can be observed that the *vanA* ligase genes are found grouped towards the upper portion of the tree. The two *vanD* ligase genes are grouped together and are also grouped with the *vanA* genes. The rest of the sequences are from the *vanB* ligase genes and forms the bottom portion of the tree. The MIC values of the strains shown in the tree are above 4 µg/ml implying that they are resistant (Pootoolal *et al.*, 2002). The MIC values which could not be obtained are shown as unknown in the table. It is also known that these genes produce the D-Ala-D-Lac termini therefore these genes should be similar. This can be confirmed in the distance matrix.

Distance Matrix 11 (refer to Appendix A) shows that the values obtained for the "within species group" are generally very low. In the "between species group" it can be noticed that the highest value obtained is 0.34294. The only strain that produces distance matrix values higher than 0.34294 is the *E. faecium* strain A902 VanD, with accession number AF215736. This strain produces high distances values with the other Van ligase sequences, it even produces a high of 0.53985 with the VanD ligase gene, from *E. raffinosus,* accession number AB183866. This result is unexpected since we expect this value to be low, due to the fact that these genes have the same function of producing the D-Ala-D-Lac in preference to D-Ala-D-Ala (Leclercq & Courvalin, 1997; Cetinkaya *et al.*, 2000). It is not entirely understood why this is so.

The alignment 11 (refer to Appendix B, Alignment 11 showing the *vanA, vanB* and *vanD* ligase genes) shows that there are many homologous regions found throughout the gene sequence when the "within species" are compared. However, when the strain *E. faecium* strain A902 VanD with the accession number AF215736 is compared, it can be noticed that there are several changes found throughout the gene sequence which supports the result given above. Indicating that, this strain is perhaps distantly related. However, it is not understood whether, it is due to its contribution to resistance or not.

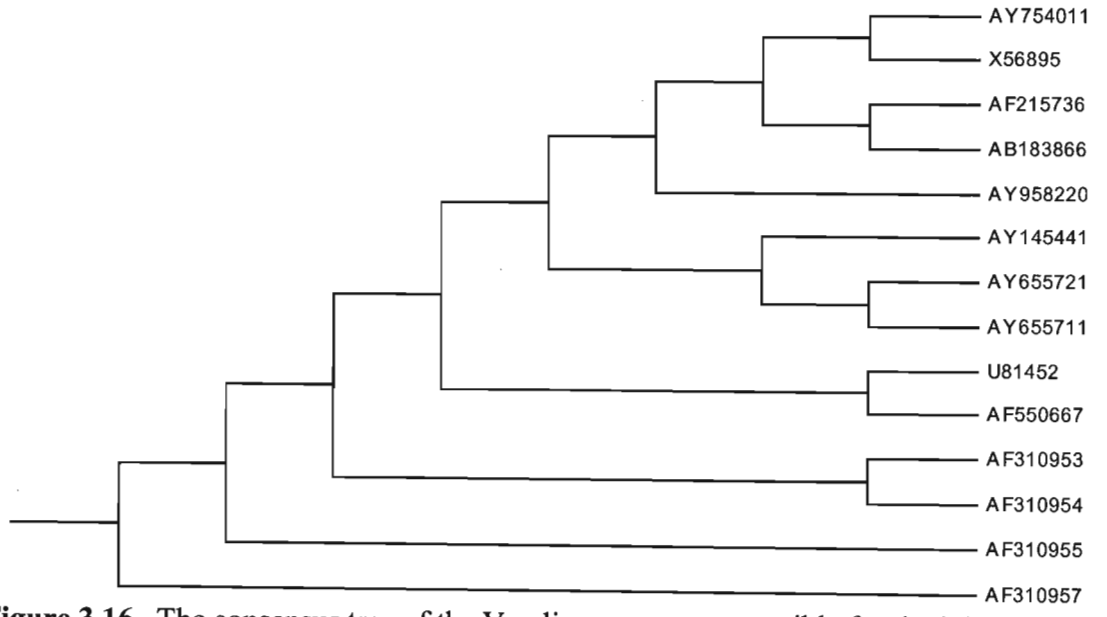


Figure 3.16. The consensus tree of the Van ligase gene, responsible for the joining of the D-Ala-D-Ala and the D-Ala-D-Lac, from the *vanA, vanB* and *vanD* phenotypes.

Table 3.12. The Accession numbers and the description of the Van ligase gene, indicated are also the level of resistance.

Accession Number	Description	Resistance MIC (µg/ml)	Reference
AY754011	*E. faecium* - VanA	Unknown[a]	Khudaier *et al.*, 2004
X56895	*E. faecium* BM4147 - VanA	Unknown[a]	Dukta-Malen *et al.*, 1990
AF310957	*E. faecium* CG4248 - VanB2	32	Lu *et al.*, 2001
AF310955	*E. faecalis* T4059 - VanB2	Unknown[a]	Lu *et al.*, 2001
AF310954	*E. faecium* SLH475 - VanB2	>256	Lu *et al.*, 2001
AF310953	*E. faecium* VRE-1 – VanB2	4	Lu *et al.*, 2001
U81452	*E. faecium* TSGH1 – VanB2	>256	Lu & Perng, 1996
AF550667	*E. faecium* BM4524 – VanB	Resistant	Depardieu *et al.*, 2003
AY958220	*E. faecium* - VanB	Unknown[a]	Libisch *et al.*, 2005
AY145441	*E. faecium* UI709 – VanB2	Unknown[a]	Lorenzo-Diaz *et al.*, 2004
AY655711	*E. faecium* MLG856-2 – VanB2	32	Ballard *et al.*, 2005
AY655721	*E. faecium* MLG229 – VanB2	24	Ballard *et al.*, 2005
AB183866	*E. raffinosus* - VanD	Unknown[a]	Tanimoto *et al.*, 2004
AF215736	*E. faecium* A902 - VanD	Resistant	Gold *et al.*, 1999

[a] MIC values were unobtainable.

Due to the limited number of van genes available from the databases only *vanB* were used. The *vanB* genes that were used in Figure 3.16 were acquired and used to make comparisons in Figure 3.17. Table 3.12 was used to supply the information about the strains used in Figure 3.17. This figure indicates that all the strains that have a known affinity for the antibiotic shows resistance. Furthermore in the figure it is noticed that the strain *E. faecium* VRE-1, VanB2, accession number AF310953, is found at the root of the tree with a MIC value of 4 µg/ml. It is thought that all the highly resistant strains found throughout the tree might have

evolved from this common strain. It is observed that strains with different levels of resistance are found throughout the tree.

In Distance Matrix 12, (refer to Appendix A) the values obtained are considerably low. This therefore, shows that this set of vanB ligase genes are similar to each other, probably due to the fact that they are all found within the resistant strains. In addition, this is confirmed by Alignment 12 (refer to Appendix B, Alignment 12 showing the alignment of the *vanB* ligase genes), which shows that there are very few changes within the sequences. Therefore, indicating that the strains are very similar to each other. The results in this section, thus shows that this set of vanB ligase genes are similar to each other. It is probably due to the fact that this set of genes has the same function, production of the D-Ala-D-Lac termini, typical of a resistance phenotype.

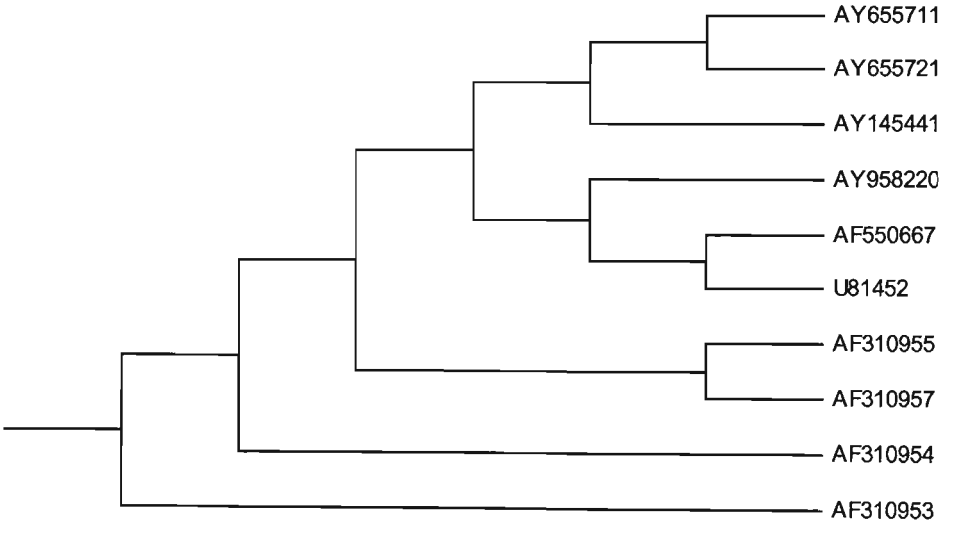


Figure 3.17. The consensus tree of the vanB genes responsible for the ligation of D-Ala-D-Ala to D-Ala-D-Lac.

The nucleotide sequences used in Figure 3.17 were translated into protein sequences and used in the analysis in Figure 3.18, in order to evaluate the differences between the nucleotide and protein sequences. The result obtained in Figure 3.18, with reference to Table 3.12, shows that the overall 'shape' of the tree is still maintained. *E. faecium* VRE-1 VanB2, with the accession number AF310953, is found at the root of the tree. Noticeably that there are some position changes found in the tree. For example, the AY145441 strain is grouped with the AY655711 and AY655721 in Figure 3.17, while in Figure 3.18 the AY145441 strain is grouped with these two strains as well as with AF310955 and AF310957.

When the Distance Matrix 13 (refer to Appendix A) is examined it can be seen that there are few changes between the sequences, with the highest total character difference being six. Also, the Alignment 13 (refer to Appendix B) indicates a similar finding, with the translated vanB ligase regions having almost identical regions of homology between all the strains studied.

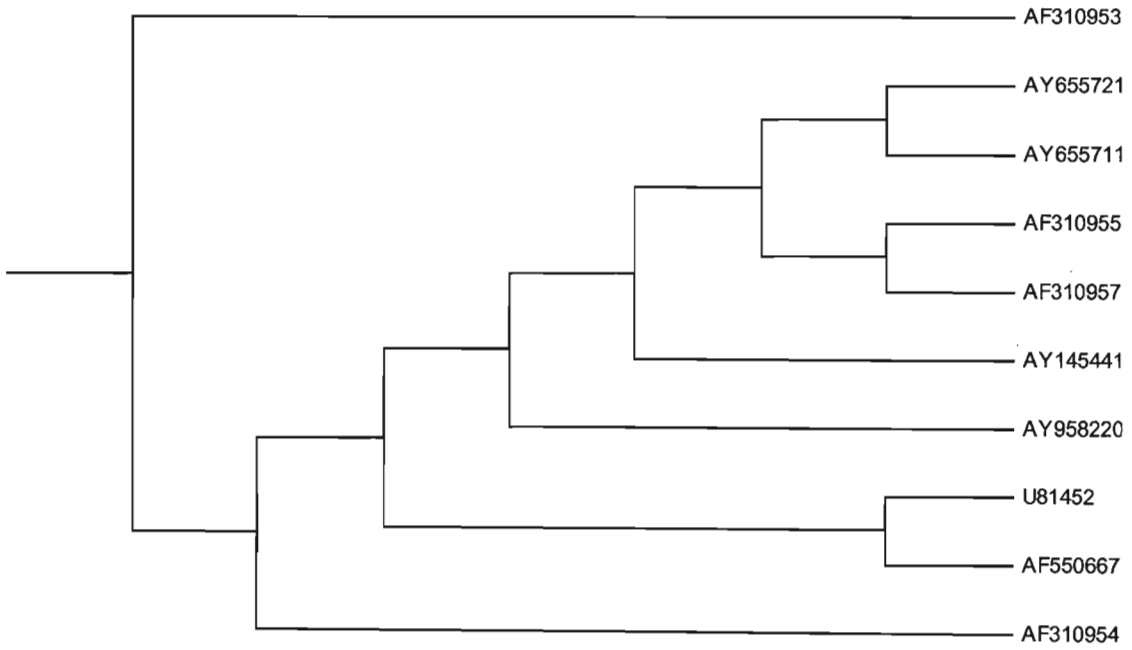


Figure 3.18. The consensus tree produced using the translated nucleotide sequences of the vanB gene used in Figure 3.27.

3.2.2. Protein Modelling

Three dimensional structures were obtained in order to determine the configuration of the enzymes that confer resistance and the effect that an amino acid substitution might have on the structure and function of the protein. The active sites were analyzed to observe the changes that occur. Figures 3.19 and 3.20 show the 3D structures of the VanX and the VanA proteins genes, their actives sites are shown in color and each binding motif are shown in a different color.

Figure 3.19 shows the 3D structure of the VanX protein, which is a zinc-dependant (the zinc is shown in yellow in the figure) D-Ala-D-Ala dipeptidase that is an essential constituent in a system that mediates resistance in enterococci. This figure shows the binding pocket of the VanX protein as well as the essential amino acids found in the active site. The binding pocket is quite small and is able to hold only the dipeptide. Thereby, performing its function to hydrolyse the D-Ala-D-Ala peptide with considerable efficiency (Bussiere *et al.*, 1998)

Figure 3.20 shows the VanA ligase 3D structure. This protein is responsible for joining the D-Ala to the D-Lac. The structure shows the amino acids necessary for this function, i.e. the active site (shown as an enlarged section in the figure). The figure also shows the phosphinophosphinate inhibitor (white) and the magnesium ions (are shown as white dots), necessary for activity.

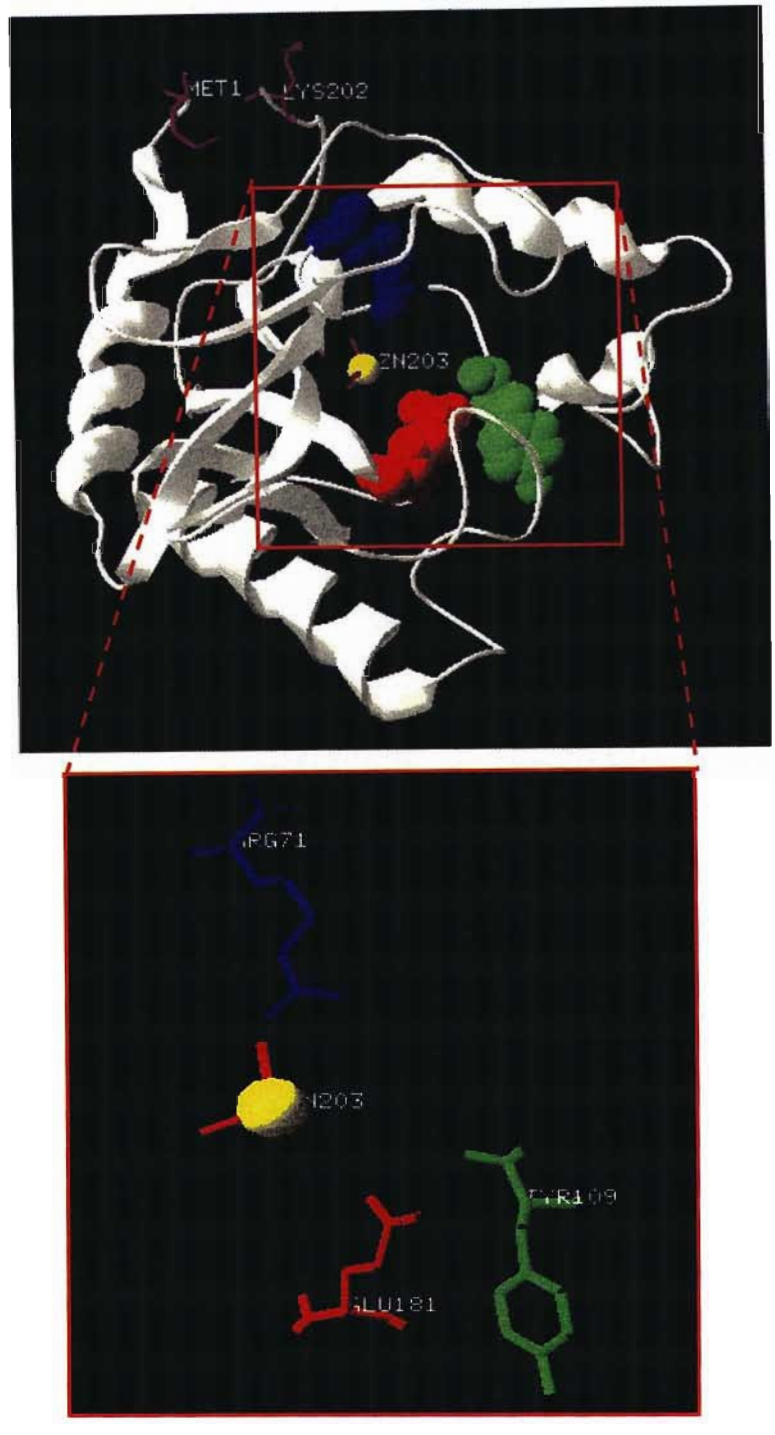


Figure 3.19. The 3D structure of the vanXB2 protein used for resistance in *E. faecium* from position MET1 to LYS202 (shown in purple), with the accession number Q9EYV8. The amino acids involved in the active site is also highlighted, Arg at position 71 (shown as blue), Typ position 109(shown as green), Glu position 181 (shown as red), the Zn atom is as seen (shown as yellow) (Bussiere *et al.*, 1998).

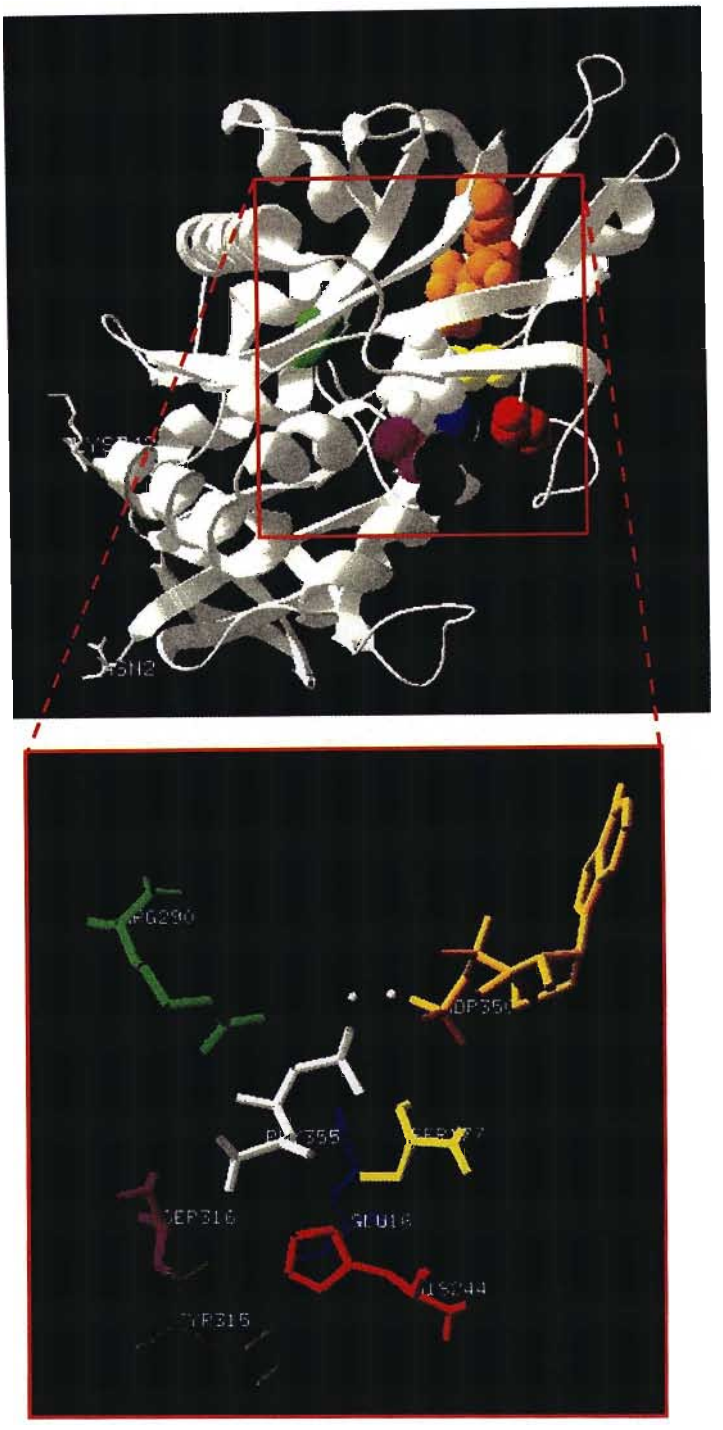


Figure 3.20. The 3D structure of the vanA ligase protein of the *E. faecium* strain BM41417 from position Asn2 to position Lys342. The amino acids used in the active site are Glu16 (shown in blue), Ser177 (yellow), His244 (red), Arg290 (green), Tyr315 (grey), Ser316 (purple), ADP350 (gold), phosphinophosphinate inhibitor (white) and the magnesium ions (are shown as white dots). Model was obtained from Swiss-PDB Viewer 3.7 (Roper *et al.*, 2000).

3.3. Methicillin resistant *Staphylococcus*

3.3.1. Sequence Analysis

Some of the genes that are responsible for playing a role in resistance to methicillin in staphylococci were obtained and analyzed. The *SCCmec, mecA, mecI, mecR* and *fem* genes were used in this study. In addition, several species were used in this section; *S. aureus, sciuri, haemolyticus, epidermidis, intermedius, warneri, cohnii, gallinarum, schleiferi, xylosus, capitis, lugdunensis* and finally *saprophyticus*. The MIC values could not be obtained for all the strains and those strains that do have resistance levels are classified.

In Figure 3.21 with reference to Table 3.13 it can be noticed that the *S. aureus* Type - IVg *SCCmec*, strain M03-68 with the accession number DQ106887, is found at the root of the tree. It can also be noticed that the different types of *SCCmec* are scattered throughout the tree with no distinguished groupings. When Distance Matrix 14 (refer to Appendix A) is examined it can be noticed that the values obtained are very low, the highest value obtained is 0.00199, therefore, indicating that this set of *SCCmec* genes analysed are very similar to each other, if not identical. Even when the Alignment 14 (refer to Appendix B) is examined, one would notice that there are very few changes between the different strains. This confirms the result that this set of SCCmec sequences is almost identical. The proposed reason for this could be that different strains found in the same species of *S. aureus* could have therefore originated from a single strain being closely related with very little substitutions. To make more accurate assumptions, a different set of *SCCmec* genes need to be obtained from varied sources of different species with varied levels of resistance.

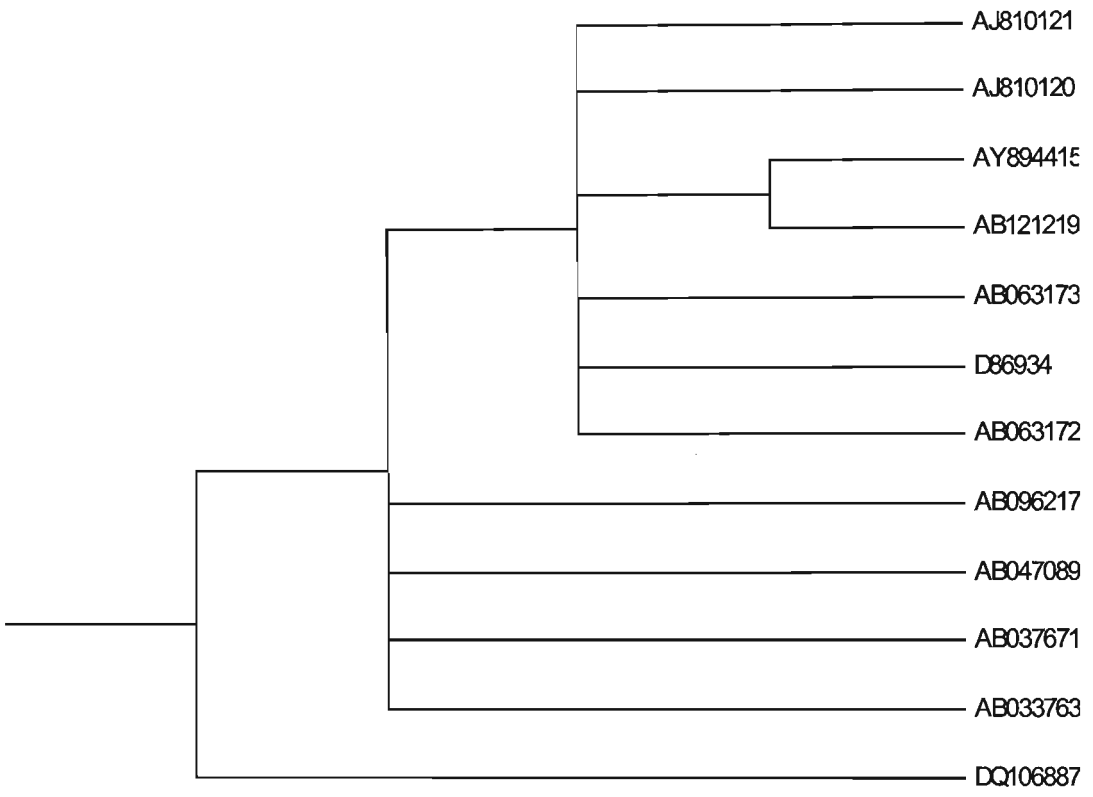


Figure 3.21. The consensus tree of the *SCCmec* gene of the *S. aureus* species using the nucleotide sequences.

Table 3.13. Information relating to the *SCCmec* gene of the *S. aureus* species.

Accession number	Description	Resistance	Reference
AB033763	*S. aureus* type-I SSCmec, Strain NCTC10442	Resistant	Ito *et al.*, 2003
AB037671	*S. aureus* type III SSCmec, strain 85/2082	Resistant	Ito *et al.*, 2001
AB047089	*S. aureus* Type-III SCCmec, strain 85/3907	Unknown[a]	Ito *et al.*, 2001
AB063172	*S. aureus* type-IV SSCmec, strain CA05 (JCSC1968)	Resistant	Ma *et al.*, 2002
AB063173	*S. aureus* type-IV SSCmec strain JCSC1978 (8/6-3P)	Resistant	Ma *et al.*, 2002
AB096217	*S. aureus* type-IVc SSCmec, strain MR108	Unknown[a]	Ito *et al.*, 2003
AB121219	*S. aureus* type-V SSCmec, strain JCSC3624 (WIS)	Unknown[a]	Ito *et al.*, 2004
AJ810120	*S. aureus* type-IIE SCCmec, strain AR13.1/3330.2.	Unknown[a]	Shore *et al.*, 2005
AJ810121	*S. aureus* type-IVE SCCmec, strain AR43/3330.1	Unknown[a]	Shore *et al.*, 2005
AY894415	*S. aureus* mec complex C2 region of SCCmecVa, strain TSGH17	Unknown[a]	Boyle-Vavra *et al.*, 2005
D86934	*S. aureus* Type-II SCCmec, Strain N315	Resistant	Ito *et al.*, 2003
DQ106887	*S. aureus* type-IVg SCCmec, strain M03-68	Unknown[a]	Kwon *et al.*, 2005

[a] MIC values were unobtainable.

Additional nucleotide sequences could not be obtained because they did not meet the requirements of this investigation. The sequences should be complete lengths of the gene and not partial sequences. Therefore, the protein sequences were considered for the *mecA, mecI, mecR* and the *fem* genes. The *mecA* genes were obtained and thereafter the analysis was performed on them and the results are shown as follows.

In Figure 3.22 with reference to Table 3.14, it can be observed that in this tree the *S. aureus* strain MW2, accession number Q7A209, is found at the root of the tree. While the only susceptible strain characterised, *S. sciuri* strain K11, accession number O54272, and is found

on the upper portion of the tree. The tree also shows that the *S. aureus* species are dispersed throughout the tree in-between the other species used. The *S. sciuri* species can be seen to form a group toward the upper section of the tree. However, the other species used in the tree seem to be spread throughout the tree.

In the Distance Matrix 15 (refer to Appendix A) it can be noticed that the *S. sciuri* group found at the top of the tree, *S. sciuri* strain K11 accession number O54272; *S. sciuri* MecA1 strain K3 accession number O54277; and *S. sciuri* strain ATCC29062 accession number P96018, have total character differences of greater than 120 when compared with the same and different species. However, when these species are compared to each other they have a high value of 16. It is unknown why these strains have such high values when compared to other strains, while the rest of the matrix shows very low values obtained when even different species are compared.

Alignment 15 (refer to Appendix B) shows that when, the different species are compared they have sequences with large regions of homology. However, when the group of *S. sciuri* are compared to the rest of the strains, there are a number of changes found throughout the length of the protein. It could be assumed that these strains might have evolved away from the other strains. If we look at the resistance levels, it is noted that there is a susceptible strain present together with the resistant strains, within this group. Therefore we can reject this assumption. More work needs to be done on these strains i.e. re-isolate the strains and determine if the MIC values are correct in order to draw logical conclusions.

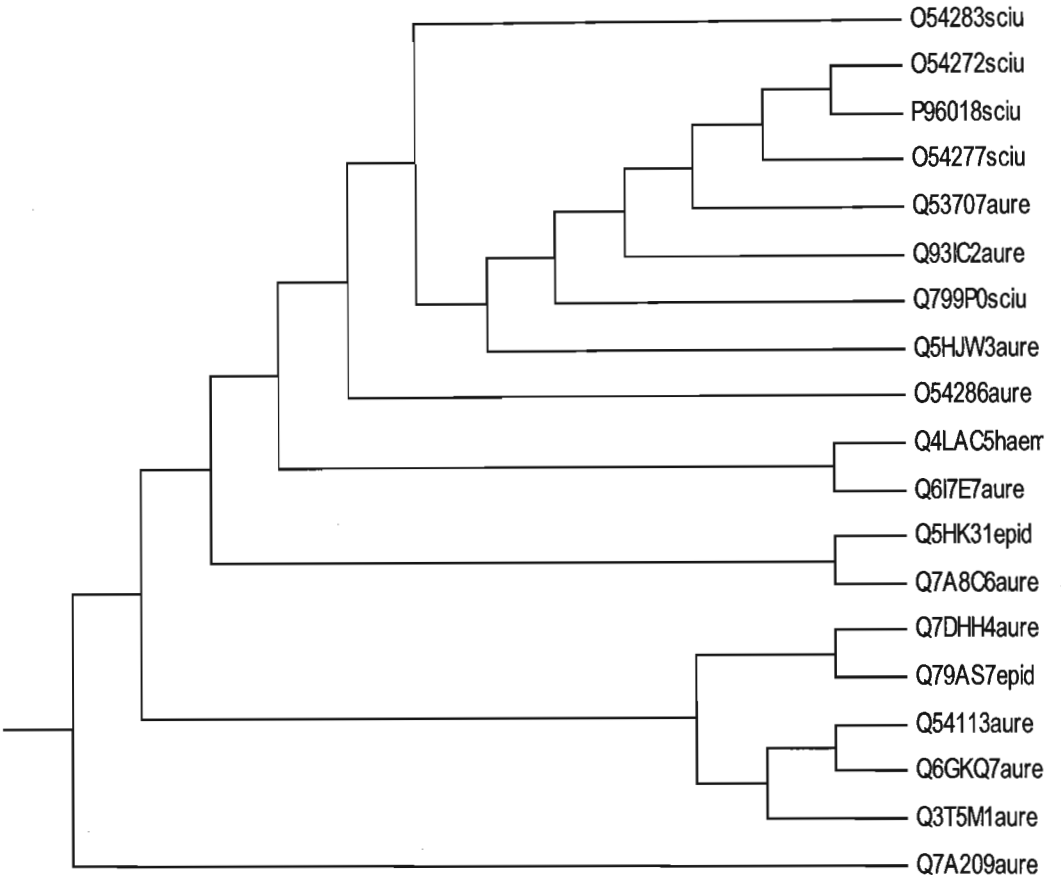


Figure 3.22. The phylogram of the mecA gene from different *Staphylococcus* genera, constructed with the translated protein sequences.

Table 3.14. The information regarding the *mecA* gene using various staphylococci species

Accession number	Description	Resistance	Reference
O54272	*S. sciuri* Strain K11	Susceptible	Wu *et al.*, 1998
O54277	*S. sciuri* MecA1 Strain K3	Resistant	Wu *et al.*, 1998
O54283	*S. sciuri* MecA2 Strain K3	Resistant	Wu *et al.*, 1998
O54286	*S. aureus* Strain NCTC10442	Resistant	Ito *et al.*, 2001
P96018	*S. sciuri* Strain ATCC29062	Unknown[a]	Wu *et al.*, 1996
Q3T5M1	*S. aureus*	Unknown[a]	Bressler *et al.*, 2005
Q4LAC5	*S. haemolyticus* Strain JCSC1435	Unknown[a]	Takeuchi *et al.*, 2003
Q5HJW3	*S. aureus* EC 3.4.16.4 strain COL	Unknown[a]	Gill *et al.*, 2005
Q5HK31	*S. epidermidis* EC 3.4.16.4 Strain ATCC 35984 / RP62A	Unknown[a]	Gill *et al.*, 2005
Q6GKQ7	*S. aureus* Strain MRSA252	Resistant	Holden *et al.*, 2004
Q6I7E7	*S. aureus* Strain JSCC 3624	Unknown[a]	Ito *et al.*, 2004
Q7A8C6	*S. aureus* Strain N315	Unknown[a]	Kuroda *et al.*, 2001
Q7A209	*S. aureus* strain MW2	Resistant	Baba *et al.*, 2002
Q7DHH4	*S. aureus* Strain JCSC 1968, and JCSC1978	Unknown[a]	Hiramatsu *et al.*, 2001
Q79AS7	*S. epidermidis* Strain WT55	Unknown[a]	Ryffel *et al.*, 1990
Q93IC2	*S. aureus* Strain 85/2082	Resistant	Ito *et al.*, 2001
Q799P0	*S. sciuri* Strain K8	Resistant	Wu *et al.*, 1998
Q53707	*S. aureus* Strain BB270, and BMS-1	Resistant	Wu *et al.*, 1998
Q54113	*S. aureus* Strain Mu50 / ATCC 700699	Unknown[a]	Kuroda *et al.*, 2001

[a] Refers to the MIC values that could not be obtained.

Figure 3.23 shows the *mecI* genes of the different staphylococcal species. When the figure is examined with reference to Table 3.15, it is seen that the *S. aureus* species is found spread throughout the tree between the other species. When the Distance Matrix 16 (refer to Appendix A) is studied, it can be seen that when the different species are compared the result obtained shows that there are no differences between some of the species. All the species show this quality with the exception of two strains *S. sciuri* subsp. *rodentium* strain K3, and K8 with the accession number O54281, *S. aureus* strain Mu50 / ATCC 700699 with the accession number Q932L5. This, therefore, indicates that the different species have the same *mecI* protein sequence. Alignment 16 (refer to Appendix B) obtained also confirms this result.

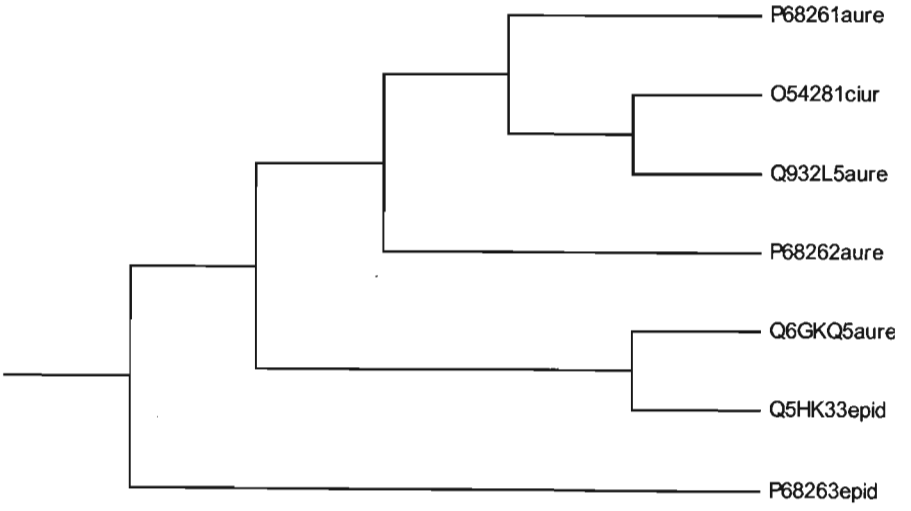


Figure 3.23. The consensus tree of the *mecI* gene from different staphylococci using the translated protein sequences.

Table 3.15. The accession number and information regarding the *mecI* gene used.

Accession number	Description	Reference
O54281	*S. sciuri* subsp. *Rodentium* Strain K3, and K8	Wu *et al.*, 1998
P68261	*S. aureus* strain N315	Hiramatsu *et al.*, 1992
P68262	*S. aureus* Strain BMS-1	Archer *et al.*, 1994
P68263	*S. epidermidis* Strain WT55	Ryffel *et al.*, 1990
Q5HK33	*S. epidermidis* strain ATCC 35984 / RP62A	Gill *et al.*, 2005
Q6GKQ5	*S. aureus* strain MRSA252	Holden *et al.*, 2004
Q932L5	*S. aureus* strain Mu50 / ATCC 700699	Kuroda *et al.*, 2001

The result obtained for the *mecR* shown in Figure 3.24 with reference to Table 3.16, shows a similar result to the *mecI* result. It can be noticed that although the *S. aureus* species are spread throughout the tree, when the Distance Matrix 17 (refer to Appendix A) and the Alignment 17 (refer to Appendix B) are considered it is seen that there are very few changes in some of the strains used. Therefore, this shows the different species have identical sequences. For most of the values obtained, the distance matrix is zero, with the highest value obtained being 3 for the total character differences, calculating the mean character differences to be 0.01119. This level of similarity between the different species, therefore, means that similar sequences are required in most species for resistance to be effective.

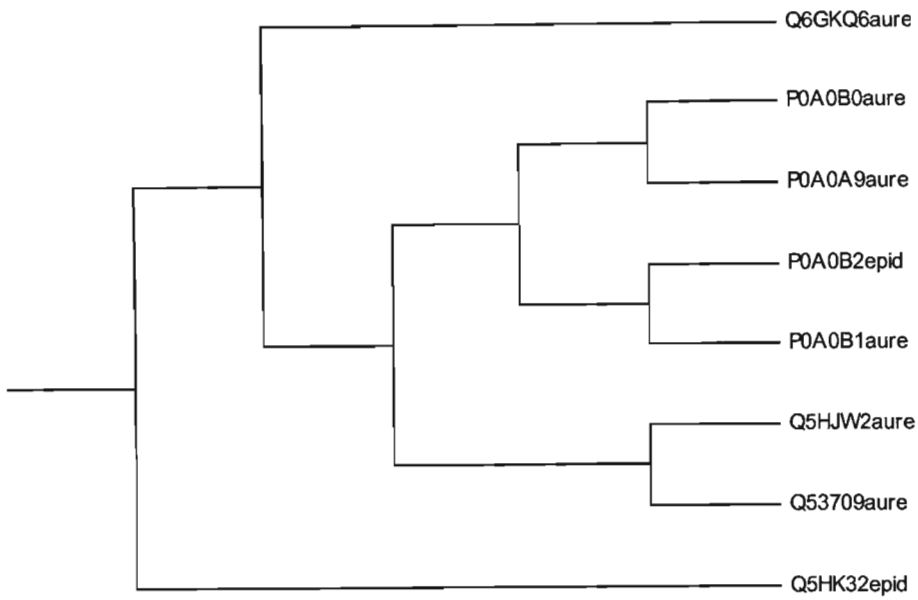


Figure 3.24. The consensus tree of the *mecR* gene obtained from different staphylococci species constructed with the translated protein sequences.

Table 3.16. The information of the *mecR* gene obtained from different staphylococci species

Accession number	Description	Reference
Q53709	*S. aureus* Strain MR108	Ito *et al.*, 2003
P0A0B0	*S. aureus* strain N315	Hiramatsu *et al.*, 1992
P0A0B1	*S. aureus* Strain BMS-1	Archer *et al.*, 1994
P0A0B2	*S. epidermidis* Strain WT55	Ryffel *et al.*, 1990
Q5HK32	*S. epidermidis* strain ATCC 35984 / RP62A	Gill *et al.*, 2005
Q6GKQ6	*S. aureus* Strain MRSA252	Holden *et al.*, 2004
P0A0A9	*S. aureus* strain Mu50 / ATCC 700699	Kuroda *et al.*, 2001
Q5HJW2	*S. aureus* strain COL	Gill *et al.*, 2005

The results obtained for Figure 3.25 with reference to Table 3.17, shows that the different strains and species are found spread throughout the tree with no specific grouping. When the Distance Matrix 18 (refer to Appendix A) and the Alignment 18 (refer to Appendix B) are examined it can be noticed that there are a number of changes between the different species, the four species that shows the most amount of changes are: *S. schleiferi* strain ATCC 43808 with accession number Q9ZFG2; *S. sciuri* Strain ATCC 29062 with accession number Q9ZFG3; *S. intermedius* Strain ATCC 20273 with accession number Q9X6T0; and finally *S. haemolyticus* strain JCSC1435 with accession number Q4L685. It can also be noted that these species form the root of the tree.

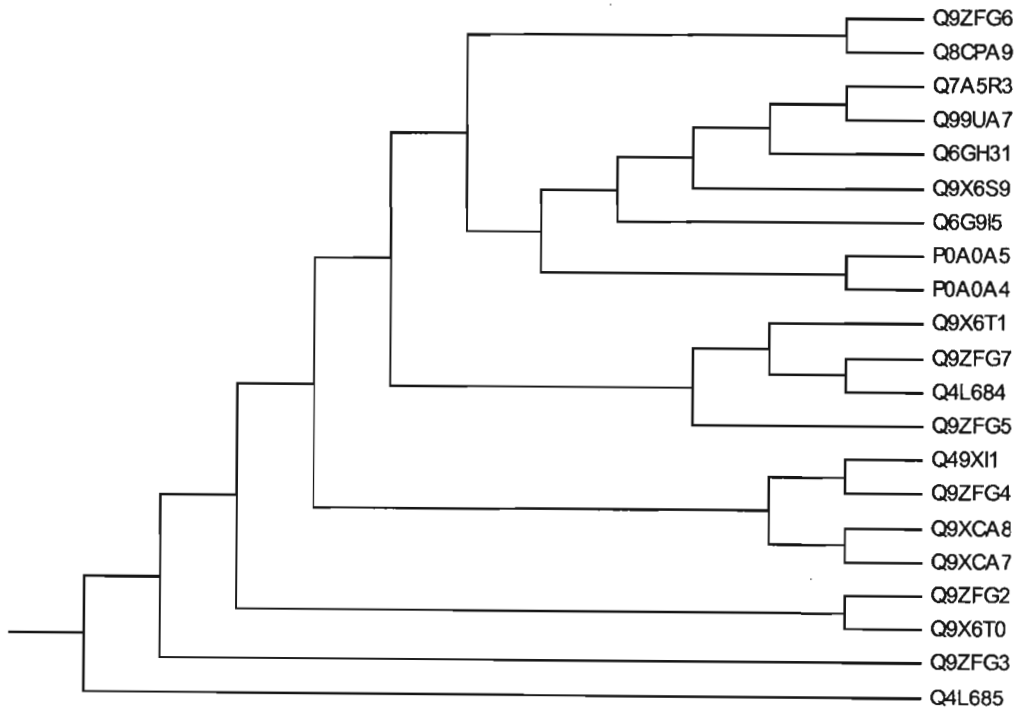


Figure 3.25. The consensus tree of the *fem* gene from various staphylococci species using the protein sequences.

Table 3.17. The different strains used for the *fem* gene analysis, the description and their accession numbers.

Accession number	Description	Reference
Q8CPA9	*S. epidermidis* Strain ATCC 12228	Zhang *et al.*, 2003
P0A0A4	*S. aureus* strain MW2	Baba *et al.*, 2002
P0A0A5	*S. aureus*	Berger-Baechi *et al.*, 1989
Q4L684	*S. haemolyticus* strain JCSC1434	Takeuchi *et al.*, 2003
Q4L685	*S. haemolyticus* strain JCSC1435	Takeuchi *et al.*, 2003
Q6G9I5	*S. aureus* strain MSSA476	Holden *et al.*, 2004
Q6GH31	*S. aureus* strain MRSA252	Holden *et al.*, 2004
Q7A5R3	*S. aureus* strain N315	Kuroda *et al.*, 2001
Q9X6S9	*S. aureus* subsp. *Anaerobius* Strain ATCC 20714	Vannuffel *et al.*, 1999
Q9X6T0	*S. intermedius* Strain ATCC 20273	Vannuffel *et al.*, 1999
Q9X6T1	*S. warneri* Strain ATCC 20316	Vannuffel *et al.*, 1999
Q9XCA7	*S. cohnii* subsp. Urealyticus Strain ATCC 49330	Vannuffel *et al.*, 1999
Q9XCA8	*S. gallinarum* Strain CCM 3572	Vannuffel *et al.*, 1999
Q9ZFG2	*S. schleiferi* strain ATCC 43808	Vannuffel *et al.*, 1999
Q9ZFG3	*S. sciuri* Strain ATCC 29062	Vannuffel *et al.*, 1999
Q9ZFG4	*S. xylosus* Strain ATCC 35663	Vannuffel *et al.*, 1999
Q9ZFG5	*S. capitis* Strain ATCC 27840	Vannuffel *et al.*, 1999
Q9ZFG6	*S. lugdunensis* Strain ATCC 43809	Vannuffel *et al.*, 1999
Q9ZFG7	*S. haemolyticus* Strain ATCC 29970	Vannuffel *et al.*, 1999
Q49XI1	*S. saprophyticus* subsp. saprophyticus strain ATCC 15305 / DSM 20229	Kuroda *et al.*, 2005
Q99UA7	*S. aureus* strain Mu50 / ATCC 700699	Kuroda *et al.*, 2005

3.4. Conclusions and Directions

It is important to note that several research groups, which may have used different methods for the determination of the MIC values, which are given in this study. The different methods of MIC determination does have an effect of the actual MIC which in turn may effect the classification of a strain as been resistant or intermediate.

The results obtained, for the penicillin resistance in streptococci, are consistent throughout the different studies. It can be seen that the three genes, PBPs 1A, 2B, and 2X play an important role in the development of resistance to β-lactam antibiotics. The alteration of these three genes results in a lowered affinity to β-lactam antibiotics. This result is consistent with previous studies, which implicate the same genes playing a role in resistance (Hakenbeck, 1999; Machebouf *et al.*, 2005; Massova & Mobashery, 1998; Pernot *et al.*, 2004; Nagai *et al.*, 2002; Pagliero *et al.*, 2004). The alteration ratios for the other three PBPs 2A, 1B, and 3 were low, regardless of whether the strain was susceptible or resistant to β-lactams. When the nucleotide and protein sequences are analyzed it can be seen that the resistant and susceptible strains form separate groups for only the PBPs 1A, 2B, and 2X.

The study shows that active site mutations are important in the development of resistance. We can conclude, from this study, that the SXXK active site is the critical active site for the development of resistance. It is found in all the resistant strains used in this study. The changes that are most common are the T337A in the STMK (337) active site, and L546V in the LKSG (546) active site. This was also observed in other studies (Hakenbeck *et al.*, 1999a; Hakenbeck *et al.*, 1999b; Nagai *et al.*, 2002). These amino acids substitutions are very important to resistance, as they change the conformations of the active site, therefore, reducing the affinity penicillin so it cannot bind.

Future work, for the penicillin resistance in *Streptococcus* section, would involve the determination of the affinity for penicillin that these proteins have and subsequently the formation of informed comparisons. Also we can determine the number substitutions that are present in a resistant strain for the promoter regions and also determine if they play a role in

the resistance of the gene. There after, we could conclusively determine the relationship between the resistant and susceptible strains. In this study we have shown that there is a relationship between the genes PBP 1a, 2b, 2x and with resistance. We have also shown the phylogenetic relationship between the different streptococcal species using the PBP genes.
In the result obtained for the vancomycin resistance in *Enterococcus* section, it can be noticed that the VanA and VanB phenotypes are similar to each other, while the VanC and the VanE phenotypes are grouped with each other. This result is similar to the results obtained by Reynolds & Courvalin in 2005. It could be speculated that VanE could have evolved from VanC. The 3D structures also showed the active sites involved in the VanX as well as the VanA genes. The VanX 3D structure shows the position of the critical amino acids responsible for the breakdown of the D-Ala-D-Ala precursors. The VanA ligase 3D structure shows the amino acids responsible the ligation of the D-Ala-D-Lac precursors. Future work in this field of study would entail obtaining all the respective MIC values and performing a conclusive analysis on resistant and susceptible strains, as well as the comparison between and within species.

The methicillin resistance in staphylococcal strains showed that there are many genes involved in the development of resistance. The results obtained when the phylogenetic analysis was performed within the genes implicated in resistance showed that the different strains used are very similar to each other. Therefore, proving these genes are highly conserved across different strains as well as in some cases across different species. Future work would involve obtaining all the MIC values for all the strains of both the resistant and susceptible phenotypes.

CHAPTER FIVE – REFERENCES

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