THE ELUCIDATION OF THE POSSIBLE MECHANISM OF VANCOMYCIN-RESISTANCE IN SELECTED *STREPTOCOCCAL* AND *ENTEROCOCCAL* SPECIES

By

RIZWANA DESAI

Submitted In Fulfilment of The Academic
Requirements of The Degree of
Master of Science
In The Discipline of Genetics,
School of Biochemistry, Genetics, Microbiology and Plant Pathology,
University of KwaZulu – Natal
Pietermaritzburg
2005

PREFACE

The experimental work described in this dissertation for M.Sc was carried out from January 2004 to December 2005 in the School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Dr. Mervyn Beukes.

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

Rizwana Desai (Miss)

Dr. M. Beukes (Supervisor)

ACKNOWLEDGMENTS

I would like to express my sincere thanks and gratitude to the following people for their contribution to this thesis:

First and foremost, to my supervisor and mentor Dr. Mervyn Beukes, for his guidance, advice, supervision and the invaluable knowledge and skills that I have acquired through his teachings.

My dear friends, most especially, Faiza Peer, Kershney Naidoo, Kovashni Pillay (Nair), Mohammed Karloo, Rizwana Mia and Veron Ramsuran, who were always there with their moral support, encouragement and for the fun and laughter they have provided throughout my studying career.

My fellow colleagues in the Microbial Genetics Research Group (MGRG), who were there with their ever-willing kindness, support, good camaraderie and assistance. A special thanks to Duran Ramsuran, for his generous assistance and willingness to learn; and Paradise Madlala, for his advice and support on student issues.

My mum, Fatema Bibi Desai, sisters, Razia, Saajida, and Sumaiya and the rest of my family for their unending love, support, encouragement, understanding and faith in me.

My darling, Yaseen Badat, for always motivating me, inspiring me, believing in me, loving me and listening to me, as well as for all the wonderful respites from work.

Dr. C. Southway, from the Department of Chemistry, University of KwaZulu – Natal, Pietermaritzburg, for the use of and help with the HPLC machine.

The University of Stellenbosch, for use of their mass spectrometry equipment.

The National Research Foundation, South Africa, for their financial support throughout this study.

ABSTRACT

Three Streptococcal strains: *S. milleri* P213, *S. milleri* P35 and *S. milleri* B200 and three enterococcal strains: *E. faecalis* 123, *E. faecalis* 126 and *E. faecium* were used to test for vancomycin resistance. Two strains were used as reference strains that were already characterized as vancomycin resistant. *E. faecium* BM4147 was used as a VanA control and *E. faecalis* ATCC was used as a VanB control. Susceptibility of each strain to this antibiotic was tested by disk-diffusion assay and the MIC values for the strains were found to be between 5 – 10 μg/ml and for the VanA control, the MIC was > 64 μg/ml and for the VanB control was 32 μg/ml. These MIC values indicate that *S. milleri* P213, *S. milleri* P35, *S. milleri* B200, *E. faecalis* 123, *E. faecalis* 126, and *E. faecium* are all of the VanC phenotype. All strains were tested for lysis by means of addition of vancomycin (10 μg/ml) to the bacterial cultures. Lytic curves were constructed and the VanB control was found to be most autolytic upon addition of vancomycin and *E. faecalis* 123 was the least autolytic. However, under normal conditions in phosphate buffer, lytic curves showed that *S. milleri* P213 was the most autolytic and the VanA control, the least autolytic. PCR assays were performed to detect specific antibiotic resistant genes. Primers were selected from Dukta-Malen *et al.*, 1995. The VanA primer yielded amplification of 732 bp for only the VanA control DNA and the VanB primer set yielded products for the VanB control DNA. *S. milleri* P213, P35, B200 and *E. faecalis* 123 and 126, and *E. faecium* DNA were amplified with the VanC primers. This supports the results obtained in MIC that these strains are possibly VanC resistant strains. Amplified VanA control and that of *E. faecalis* 126 were thereafter sequenced. VanA control amplicon was correctly amplified since it showed homology to *E. faecium* BM4147 as well as the VanB amplicons which was found to be homologous to the transposon Tn*1549* found on the well-characterized *E. faecalis* strain which is known to harbour the VanB vancomycin-resistant genes. Whilst *E. faecalis* 126 which represented the VanC phenotype showed 96% homology to *E. gallinarum* BM4147 which is a well-characterized glycopeptide-resistant enterococci belonging to the VanC phenotype. Southern blots were performed using specific primers as a probe to verify whether the gene sequences for the specific genotype were present in these strains and results confirmed those found in the PCR assays and in DNA sequencing. The peptidoglycan precursors of each strain were arrested in vancomycin (20 μg/ml) to block transpeptidation and transglycosylation steps of peptidoglycan synthesis and bacitracin

(100 μg/ml) was used to amplify precursors at the transglycosylation step. Precursors were extracted and analysed by reverse-phase HPLC. UDP-MurNAc-tetrapeptides cell wall precursors, which are found abundantly in vancomycin-resistant strains, were found in large proportions in all strains, except in *E. faecalis* 123 when arrested with vancomycin. This precursor has a noticeably decreased affinity for vancomycin, hence contributing to its resistance. The precursor accumulated when arrested with bacitracin, was, UDP-MurNAc-tetrapeptide in all strains except in *E. faecalis* 126. UDP-MurNAc-pentapeptides were also found in moderate amounts in most strains. The molecular masses of the peptidoglycan precursors obtained from mass spectrometry correctly identified them. This confirmed that the bacterial strains investigated were in fact resistant to the antibiotic vancomycin and this study shows that results obtained from conventional phenotypical screening methods reliably correlated with the genotypes classified using more advanced techniques such as PCR, southern blot/hybridisation and DNA sequencing.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BSA	bovine serum albumin
CO_2	carbon dioxide
D – Ala	D – Alanine
D – Lac	D – Lactate
D – Ser	D – Serine
D – Ala-D-Ala	D – Alanyl-D-Alanine
D – Ala-D-Lac	D – Alanyl-D-Lactate
D – Ala-D-Met	D – Alanyl-D-Methionine
D – Ala-D-Phe	D – Alanyl-D-Phenylalanine
D – Ala-D-Ser	D – Alanyl-D-Serine
EDTA	ethylene diamine trifluoro acetate
HPLC	high performance liquid chromatography
MIC	minimum inhibitory concentration
$MgCl_2$	magnesium chloride
MRSA	methicillin – resistant *Staphylococcus aureus*
NBT/BCIP	nitro blue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt
NCCLS	National Committee for Clinical Laboratory Standards
OD	optical density
PBPs	penicillin-binding proteins
PCR	polymerase chain reaction
PFGE	pulse-field gel electrophoresis
PO_4	phosphate buffer
SDS	sodium dodecyl sulphate
SSC	sodium citrate buffer
T_a	annealing temperature
TAE	tris acetate EDTA
TE	tris/EDTA
TCA	trichloro acetic acid
TSA	tryptone soy agar
TSB	tryptone soy broth

UDP-MurNAc-pentapeptide	UDP-*N*-acetylmuramyl-pentapeptide
UDP-MurNAc-tetrapeptide	UDP-*N*-acetylmuramyl-tetrapeptide
UDP-MurNAc-tripeptide	UDP-*N*-acetylmuramyl-tripeptide
VISA	vancomycin-intermediate *Staphylococcus aureus*
VRE	vancomycin-resistant Enterococci

CHAPTER ONE

LITERATURE REVIEW

1.1. INTRODUCTION

In 1958 the glycopeptide antibiotic vancomycin was introduced for the treatment of Gram-positive bacteria because of the increasing prevalence of methicillin-resistance in coagulase negative *Staphylococcus aureus* (MRSA) (17, 85, 96, 100, 105). Vancomycin and its structurally related antibiotic, teicoplanin was also thought to be the last resort in the defence against severe infections caused by other gram-positive organisms such as *Enterococci*, *Streptococci* and *Clostridium difficle* (5, 10, 13, 30, 33, 45, 46, 48-50, 69, 70, 79, 80, 85, 95, 106). However, Gram negative organisms are intrinsically resistant since they have impermeable outer membranes (54).

In general, glycopeptides consist of a heptapeptide backbone that is substituted with five to seven aromatic rings and different sugars. Vancomycin is a large molecule comprising of two hexoses and five aromatic rings (Figure 1.1.) (46, 72). Vancomycin binds to the substrates of transglycosylases and transpeptidases involved in the cell wall biosynthesis, the D-Alanyl-D-Alanine (D-Ala-D-Ala) terminus of the lipid-PP-disaccharide-pentapeptide. This is in contrast to penicillin, which directly binds to and inhibits these enzymes. By binding to these enzymes, vancomycin is thought to sterically prevent the consequent action of both activities of transglycosylases and transpeptidases. This is achieved by adding disaccharyl pentapeptide units to the growing strand and then cross-linking peptides within and between peptidoglycan strands on the external face of the cytoplasmic membrane. The bacterial cell then becomes susceptible to osmotic lysis since the rigidity of the cell wall is lowered because of the failure to form cross-links between peptidoglycan intermediates. Hence vancomycin has become the leading therapy for treating infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and enterococci (54, 62, 71, 72, 95, 99).

In 1979 the first case of vancomycin resistance in a clinical setting was reported and in 1986 the first plasmid-mediated resistance to glycopeptide antibiotics such as vancomycin was discovered (45, 46, 59, 61, 70, 85, 88, 96, 100, 102, 106). Vancomycin was until very recently one of the most powerful antibiotics that no bacterial cell had resistance to. The swift emergence of vancomycin resistant strains as life threatening organisms in hospital settings worldwide, has led to a surge of exhaustive investigations of the molecular determinants of glycopeptide resistance (24, 25, 45, 46, 62, 63, 93).

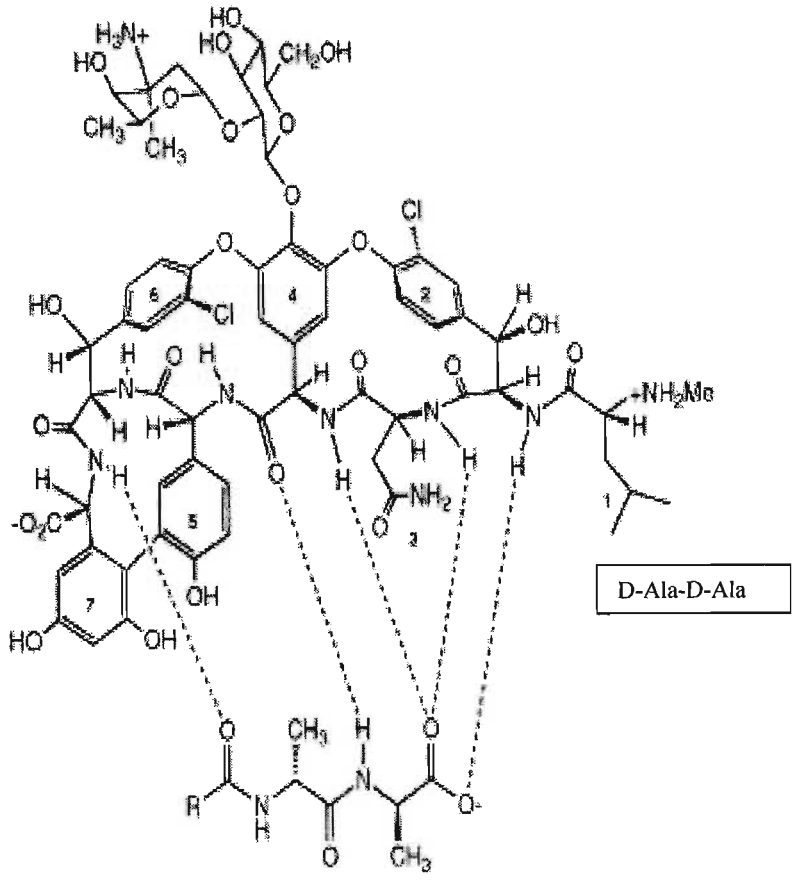

Figure 1.1. Model of the antibiotic, vancomycin (top) connected to a portion of bacterial cell wall precursor (bottom) by hydrogen bonds indicated by the dashed lines. The heptapeptide backbone of the antibiotic can be seen running through the centre of the molecule. The hydrogen bond formed between the NH and CO groups of the binding pocket along the peptide backbone of the D-Ala-D-Ala motif of the expanding cell wall, Me, methyl (5, 64).

Medical practitioners and their patients are becoming increasingly concerned of the rapid emergence and spread of vancomycin resistance, since strains are now intrinsically resistant to glycopeptides even though some of these strains were previously considered uniformly susceptible to glycopeptides (22, 57, 65). Those patients who are immunocompromised, severely debilitated and undergo antimicrobial therapy, usually suffer with infections caused by vancomycin-resistant Enterococci (VRE) (58, 61). There has been an increase in the number of reported incidence of infection and colonization of VRE hence there is added pressure placed on researchers to find antimicrobial drugs to treat these infections. The ease

with which bacteria acquire resistant genes is a major factor behind the dramatic increase in resistant strains (93).

Since most VRE already have resistance to multiple drugs and have acquired additional resistant determinants through mutation and transfer of resistant genes from other species. This emergence of glycopeptide resistance in Enterococci poses a serious threat since the efficacy of antibiotic therapy is compromised because there are few alternatives in cases of resistance or allergy to β-lactams (10, 41). The widespread of glycopeptide resistance may also be due to an epidemic of genes that are mobile to varying degrees and an epidemic of clone carrying those resistant genes (28, 50). Another serious public health concern is the possibility that vancomycin–resistant genes may be transferred to other gram-positive organisms since vancomycin-resistant Enterococci may serve as a reservoir for vancomycin resistant genes. In the future these genes may make their way to more virulent organisms such as those of the genus *Staphylococcus* (18, 64).

The high incidence of MRSA and vancomycin use, which are thought to be risk factors for vancomycin-resistant *S. aureus*, make the extensive distribution of these organisms an alarmingly realistic possibility even though there have been only a few reports of *S. aureus* isolates with reduced susceptibility to vancomycin (28, 96). In all cases of glycopeptide resistance reported, resistance seems to be inducible (52). There is a new urgency to search for novel antibiotics and through these investigations one of the most sophisticated molecular systems of acquired resistance and a paradigm of genetic adaptation has been discovered (24, 25, 29, 63). Bacteria that produce antibiotics have evolved strategies and mechanisms that provide immunity to the action of the antibiotic, and there is a general consensus that immunity mechanisms may have evolved with antibiotic biosynthetic genes to protect the producing organisms. In nature evolution of bacteria towards resistance to antibiotics and even multiple drug resistance exists hence it is unavoidable. The basic criteria of bacterial resistance are: (i) genetic and involves genetic differences from the parental strain; (ii) biochemically based on the presence or absence of a resistance mechanism; (iii) microbiological which results from an increased minimum inhibitory concentration (MIC) for an antimicrobial agent; (iv) clinically based on the outcome of therapy; (v) intrinsic whereby the type of resistance is present in all members of a given species or genus; and (vi) acquired where only certain isolates of a species or genus have a type of resistance present. There are two phenomena that can be

associated with the emergence and efficient spread of resistance, i.e., mutations in the house-keeping structural or regulatory genes or an alternative is through horizontal acquisition of foreign genetic information (31, 57, 58, 66, 93, 100). The only hope we have is to delay the emergence and ensuing widespread of resistant bacteria or resistant genes (31).

Three glycopeptide resistance phenotypes can be distinguished on the basis of the level and inducibility of resistance to vancomycin and teicoplanin (37). There are two classes of acquired resistance to glycopeptide antibiotics in Enterococcus, VanA and VanB whilst VanC is thought to be intrinsic (22, 50, 84). The most extensively studied phenotype is the VanA resistant phenotype which led to the elucidation of a mechanism of resistance to glycopeptides and an insight into the regulation and mode of dissemination of the corresponding genes (9, 45, 70). Phenotypes VanA and VanB are of the highest clinical importance since they are the most frequently observed (47, 58, 88, 102, 103). In Enterococci, acquired resistance is due to the production of peptidoglycan precursors ending in the depsipeptide D-Alanyl-D-Lactate (D-Ala-D-Lac) instead of the dipeptide D-Alanine-D-Alanine (D-Ala-D-Ala) found in susceptible bacteria (14, 18, 32, 52, 72, 103).

1.2. Peptidoglycan Biosynthesis

Peptidoglycan or murein is the major component of the cell wall of the bacteria and is essential for an organisms survival. It is a macromolecule that surrounds the cell, determining the shape of the cell as well as maintaining cell integrity (46, 69, 78, 90, 101, 104). Bacterial peptidoglycan may vary between species although some functional elements are conserved. The glycan strands of all bacterial peptidoglycan are made up of repeat disaccharide units known as *N*-acetylglucosamine-*N*-acetylmuramic acid (GlcNAc-MurNAc). Glycan chains are cross-linked by short cell wall peptides. The amino group of the first amino acid in the peptide moiety is linked to the carboxyl group of the lactic acid of *N*-acetylmuramic acid by an amide bond. This process occurs in the cytoplasm, membrane and extracellular cell wall compartments. After synthesis in the cytoplasm, nucleotide precursors form a phosphodiester bond with an undecaprenol carrier (lipid I) and then lipid II is formed when lipid I is attached to *N*-acetylglucosamine. Lastly, penicillin-binding proteins catalyse the polymerisation of lipid II subunits by the transglycosylation and

transpeptidation reactions. Many of the chains run parallel to one another, and are connected with each other via a peptide moiety of the five amino acids: L-Alanine-D-Glutamic acid-L-Lysine-D-Ala-D-Ala. The peptide cross-bridges connect the MurNAc residues on the glycan chains. Cross-links, which makes up the most important component of the cell wall are then generated (20, 101, 104). Peptidoglycan is closely involved in cell division hence peptidoglycan biosynthesis is a target for antibiotics although inhibition of its production is bactericidal (Figure 1.2.) (90). Resistance occurs when the antibiotic does not bind or reach to its bacterial target and thereby leading to multiple replication of the bacteria (56). Glycopeptides and moenomycins are known to interfere with the transglycosylation reactions of peptidoglycan synthesis (23, 104).

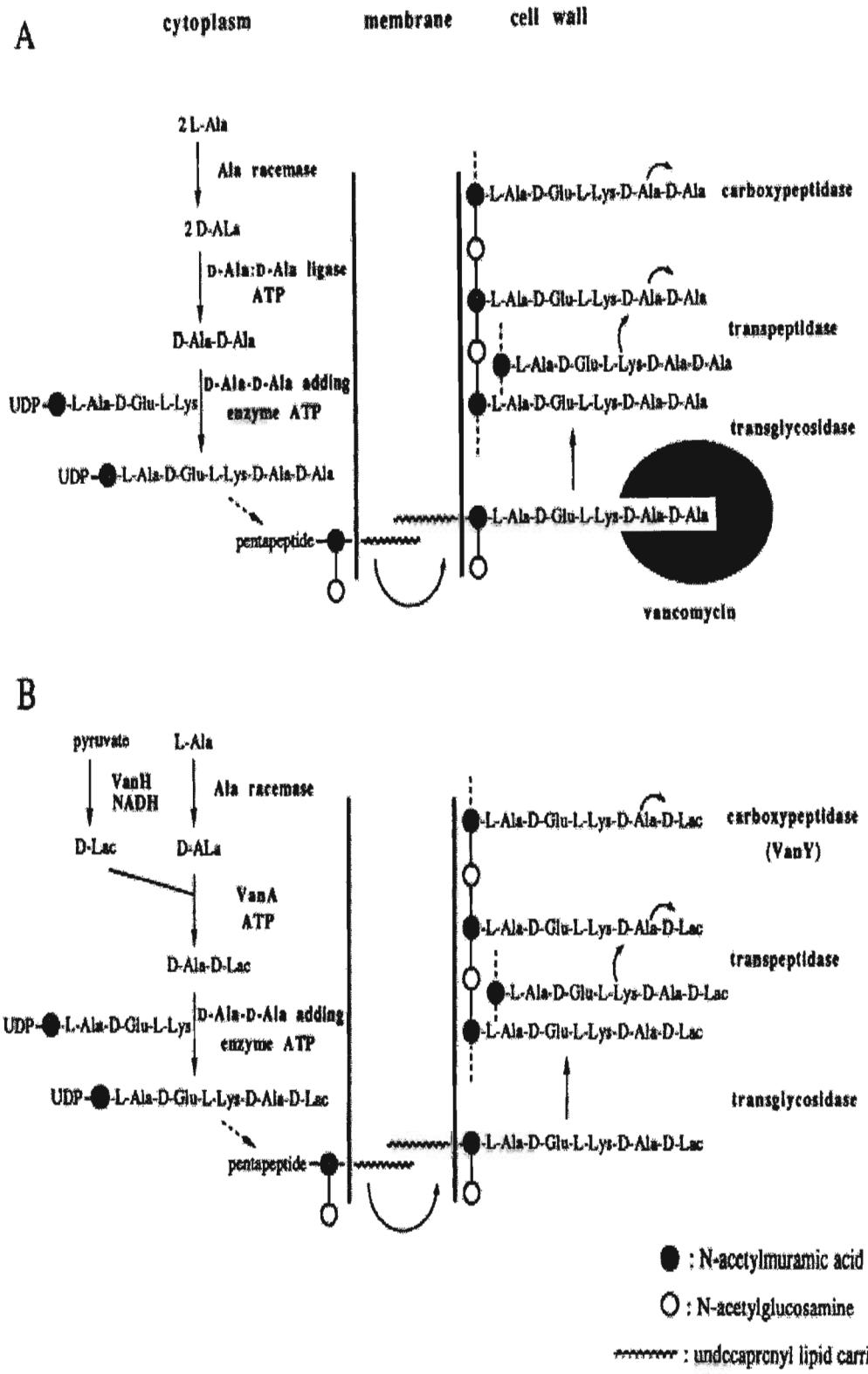

Figure 1.2. Schematic representation of peptidoglycan biosynthesis in glycopeptide-susceptible (A) and –resistant cells (B) (5).

1.3. Glycopeptide Resistance Mechanisms

For vancomycin to be effective it must reach the cytoplasm and bind with cell wall precursors thereby inhibiting their incorporation into the growing cell wall (54, 96). Hence vancomycin is thought to be an effective antibacterial agent as it inhibits peptidoglycan synthesis (Figure 1.3.) (13, 57, 84). There are two factors which are shown to be critical for antibacterial activity. These are: dimerization of the vancomycin-group of antibiotics which results in an enhanced affinity of the antibiotic for cell wall analogs in free solution, and allows for a chelate effect enhancement of affinity for the bacterial cell wall peptidoglycan. This is critical since antibiotics, which dimerize weakly are less active than strongly dimerizing antibiotics. However, if they have initially a greater affinity for bacterial cell wall analogs then they might be more active. Dimerization of vancomycin-group antibiotics is an important factor affecting antibiotic activity (17). However there is an escalating problem since bacteria are now becoming resistant since they produce different cell wall intermediates (54).

Resistance to vancomycin found in clinical isolates was shown to be inducible and in most cases transferable, and at times, plasmid mediated (2). Resistance to glycopeptides such as vancomycin is thought to be phenotypically and genetically heterogenous (30). Vancomycin alone is bacteriostatic against organisms such as Enterococci (35). Bacteriostasis or bacterial cell death occurs as a result of disruption of the transglycosylases and or transpeptidases activity of enzymes responsible for polymerisation and cross-linkage of the cell wall (17). An alternate biosynthetic pathway for the production of cell wall precursors that bind vancomycin poorly was found to be the mechanism of acquired glycopeptide resistance in the VanA and VanB Enterococci (12, 30, 45, 63, 103). Resistance is acquired from the transfer of mobile genetic elements that encode the enzymes responsible for the synthesis of low-affinity precursors and the removal of the high-affinity precursors that are normally produced by the host (13). This is achieved by forming specific hydrogen bonds and other non-covalent interactions (17). A strategy of reprogramming the terminal peptidoglycan intermediates in cell-wall cross-linking steps is employed by vancomycin resistance (96).

Glycopeptides inhibit cell wall synthesis due to formation of complexes between the antibiotic and the C-terminal D-Alanine (D-Ala) residue of peptidoglycan precursors (3-5, 12, 13, 30, 33, 36, 48, 53, 69, 72, 74, 81, 92, 95, 96, 106). Hence, vancomycin prevents two steps in peptidoglycan synthesis: transglycosylation and transpeptidation (Figure 1.4.) (46,

72, 81). Contact with the target can only take place after translocation of the precursors bound to the lipid carrier to the outer surface of the cytoplasmic membrane since these antibiotics do not penetrate into the cytoplasm.

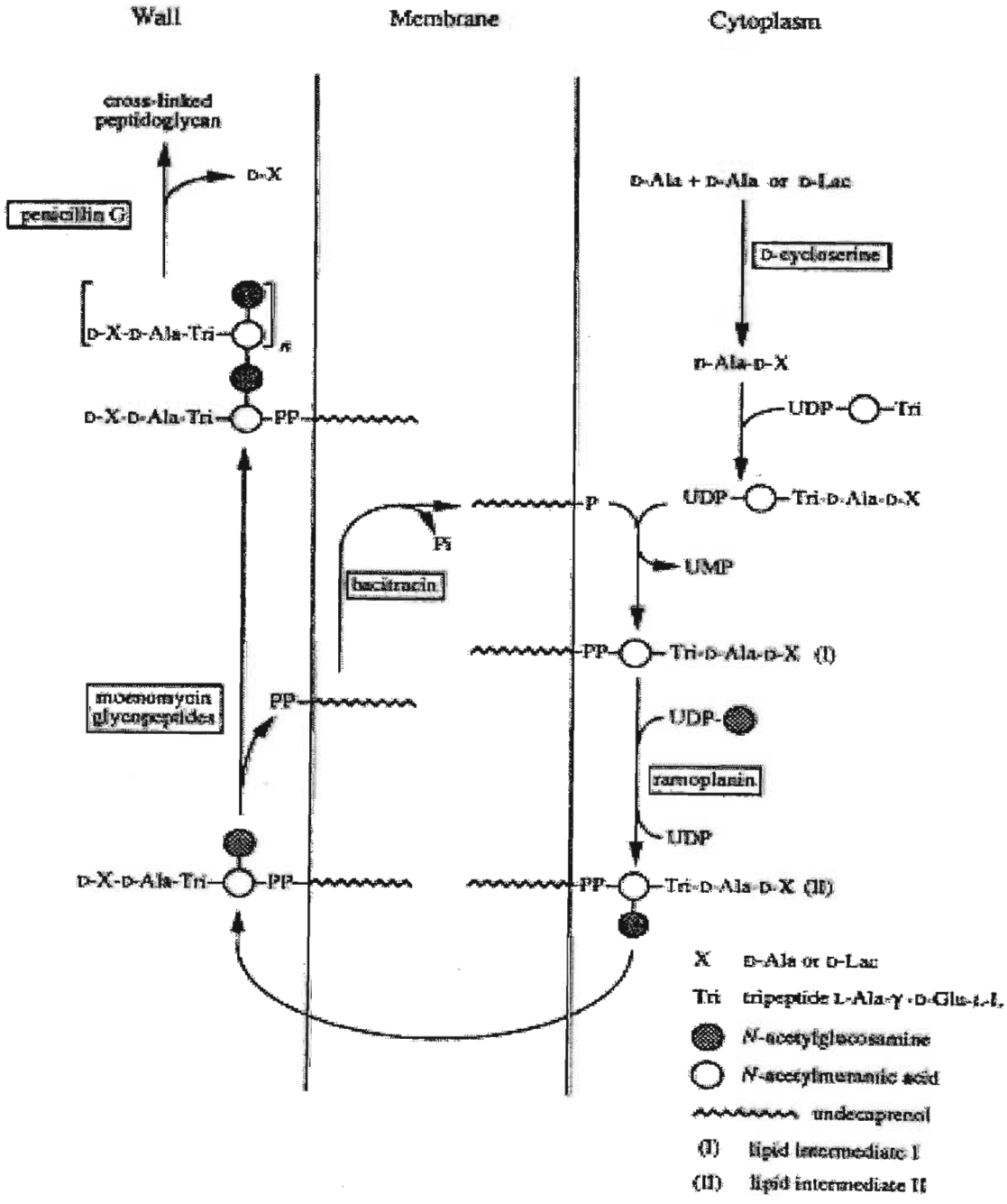

Figure 1.3. Mode of action of inhibitors of peptidoglycan synthesis (14).

The transfer of precursors from the lipid carrier to the peptidoglycan is prevented by transglycosylases by formation of complexes at the outer surface of the cytoplasmic membrane. Transglycosylases then block the incorporation of the disaccharide pentapeptide subunits into the growing peptidoglycan (13). Other reactions that are catalysed by transpeptidases and D, D- carboxypeptidases are also inhibited. It has been revealed by analysis of cell wall components that various bacterial species show conservation of the C-terminal D-Ala-D-Ala sequences required for vancomycin binding (13, 96).

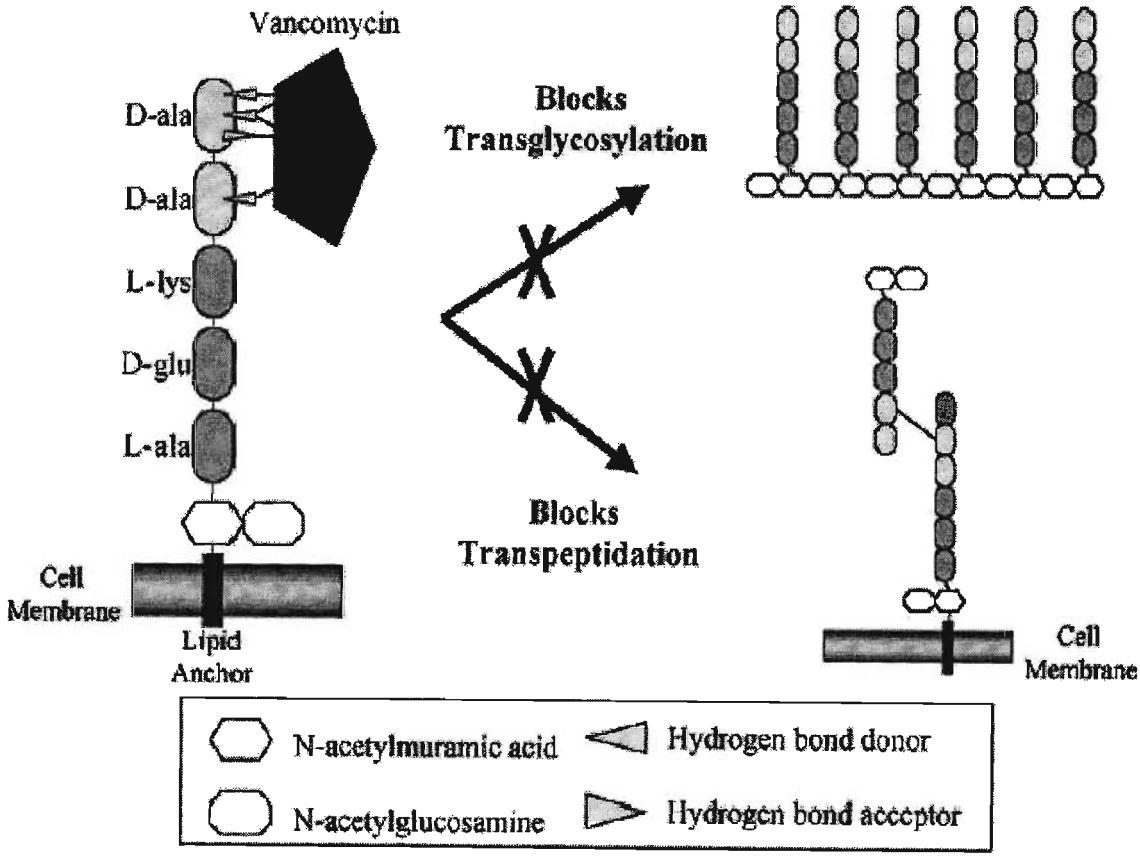

Figure 1.4. The bactericidal effect of vancomycin. Vancomycin binds noncovalently via five hydrogen bonds to the D-Alanine-D-Alanine terminus of the peptidoglycan pentapeptide linker. This tight binding of vancomycin inhibits the cross-linking of the peptidoglycan, lowering the strength of the bacterial cell wall. Transglycosylation is also inhibited, thus stopping peptidoglycan growth by blocking the addition of N- acetylmuramic acid and N-acetylglucosamine (85).

Under normal conditions of peptidoglycan synthesis a ligase enzyme joins two molecules of D-Alanine to form a D-Ala-D-Ala complex. This complex is then added to the UDP-*N*-acetylmuramyl-tripeptide to form the UDP-*N*-acetylmuramyl-pentapeptide. Glycopeptides cause the increase of the UDP-*N*-acetylmuramyl pentapeptide. During transglycosylation the UDP-*N*-acetylmuramyl-pentapeptide is incorporated into the nascent peptidoglycan and permits the formation of cross-bridges during transpeptidation contributing to the strength of the peptidoglycan layer.

Vancomycin then binds to the D-Ala-D-Ala termini of the pentapeptide precursor units in the outer surface of the bacterial membrane with high affinity thereby blocking their addition to the growing peptidoglycan chain. Thus preventing successive cross-linking by blocking the transglycosylation and transpeptidation reactions resulting in a weaker cell wall (27, 30, 52). Hence, the D-Ala-D-Ala terminus of peptidoglycan which is a rigid polymer that protects bacterial cells from osmotic lysis is the molecular target of glycopeptide antibiotics (12, 65, 99). Vancomycin therefore inhibits production of the bacterial cell wall since it acts by binding irreversibly to terminal D-Ala-D-Ala of the cell wall disaccharide pentapeptide precursors which eventually results in cell death (27, 46, 65).

Organisms that are glycopeptide resistant avoid cell death by modifying the drug's peptide target. This is done by specifically modifying it to the depsipeptide D-Ala-D-Lac (16, 34, 65). The substitution of D-Ala-D-Ala for D-Ala-D-Lac in VanA, VanB and VanD phenotypes of Enterococci prevents binding of glycopeptides to cell wall components and allows peptidoglycan polymerisation in the presence of the antibiotics (27, 33, 35, 88, 98). Hence there is a greater than 1000-fold decrease in vancomycin binding when a substitution of the NH group of the amide D-Ala-D-Ala linkage occurs by an oxygen in a D-Ala-D-Lac ester linkage (Figure 1.5.) (6, 9, 15, 33, 37, 53, 57, 80, 85, 86, 95, 99). This replacement of D-Ala-D-Ala for D-Ala-D-Lac removes a hydrogen bond, which is imperative for antibiotic binding, thus reducing the glycopeptide's affinity considerably (36, 99, 106). However, the substitution of D-Ala by D-Ser does not alter the H-bond, but is responsible for conformational changes which reduce the affinity for vancomycin slightly (30). Because it binds to the D-Ala-D-Lac terminus with a lower affinity, it is unable to sterically block the transpeptidation and transglycosylation steps needed for the formation of the peptidoglycan cell wall(54). The transposable element, Tn*1546* present in VRE has the biosynthetic machinery required to effect such a transformation (65).

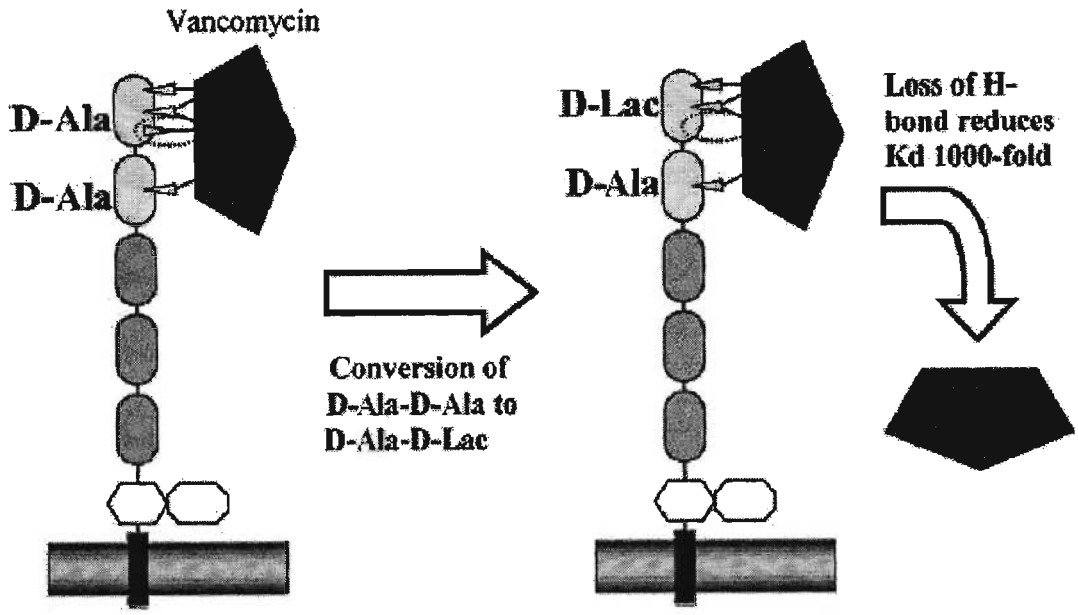

Figure 1.5. Resistance mechanism of vancomycin by *vanHAX* type resistance. Conversion of the terminal D-Alanyl-D-Alanine to D-Alanyl-D-Lactate occurs through the action of VanH, VanA, and VanX. Conversion to D-Lactate results in vancomycin binding lower by a 1000-fold (85).

In resistant strains, the following genes are expressed, viz., *vanA, vanH, vanX, vanR,* and *vanS.* The gene products VanR and VanS are members of a two-component regulatory system required for the vancomycin-induced resistance response by directing transcription of v*anH, vanA* and v*anX.* These genes are responsible for encoding the three proteins necessary for the synthesis of abnormal peptidoglycan precursors terminating in D-Ala-D-Lactate (D-Lac) for incorporation into peptidoglycan precursors (5, 6, 9, 15, 16, 24, 25, 33, 45, 46, 53, 65, 84, 85, 103). Several proteins that sense the drug or effect of the drug, produce a drug-resistant target. Thus eliminating the drug susceptible target in a coordinated manner responsible for this alteration of the target site for glycopeptide antibiotics (63, 103). The collective action of the three enzymes VanH, VanA and VanX ensures this alternate pathway. The origin of these resistant cassettes may have come about from the intrinsic vancomycin resistant lactic acid bacteria or glycopeptide-producing organisms. An alternative is that the genes may have arisen through mutations in homologous genes within Enterococci or that they might have evolved from a common ancestor (64).

The VanA protein is a ligase of altered substrate specificity which synthesizes D-Ala-D-Lac in preference of D-Ala-D-Ala whilst the VanH (or $VanH_B$, $VanH_D$) protein is known as a D-hydroxyacid dehydrogenase which creates a pool of D-Lactate by reducing pyruvate. The VanX ($VanX_B$) protein is a D, D-dipeptidase lacking activity against D-Ala-D-Lac. This enzyme minimizes the competing synthesis of normal pentapeptide since it reduces the pools of D-Ala-D-Ala produced by the native ligase hence inhibits production of glycopeptide-susceptible precursors (84).

D-hydroxy acids such as D-Lac are neither natural products present in the environment of some organisms or are not normally produced by organisms such as Enterococci therefore VanA alone cannot confer resistance to vancomycin. Hence these organisms must acquire the genes within the *vanA* operon required to synthesize D-Lac in order to produce the substrate for VanA (27). The VanA mediated incorporation of D-2-hydroxybutyrate at the C-terminal position of peptidoglycan precursors allows cell wall synthesis in the presence of vancomycin (9).

Through investigations, it has been established that vancomycin resistance is due to the production of peptidoglycan precursors that bind the antibiotic with reduced affinity. Due to the broad substrate specificity of VanA, VanH and the D-Ala : D-Ala ligase adding enzyme, the actual binding of the D-Ala substitute incorporated *in vivo* could not be identified. The most likely pathway was considered to be the synthesis of a depsipeptide precursor since it does not catalyse ester bond formation compared to the D-Ala : D-Ala ligase from susceptible Enterococci (9). Arthur *et al.* found in their studies that D-Lactate is the most likely substituent for D-Ala present at the C-terminal position of peptidoglycan precursors that show low affinity to vancomycin.

VanY and VanZ acts as accessory proteins (13, 70). Even though these two proteins are not essential for resistance, their production increases the level of resistance to vancomycin and teicoplanin (13). VanY removes the terminal D-Ala residue from peptidoglycan precursors and removes vancomycin binding sites by its carboxypeptidase activity (63, 84). Vancomycin induces the synthesis of two proteins that are readily detectable in Enterococcal membrane fractions. This is a 39 kDa protein, which was identified as the VanA ligase that is necessary for cell wall synthesis in the presence of glycopeptides. The other protein is a D,D-carboxypeptidase of 39.5 kDa, which is required for resistance (5). These two proteins were only produced after exposure to vancomycin (106). Another model proposes that the

ligase plays a role in the formation of new pentapeptide precursors which would not bind vancomycin (52). It has been hypothesized that an inducible carboxypeptidase is involved in glycopeptide resistance. Gutmann *et al.* found that induced carboxypeptidase activity of the high-level resistant strains were greater than that of the two-level resistant strains. In clinical isolates, Gutmann *et al.* observed that there is a correlation between the level of resistance and the carboxypeptidase activity and therefore suggested that the carboxypeptidase is a functional component of the resistant mechanism.

It has also been suggested that the role of carboxypeptidase could be to decrease the normal UDP-MurNAc-pentapeptide pool either by cleaving the terminal alanine of this molecule or by hydrolysing the D-Ala-D-Ala normally added to the UDP-MurNAc-tripeptide (19). Therefore the low ligase and the high carboxypeptidase activities contribute to vancomycin resistance since it results in the scarcity of the D-Ala-D-Ala containing precursors (10). Al-Obeid *et al*, has shown that crude walls from non-induced cells or from induced cells treated with sodium dodecyl sulfate (SDS) to remove the inducible proteins were shown to bind vancomycin, however cell walls containing cytoplasmic membrane-associated proteins did not bind vancomycin. Cytoplasmic membranes from vancomycin-induced cells were found to protect the glycopeptides from being bound to synthetic pentapeptides, however did not bind vancomycin or teicoplanin. Any subsequent enzymatic modifications of the pentapeptide precursor of peptides which is considered to be natural targets of glycopeptides is facilitated by inducible proteins that are responsible for glycopeptide resistance (2).

In a previous study, it has been proposed that cytoplasmic membrane proteins restrict access of vancomycin to its target. Cell wall-associated membranes which were induced by vancomycin at low concentrations did not bind vancomycin however it was shown that cell walls from non-induced cells after being boiled in 4% SDS (w/v) were able to bind vancomycin. It was used to distinguish between protection of the target site and protection against non-specific binding to the cell wall. It was also shown experimentally that only membranes from induced cells prevented binding of vancomycin to exogenous pentapeptides. From results in some studies it was observed that a component of the cytoplasmic membrane from vancomycin-induced cells was able to reduce the access of glycopeptides to the pentapeptide in the natural cell walls of these organisms. It was also inferred that these proteins are involved in the absence of binding to the glycopeptide target sites (2).

The most plausible mechanism of glycopeptide resistance is the binding of the induced proteins to the pentapeptide and its subsequent modification in such a way that glycopeptide binding is prevented. Increased susceptibility to penicillin has also been shown to be accompanied with glycopeptide resistance after the synthesis of these proteins. It is likely that the proteins after binding and modifying the endogenous pentapeptide, do not allow normal synthesis of the peptidoglycan by the cell wall enzymes. This might also be inferred from the altered cell shape of vancomycin-induced strains (2).

The combined substrate specificities of the D-Ala : D-Ala ligases (Ddl), the D-Ala-D-Ala adding enzymes and the D-Ala-D-Ala termini that interact directly with penicillin-binding proteins (PBPs) contribute to the incorporation of D-Ala residues into peptidoglycan precursors (5, 27). Penicillin-binding proteins is involved in the process of cross-linking the precursors to the growing peptidoglycan, however the replacement of the D-Ala by D-Lac does not impair cross-linking of the modified precursors to the growing peptidoglycan chain (27). Analysis of the cell wall peptidoglycans of resistant strains reveal the presence of altered cross-links compared to that of susceptible strains. Therefore, PBP2A, which is involved in the biosynthesis of the cell wall, is essential in vancomycin resistance cells especially in *S. aureus* strains. This penicillin-binding protein is unable to use D-Ala-D-Lac as a substrate (94).

The relative pool sizes of the new UDP-MurNac-tetrapeptide-D-Lac precursor and the normal residual UDP-MurNAc-pentapeptide determines the level of resistance to vancomycin. When vancomycin resistance was induced in the presence of vancomycin, the major impact was a significant decrease in oligomers. After the induction of vancomycin resistance, an outstanding amount of the normal UDP-MurNAc-pentapeptide precursors were present during the production of the new UDP-MuNAc-tetrapeptide D-Lac precursor (54). Billot-Klein *et al.* found that it is possible that the presence of this pentapeptide precursor would allow some specific binding of vancomycin, thus impairing the synthesis of oligomers. Another hypothesis would be that the non-specific binding of vancomycin could interfere with the synthesis of oligomers (19, 52).

Since resistance in VanA is inducible, it might be that the expression is regulated at the genetic level. In the VanA Enterococci, the VanR and the VanS proteins are histidine kinase sensors and forms part of a two-component regulatory system that sense and respond to environmental stimuli (103, 106). This system regulates the expression of resistant *vanA* and

vanB clusters (Figure 1.6.). The VanS protein is autophosphorylated, which in turn phosphorylates VanR when it detects the presence of vancomycin or teicoplanin or, if it detects disturbance of cell wall precursors which are elicited by these drugs. When the system is induced by the presence of vancomycin or teicoplanin not only is there increasing expression of *vanR* or *vanS*, but also the autophosphorylated protein binds to the promoter region of *vanA, vanH,* and *vanX*. This drives transcription of the genes that encode the essential structural molecules of the gene cluster (19, 54, 55).

In a laboratory – derived strain, the inactivation of the *vanS* gene had no effect on the level of resistance. However, it was shown that the actual level of transcription of resistance genes decreased. In comparison, the insertional inactivation of the *vanR* gene was shown to cause susceptibility to glycopeptides in a vancomycin-resistant lab derived strain and a clinical isolate. This confirms that v*anR* is a transcriptional activator and is stimulated by v*anS* (106).

For the VanC and VanE phenotype in Enterococci, glycopeptide resistance results from synthesis of modified precursors ending in D-Alanine-D-Serine (D-Ala-D-Ser). Glycopeptides exhibit low binding affinities to these modified precursors (13, 35, 84). Therefore, it can be said that it is the substrate specificity of the enzymes that determine the structure of the peptidoglycan is responsible for the activity of glycopeptides, and not the affinity of target enzymes for the molecule (13).

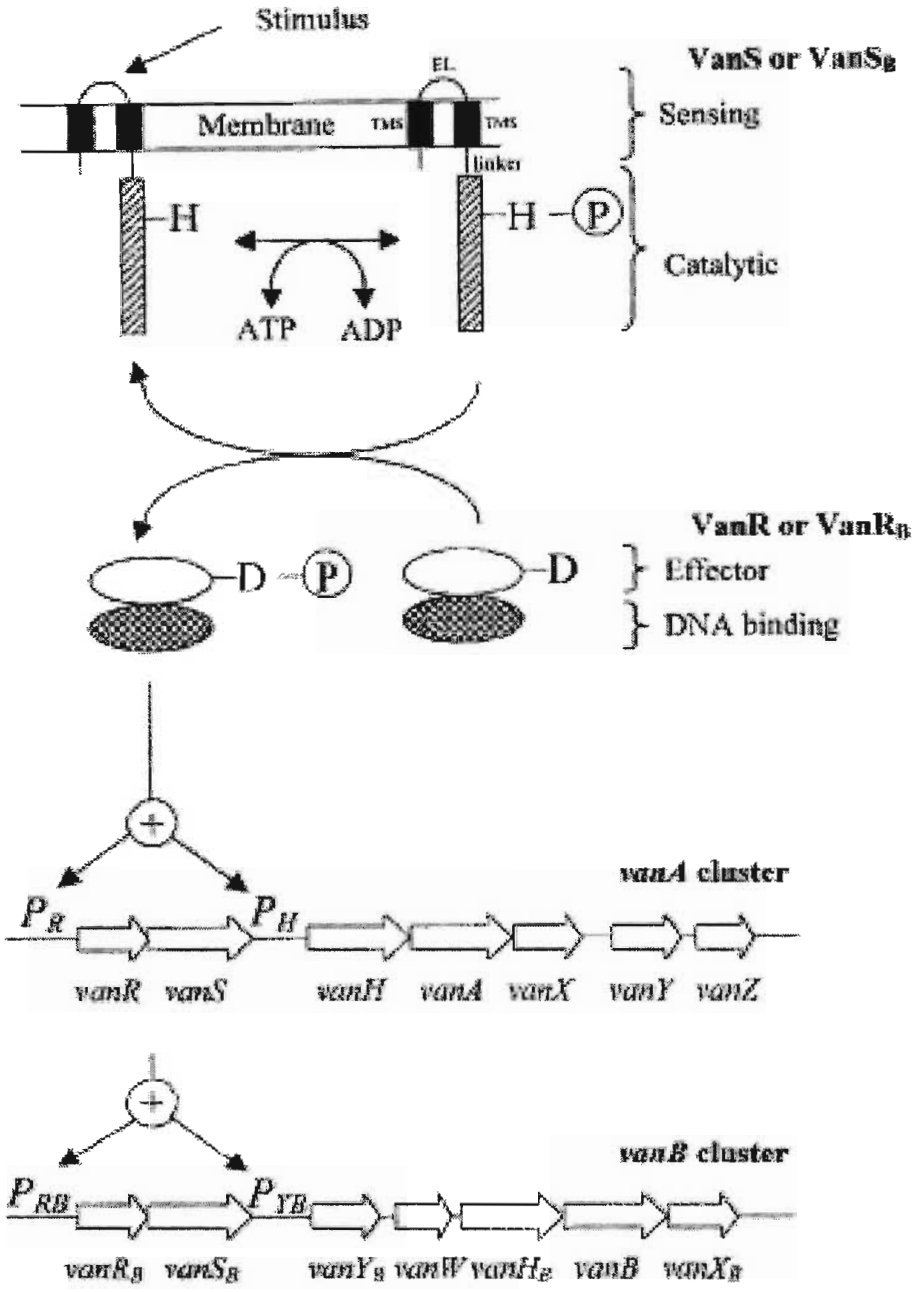

Figure 1.6. Sequence of events that lead to transcriptional activation of the *vanA* and *vanB* clusters (12).

Another hypothesis is that multiple genes or multiple metabolic pathways are altered because of long clinical vancomycin exposure times that are required to generate resistance. Some abnormalities associated with strains such as vancomycin-intermediate *S. aureus* (VISA) are: (i) reduced rate of growth, decreased cell wall cross-linking, (ii) increased cell wall thickness, (iii) decreased autolysis, (iv) changes in PBP's and alterations in glutamate amidation of the peptidoglycan stem peptide. Thickened, poorly cross-linked cell walls are thought to be peripheral targets for vancomycin hence trapping the antibiotic before it can reach its site of lethal action at the cell membrane (58).

1.4. Glycopeptide Resistant Genes

Therapeutic failure may occur as a result of resistant phenotypes that have emerged in response to glycopeptide therapy *in vivo* (15). It has been established that resistance to certain levels of vancomycin and teicoplanin was plasmid-mediated and inducible by sub inhibitory concentrations of glycopeptide antibiotics (22). Resistance to vancomycin in organisms such as VRE is conferred by five genes, viz., *vanA*, *vanB*, *vanC*, *vanD*, and *vanE* which are distinguished on the basis of the level and inducibility of resistance to vancomycin and teicoplanin (26). Acquired resistance to glycopeptides appears to be mediated by two common classes of genes, *vanA* and *vanB* (5, 19, 22, 47, 87, 103). The organization of *vanA*, *vanB*, and *vanD* operons are very similar and it is found that the biochemical basis of glycopeptide resistance is identical in VanA and VanB (21, 30, 36, 86).

1.4.1. VanA

VanA type resistance is the best understood, and most widely known for its genetic and biochemical basis of resistance (70). Resistance may be induced by high levels of glycopeptides and is therefore characterized by high level resistance to vancomycin at an MIC of $\geq$ 64μg/ml and to teicoplanin at an MIC of $\geq$ 16μg/ml (5, 15, 19, 30, 35, 52, 70, 96, 103). The VanA enzyme is encoded by the *vanA* gene. This enzyme is related to the D-Ala: D-Ala ligase that displays a broader substrate specificity and contributes to the synthesis of dipeptides (63). The *vanA* gene and other genes (Figure 1.7.) involved in the regulation and expression of vancomycin resistance are located on a transposon, Tn*1546*, which often resides on a plasmid (5, 27, 30, 85). The spread of high-level glycopeptide resistance among clinical isolates of Enterococci is through the widespread distribution of this transposon, rather than that of a plasmid or that of a bacterial clone (52). VanA was

found to have D-Ala : D-Ala ligase activity with an altered specificity. It produces dipeptides different from D-Ala-D-Ala and once they are part of the carboxy terminus, vancomycin is unable to bind to it. VanA displays a 28 - 36% amino acid identity with two D-Ala : D-Ala ligases of *E. coli* which have similar catalytic properties. The reduced catalytic efficiency shows that production of the dipeptide D-Ala-D-Ala by VanA was not responsible for glycopeptide resistance. VanA does interfere with binding of vancomycin to its target therefore the D-Ala : D-Ala ligase activity of VanA was not inhibited by UDP-*N*-acetylmuramyl-pentapeptide (UDP-MurNAc-pentapeptide). The incubation of VanA with UDP-MurNAc-pentapeptide alone, or in combination with D-Ala did not show signs of cleavage or exchange of the terminal D-Ala residue therefore VanA is not a D,D-carboxypeptidase (19). VanA produces mixed dipeptides such as D-Ala-D-Met, D-Ala-D-Phe and D-Ala-D-Aminobutyrate more efficiently than D-Ala-D-Ala. The enzyme uses exclusively amino acids of the D-configuration and incorporates D-Ala at the N-terminal position. It has been concluded that synthesis of a D-Ala-X compound by VanA allows cell wall synthesis in the presence of glycopeptides and therefore VanA does not possess the properties that would account for resistance by modification, overproduction or protection of the target. Ligation of the D-Ala-X with UDP-MurNAc-tripeptide would lead to the production of peptidoglycan precursors with reduced affinities for glycopeptides (5).

VanA was also shown to catalyze formation of ester bonds between D-Ala and D-2-Hydroxyxacids. Preferential substrates for VanA-mediated depsipeptide and dipeptide synthesis are D-2-Aminobutyrate which both contains an ethyl side chain. D-Ala-D-2-Hydroxybutyrate or D-Lac which are depsipeptides produced by VanA can be added onto UDP-MurNAc-tripeptide by the adding action present in crude extracts from susceptible Enterococci or by the addition of purified MurF protein from *E. coli* (5). Substrate specificity of VanA allows for the synthesis of peptidoglycan precursors which display reduced affinities for vancomycin. Incorporation of D-Lac at the C-terminal ends of precursors is mediated by VanA (65).

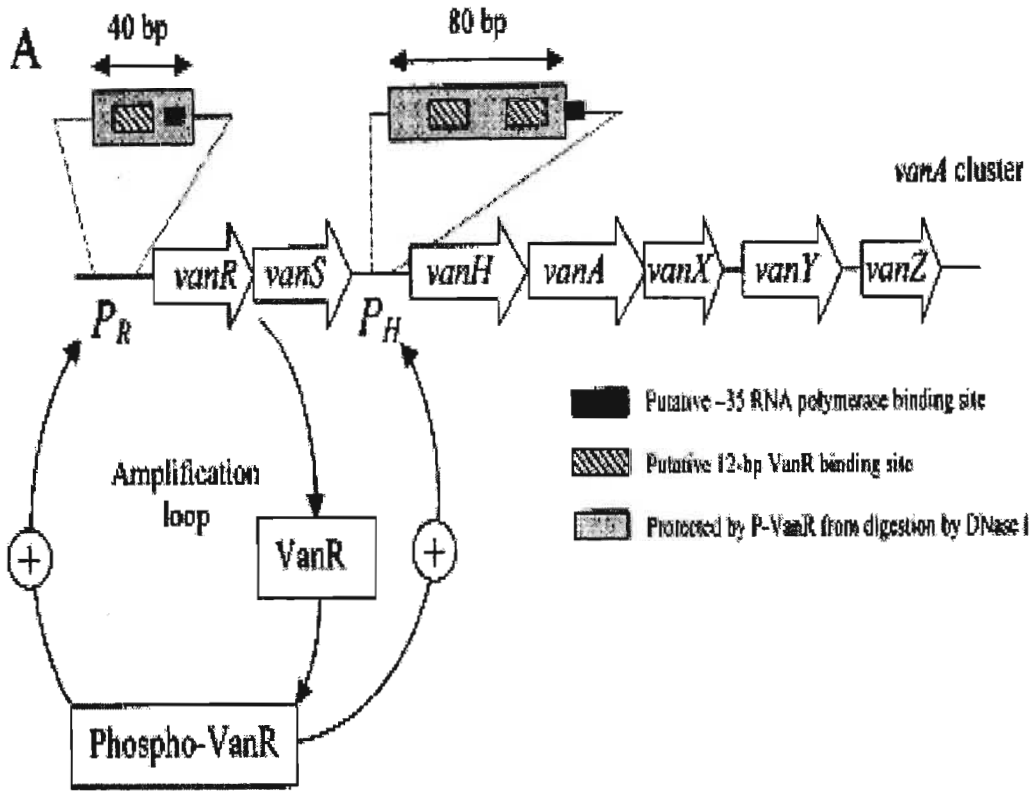

Figure 1.7. Schematic representation of the *vanA* gene cluster illustrating regulation of the P_R and the P_H promoters (12).

VanA or *vanB* genes encode related proteins for reduction of pyruvate to D-Lac by VanH or $VanH_B$ dehydrogenases, the synthesis of the depsipeptide D-Ala-D-Lac (VanA or VanB ligase) and hydrolysis of the dipeptide D-Ala-D-Ala produced by the host Ddl (VanX or $VanX_B$, D-dipeptidases). VanY ($VanY_B$) is a membrane-bound D,D-carboxypeptidase enzyme that is also required for the VanA and VanB phenotype (15, 84).

In certain isolates where the *vanA* gene was mapped, some heterogeneity was seen. This suggests that in some strains Tn*1546* existed intact and in others there was insertion like elements (27). The transposition of Tn*1546* into various self-transferable plasmids and subsequent transfer by conjugation is responsible for the spread of VanA resistance in clinical isolates. Experimental transfer of *vanA* to *S. aureus* and other Gram-positive organisms demonstrates the ability of these genes to be expressed in diverse hosts, however there has not been any transfer of these genes to *S. aureus* in nature (64).

1.4.2. Tn *1546*

This transposon was originally detected on the plP816 plasmid from *E. faecium* BM4147. It consists of 10 851 bp, which is surrounded by inverted 38 bp repeats and encodes nine polypeptides that can be assigned into four functional groups (Figure 1.8.). These are: (i) transposition functions (OFR1 and ORF2); (ii) regulation of vancomycin resistant genes (VanR and VanS); (iii) resistance to glycopeptides by production of depsipeptides (VanH, VanA, and VanX); and (iv) accessory proteins that may be involved in peptidoglycan synthesis, but not necessarily involved in glycopeptide resistance (VanY and VanZ) (5, 30). The two open reading frames that are co-transcribed in the opposite direction are thought to encode the production of putative transposase and resolvase enzymes. The presence of the *van* cluster on a transposable element is a factor in the widespread distribution of *vanA* – mediated glycopeptide resistance (11, 106). Heterogeneity of Tn *1546* may be due to the presence of different insertion sequences. These insertion sequences serve as hot spots for the rearrangement of genetic fragments and may also be associated with certain geographic locations (77). Only a single nucleotide difference in this sequence has been documented. This suggests that the VanA transposon emerged through a complex chain of events that occurred only once and was then transferred to many strains. The coding sequences of the VanA transposon are strongly conserved, the non-coding insertions are more variable. Tn *1546* also encodes a second mechanism of glycopeptide resistance which is mediated by the VanZ gene product (106).

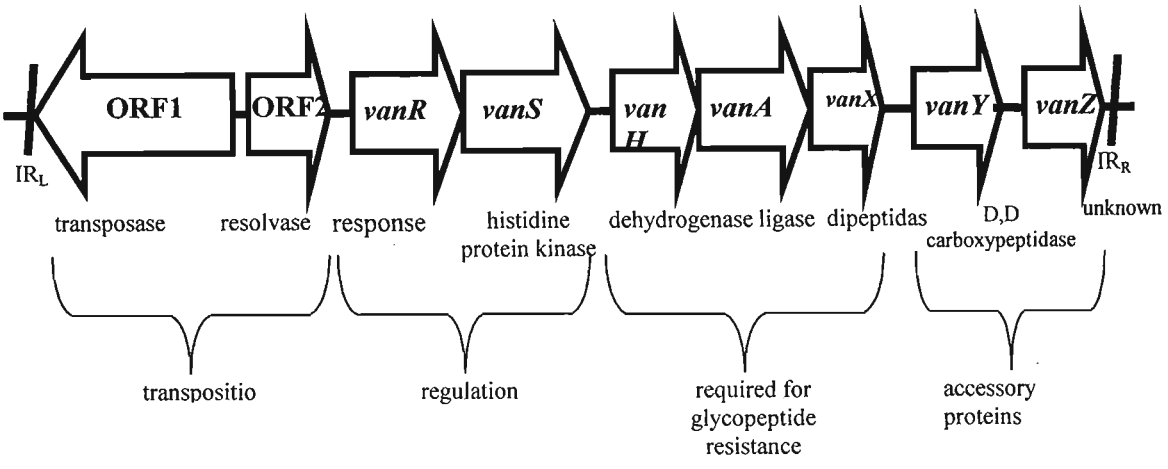

Figure 1.8. Schematic map of transposable element, Tn*1546*. Coding sequences are denoted by open arrows whilst those closed and open arrows labelled IR_L and IR_R specify the left and right inverted repeats of the transposon, respectively. The promoter found in the *vanS-vanH* intergenic region co-transcribes *vanH*, *vanA*, and *vanX* (5, 76, 106).

1.4.3. VanB

The *vanA* and *vanB* clusters have been primarily found in *E. faecalis* and *E. faecium* species and are found less commonly in other Enterococcal species. The finding of several gram-positive species carrying *vanA* and a stool isolate of *Streptococcus bovis* carrying *vanB* is evidence that there is a transfer of these genes beyond the Enterococcus genus (64, 70). Organisms exhibiting VanB phenotypes are resistant to vancomycin at an MIC of 32 – 62 μg/ml but are susceptible to teicoplanin (15, 27, 30, 35, 42, 43, 47, 58, 85). VanB is not resistant to teicoplanin since the VanB resistance pathway is not activated by teicoplanin because it is not an inducer of the *vanB* operon (42, 43, 45, 54, 103). A cluster of genes determines the VanB phenotype: $VanR_B$-S_B (Figure 1.9.). Resistance is inducible since it might be regulated in a similar fashion to the *van* gene cluster (106). Determinants reside on large mobile elements of approximately 90 - 250 kb or transposons such as Tn*1547* or Tn*1549*, that can be transferred from one strain of enterococci to another by either horizontal transmission or by plasmid conjugation (27, 58, 106). The products and expression of $vanH_B$, *-B* and X_B that are regulated by a two component regulatory system causes resistance to vancomycin (58).

Comparison of peptidoglycan precursors from VanA and VanB strains revealed a similar muropeptide precursor that may correspond to the D-Lac containing precursors identical to others. The high degree of similarity between the genes involved in VanA-and VanB- type resistance is confined to $vanH_B$, *vanB* and $vanX_B$. The distinction between the VanA and VanB phenotype can be in part be attributed to differences in the regulation of the resistant genes.

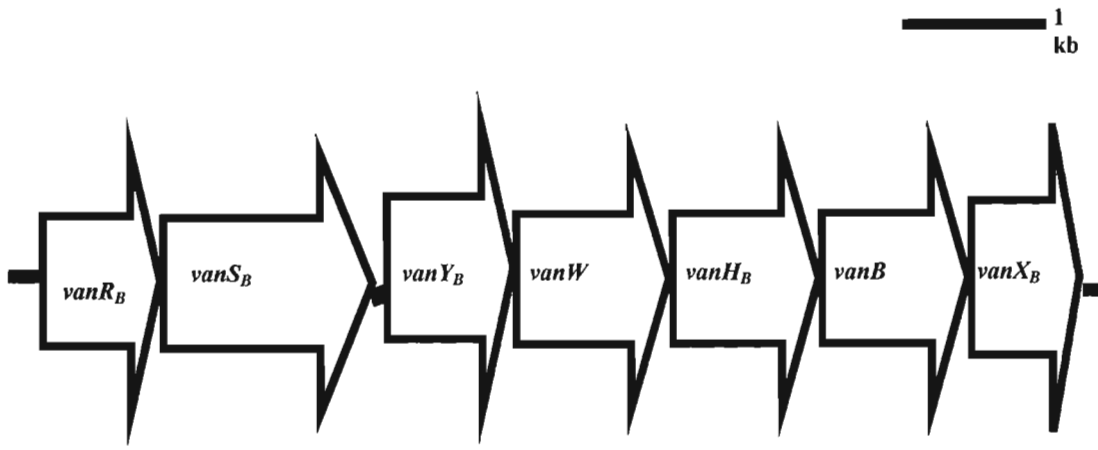

Figure 1.9. Schematic map of the *vanB* gene cluster (41).

The determinants for VanA and VanB may be similar since VanA and a portion of the D-Ala : D-Ala ligase related protein from a VanB strain displays 77% amino acid identity, therefore, *vanB* encodes a ligase of altered substrate specificity (106). Vancomycin induced production of similar D,D carboxypeptidases in both VanA and VanB strains (27). Even though the *vanB* gene cluster is functionally similar to the *vanA* cluster it differs in its regulation, however they rely on the same mechanism (63). VanB and VanC strains have shown to encode ligases which determine the synthesis of altered peptidoglycan precursors. VanB also correlates with a 39.5 kDa cytoplasmic membrane protein. The *vanB* cluster contains genes that are homologous to *vanH, vanA* and *vanX*.

Billot-Klein *et. al.,* 1990, has concluded that the constitutive expression of vancomycin resistance of the VanB type in the absence of vancomycin does not substantially affect its composition even though a new peptidoglycan precursor is synthesized. Thus, once exported this precursor seems to be perfectly well recognized by the cell wall synthetic machinery. Glycopeptide resistance by overproduction of peptidoglycan precursor that

end in the depsipeptide D-Ala-D-Lac in susceptible Enterococci is mediated by the *vanB* gene cluster. The $VanR_B$-$VanS_B$ two component regulatory system that activates transcription of the resistant genes in response to vancomycin but not to teicoplanin also controls synthesis of D-Ala-D-Lac and hydrolysis of D-Ala-D-Lac (16).

1.4.4. VanC

The organization of the *vanC* operon is different compared to the *vanA*, *vanB*, and *vanD*. It is characterized by low-level and intrinsic resistance to vancomycin at an MIC of 4 – 32 μg/ml but not to teicoplanin (30, 39, 81, 84-86). Resistance is chromosomal and generally constitutively expressed and in some strains VanC maybe inducible (30, 54, 81, 102, 106). There are three subtypes; VanC-1, VanC-2 and VanC-3. VanC favours pentapeptide ending in D-Alanyl-D-Serine (D-Ala-D-Ser), which weakens binding of vancomycin to the pentapeptide (3, 13, 39, 45, 73, 81, 85, 102, 106). Higher MIC's could be explained by a higher proportion of D-Ala-D-Ser. VanC D-Ala : D-Ser ligase, $VanXY_C$ D,D-dipeptidase or D,D-carboxypeptidase and $VanT_C$ serine racemase which is membrane-bound mediates glycopeptide resistance of the VanC phenotype (3, 4, 30, 39, 81, 84-86, 106). This results in a six-fold decrease in affinity for vancomycin since the hydroxymethyl side chain of D-Ser is thought to disrupt sterically the binding of vancomycin to the normal D-Ala-D-Ala termini (54). These three proteins are involved in replacing the terminal D-Ala-D-Ala precursor with D-Ala-D-Ser. VanX and VanY are enzymes that are also active with substrates terminating in D-Ser (84, 86). $VanXY_C$ is a bifunctional enzyme since it catalyses D,D-peptidase and D,D-carboxypeptidase activities (3, 4, 54, 83, 86).

1.4.5. VanD

In several isolates of *E. faecium* the less common phenotype of acquired glycopeptide resistance includes VanD (21, 36, 64). It has constitutive resistance to moderate levels of glycopeptides and is inhibited by vancomycin at an MIC of 64 μg/ml (30). They are located on the chromosome and are not transferable by conjugation to other cocci (30). Together with the *vanA* and *vanB* operons, the *vanD* operons all have similar organization (Figure 1.10.). *VanA, vanB* and *vanD* are ligases, which synthesize D-Ala-D-Lac. It has been shown for this phenotype precursors ending in D-Ala-D-Ala is due to a frame shift mutation in the chromosomal *ddl* gene (21, 26, 36, 85).

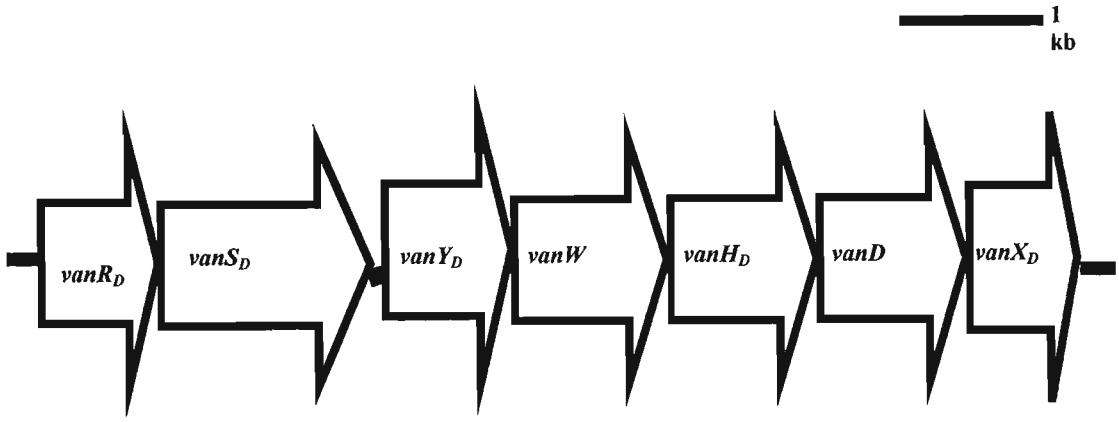

Figure 1.10. Schematic representation of the *vanD* gene cluster (26).

1.4.6. VanE

Has low levels of resistance to vancomycin at an MIC of 16 μg/ml and is susceptible to teicoplanin (30, 34, 44, 64, 85, 86). For this phenotype, the precursors normally produced by the host are eliminated and replaced by D-Ala-D-Ser (30, 32, 44, 86).

1.4.7. VanG

VanG type resistance is acquired and is susceptible to teicoplanin at an MIC of 16 μg/ml. This is due to the inducible production of peptidoglycan precursors ending in D-Alanine-D-Serine (30, 32, 34, 68, 85, 86). This cluster, which is chromosomally located, is comprised of genes from various *van* operons. As in the VanC- and VanE-type strains, the D-Ala-D-Ser ligase, $VanXY_G$, a D,D-peptidase and a serine racemase ($VanT_G$) are all implicated in the synthesis of D-Ala : D-Ser. $VanY_G$ contains a frame shift mutation which results in the premature termination of the encoded protein and accounts for the lack of UDP-MurNAc-tetrapeptide in the cytoplasm. The transmission of VanG-type glycopeptide resistance to *E. faecalis* was associated with the movement from chromosome to chromosome of genetic elements of 240 kb carrying also *erm*B-encoded erythromycin resistance (34).

1.4.8. VanH

VanH, which has an open reading frame of 983 bp overlaps *vanA* by 5 bp (106). The primary structure of the VanH protein encoded by the *vanH* gene is a clue to identify the chemical nature of compounds incorporated into peptidoglycan precursors by VanA. VanH displays a 19 - 30% amino acid identity with NAD^+ -dependent dehydrogenases that oxidize D-2-hydroxyacids to form corresponding 2-ketoacids and is located upstream from *vanA*. Characterization of VanH shows that it catalyzes the opposing reaction that produces D-2-hydroxyacid substrates for VanA. Studies have established that in the presence of glycopeptides, VanH synthesizes a D-hydroxyacid *in vivo* and indicated that incorporation of D-2-hydroxybutyrate and D-Lac into peptidoglycan precursors allows cell wall synthesis (6, 106). It has been suggested that production of VanA and VanH, in addition to enzymes encoded by the host chromosome may be sufficient for peptidoglycan synthesis in the presence of glycopeptides (6). VanH which is a dehydrogenase is also encoded by the Tn*1546* transposon and is required for the synthesis of the depsipeptide D-Ala-D-Lac by reducing pyruvate which replaces D-Ala-D-Ala in glycopeptide resistance (9, 12, 30, 54, 103). VanH synthesizes D-Lac when pyruvate is available and efficiently catalyzes the reduction in pyruvate and is also known as a reductase (106). This may be seen when VanA catalyzes ester bond formation between D-Ala and D-2-hydroxyacid products of VanH. The resulting depsispeptides are then incorporated into UDP-muramyl-tripetide by the D-Ala-D-Ala adding enzyme; this is unable to bind glycopeptides. Hence VanH, VanA, and VanX encode enzymes that are essential for resistance (96).

1.4.9. VanR and VanS

Depsipeptide production is controlled by the VanS-VanR two component regulatory system that acts as a signal-transducing system to activate transcription of genes *vanA, vanH* and *vanX* in response to glycopeptides (5, 6, 54). This is the only two component regulatory system controlling the expression of antibiotic resistant genes. In VanA, VanB and VanD the *vanR-vanS* genes are located upstream from *vanH*, however it is located downstream from *vanT* (30). The VanS-VanR system is the only system that controls the expression of antibiotic resistant genes amongst two-component systems that comprise one of the largest known families of transcriptional regulators in bacteria (6, 15, 52). Autophosphorylation of VanS is induced by vancomycin and teicoplanin in the VanA VRE

phenotype, whilst in the VanB phenotype it is activated by only vancomycin and VanC does not seem to require VanS or VanR since its resistance is constitutive (54, 55).

The two-component regulatory system consists of a sensor (VanS or $VanS_B$) and the regulator (VanR or $VanR_B$) (12, 103). VanS is thought to be a membrane associated sensor that functions to detect the early effect of vancomycin on cell wall synthesis and is necessary for high-level transcription of vancomycin resistant genes, although it is not required for phenotypic expression. In its primary sequence, vanS consists of two clusters of hydrophobic amino acids which coincide with two membrane-spanning domains, hence this gene product appears to be related to histidine protein kinases (106). Whilst VanR was found to be a transcriptional activator necessary for the expression of vancomycin resistant genes, hence it is known to be an activator (13, 34). In its response to its stimulation by vancomycin, $VanS_B$ sensor histidine kinase catalyses $VanR_B$ phosphorylation thereby switching off the resistant genes. In the absence of vancomycin, it dephosphorylates $VanR_B$ due to the phosphatase activity of $VanS_B$. (12, 13, 58). Since it is an activator, phosphorylation of VanR by VanS modulates co-transcription activity (63). In addition to their kinase activity, this system also has many histidine kinases that also act as phosphoprotein phosphatases thereby accelerating the dephosphorylation of their similar response regulators. Phosphatase activity plays an important role in controlling VanA type resistance (52).

Control of transcription by VanS-VanR is involved in promoter activation (10). DNA binding affinity at two glycopeptide inducible promoters, P_R and P_H, for transcription of the regulator VanR-VanS and resistant VanHAX genes respectively are increased by phosphorylation of VanR. Dephosphorylation of phospho-VanR is mediated by VanS. Critical for regulation is the deletion of the *vanS* sensor gene that leads to constitutive high-level transcription of the resistant gene. Mutations in the genes encoding the host Ddl ligases and the $VanS_B$ leads to an increased resistance to glycopeptides which harbour a wildtype *vanB* gene cluster (15).

The promoter P_{YB}, which is found upstream from $vanY_B$ transcribes $vanY_B$, $-W$, $-H_B$, $-B$ and $-X_B$ genes and this is activated by $vanR_B$ in its phosphorylated form (12, 58).

1.4.10. VanX

VanX is a protein encoded by the *vanX* gene that has unknown function though insertional activation of the v*anX* gene indicates that VanX is needed for resistance. VanX is not involved in assembly steps since it is not necessary for the transcriptional activation of vancomycin resistant genes *vanH*, *vanA*, and *vanX* (6). It has also been shown that VanX is not involved in the synthesis of the 2-ketoacid substrate for the VanH dehydrogenase, however, it may act at a later stage of cell wall assembly (9). VanX, $VanX_B$ and $VanX_D$ are the D,D- dipeptidases that hydrolyze the D-Ala-D-Ala dipeptide which is synthesized by the host Ddl ligase and formed by the endogenous chromosomal pathway (24, 54, 103), (12, 30, 52, 106). Therefore VanX decreases the pool level of the normal UDP-MurNAc-pentapeptide. It is also a metallo-protease with respect to its catalytic substrate-binding and zinc binding sites (46, 84).

1.4.11. VanY

The VanY protein has a moderate contribution to vancomycin resistance since it is a D,D-carboxypeptidase that cleaves the D-Ala terminal peptide (8, 33, 34). Its enzyme activity is interconnected with the cytoplasmic membrane, whilst its production is inducible by vancomycin (6, 8, 106). *VanY* and v*anZ* are encoded by a distal part of Tn*1546* and are not necessary for glycopeptide resistance by production of depsipeptides (6, 30, 54). VanY which is a membrane-bound carboxypeptidase that increases the level of expressed resistance even though it is not required for resistance (24, 106). When the dipeptide D-Ala-D-Ala, which synthesizes the pentapeptide, has escaped VanX hydrolysis, it is then hydrolysed by VanY D, D-carboxypeptidase. For this reason VanX and VanY act together in order to prevent the accumulation of the pentapeptide in the cytoplasm of glycopeptide-resistant Enterococci which favours the replacement by the pentadepsipeptide in cell wall assembly (12, 30, 103). *VanY* may also have D,D – carboxyesterase activity since it is able to remove either the terminal D-Ala or D-Lac group from peptidoglycan precursors (106).

1.4.12. VanZ

The function of VanZ is not yet understood however it contributes to teicoplanin resistance by an unknown mechanism that does not involve the incorporation of a substituent of D-Ala-D-Ala into peptidoglycan precursors (7, 12, 13, 30).

1.5. Tolerance

Tolerance and resistance are two entirely different concepts. Those organisms that are insensitive to antibiotics, and continue to grow in its presence are antibiotic-resistant micro-organisms. However, organisms that stop growing but do not die in the presence of the antibiotic are known to be antibiotic-tolerant strains (49, 67, 79, 85, 89). In both cases infection caused by the organism continues once the therapy is stopped thus the infective agent is not eliminated. Because of their tolerance, bacteria do not undergo active growth since they are not killed by the normally fatal dose of antibiotic. Nonetheless, the MIC's of tolerant strains remain identical to that of non-tolerant ones and the antibiotic binds normally to its target (56, 89). The mechanism of tolerance is unlike that of resistance (89). Antibiotic tolerance cannot be detected using conventional susceptibility tests seeing as tolerant strains remain sensitive to antibiotics in an *in-vitro* test. Phenotypic tolerance occurs when the bacteria respond to poor growth conditions (78). The genetic basis for vancomycin tolerance is due to a mutation in the two-component signal transduction system (49, 78).

The two-component regulatory system in bacteria function in controlling a variety of responses by allowing the micro-organism to sense their environment and to respond to it by adjusting gene expression. A specific response-regulator is paired with each sensor kinase in the cell and this protein controls the expression of a unique group of genes. When the sensor kinase (VncS) becomes inactivated, bacteria become tolerant, however, were not when the response regulator (VncR) was switched 'off'. The VncR response regulator represses some of the genes required for antibiotic-induced death when the phosphorylated mode was switched 'on'. Under normal circumstances, presence of the antibiotic causes VncS to remove the phosphoryl group from VncR, thereby switching it 'off' and allowing these genes to be expressed (49). The functional loss of VncS may lead

to antibiotic tolerance becoming more easily transformed into high-level antibiotic resistance (79).

Clinical tolerance may also occur as a result of the inactivation of the amidase gene *lytA* or the down-regulation of autolysin activity (56, 78). A loss of autolysin triggering occurs in the absence of VncR-VncS signal transduction (78). Vancomycin is bactericidal since the presence of the antibiotic activates autolysis which digests the cell wall exoskeleton and kills the cells. The presence of the antibiotics in strains which have a mutation in this signal transduction system no longer results in autolysis. Hence, tolerance leads to substandard lysis and greatly diminished killing of the bacteria (56). The autolysins do not rupture the cell due to differences in the cell wall or inhibition of cell wall synthesis. The absence of autolysin action in tolerant strains could be explained by the increase in turgor pressure and the membranes or wall becoming leaky in order to prevent turgor pressure from dramatically increasing. Small molecules must leak out of the cell as fast as they are pumped in to prevent the tension in the peptidoglycan layer from becoming too much. For this reason, tolerant strains should have autolytic activity upon the increase of antibiotic and there must be structural changes in the wall to make it more permeable (60). Growth is inhibited in the presence of vancomycin, nevertheless the organism survives and begins to grow when the antibiotic is removed (49, 56). Clinical isolates showing tolerance to vancomycin is very grave as this indicates the selective pressure for the emergence of vancomycin resistance (67, 89).

1.6. Detection

Vancomycin has become the front-line therapy for treating infections caused by methicillin-resistant *S. aureus* (MRSA) and by Enterococci. The recent outbreaks of vancomycin-resistant Enterococci with its high mortality rates re-enforces the need for laboratories to be able to detect the various types of glycopeptide resistance (37, 54). It is of utmost importance to determine the antimicrobial susceptibility of a clinical isolate in order to find the best antimicrobial therapy (45, 98). Regardless of modifications made to the criteria used to classify categories of susceptibility, the actual detection of some vancomycin-resistant organisms in the clinical laboratory remains challenging (106). The heterospecific expression or transfer of glycopeptide resistant genes is fast becoming a threat among bacteria under laboratory conditions. This problem is further compounded

by the mobility of the *vanA* and *vanB* gene clusters by conjugation and transposition (13). By looking at the molecular logic of the cell wall reprogramming from vancomycin-sensitive to vancomycin-resistant bacteria one can identify new targets. The two-component regulatory sensor kinase-response regulator pair VanS and VanR control the structural genes *vanHAX*, hence these five proteins are targets for inhibition of function and reversal of vancomycin-resistant Enterococci. Another possibility is to screen natural products and other compound libraries for efficacy against vancomycin-resistant Enterococci (54).

It is also recommended to speciate vancomycin resistant enterococcal isolates in order to distinguish the VanC organisms since it has implications for the treatment and infection control. For example, detection of low-level resistance in VanB enterococci has been improved by modifications (27). Vancomycin resistance in clinical isolates should be screened. This can be done by performing agar-screening plates which is a simple, sensitive test. Standard susceptibility testing procedures are used to easily detect resistance from moderate to high concentrations of vancomycin. Another method, the E-test is an accurate alternative for detection of vancomycin resistance. Other tests such as performing 24-hour incubation and the use of strong transmitted light to read plates have improved the accuracy of the disk-diffusion method (96, 98).

Most laboratories can readily detect high-level vancomycin-resistant strains, yet the problem lies in detecting low-level resistance. Laboratories are now turning to advanced molecular biology tools to remedy this problem (106). Genotypic detection is no longer dependent upon phenotypic grouping. The advantages of genetic assays are that there is no need for phenotypic expression since these can be performed on clinical specimens directly (98). Techniques such as nucleic-acid based detection systems are fast becoming the key to detect the presence of resistance genes and disseminating the elucidation of resistance mechanisms. Research laboratories have developed genetic tests for the presence of vancomycin-resistant genes usually based on PCR. This technique is now used because it is a more sensitive procedure, easily accessible, simple and rapid. PCR has many applications in medical diagnosis. It has been used in species identification of infectious agents and specific detection of antibiotic resistant genes. PCR was used for the simultaneous identification of species and glycopeptide resistant genotypes based on specific detection of genes encoding D-Ala : D-Ala ligases and related glycopeptide

enzymes (37). This technique is also now improved upon with the introduction of real-time PCR, which is used for the quantification of expression levels of certain genes (98). Pulse-field gel electrophoresis (PFGE) is used for individual strains of vancomycin-resistant enterococci and the gene clusters can be analysed and tracked by DNA-based techniques. Interesting and varied results may be seen by the molecular epidemiology of VanA and VanB vancomycin-resistant enterococci based on PFGE (64). The detection of mutational resistance has been made easier by rapid DNA sequence analysis and DNA microarray technologies also look promising (98). Developing molecular techniques can now be used to indicate the strains in which gene expression is unusual, or which contains multiple resistant genes, or even strains with potentially novel resistance genes (106).

There is an obvious need for simple and accurate phenotypic screening and confirmation tests for glycopeptide resistance. Recently it has been shown that low-level resistance cannot be detected by methods such as automated susceptibility testing systems and disk-agar diffusion. Since the emergence of vancomycin-resistant Enterococci, rapid detection, determination and efforts in controlling outbreaks in hospitals of vancomycin resistance may be beneficial to patients (96). Constant testing and screening for these organisms are necessary to assess the spread of resistant organisms or resistant genes (45).

1.7. Conclusion

The rapid emergence of multi-drug resistant strains may be explained by a simple possibility that the ability of the organism to survive the action of antibiotics appears to be linked to the ability of the organism to acquire foreign DNA, such as resistant genes (49). The understanding of the genetics as well as the biochemical basis of resistance have increased through the advent of molecular typing methods of these organisms and has given great insight into the epidemiology of these clusters. Now that vancomycin resistance is common, synthetically modified vancomycin-group antibiotics that are remarkably active against vancomycin-resistant Enterococci are under active development (17). Treatment of infections caused by organisms such as enterococci is compromised by intrinsic and acquired drug resistance. A combination of various β-lactams with vancomycin have shown synergistic activity against vancomycin-resistant Enterococci due to the potential differences in the PBPs used to cross-link the vancomycin-resistant peptidoglycan precursors.

An important determination of the ability of an antibiotic to kill vancomycin resistant organisms is that antibiotics have membrane anchors that bind tightly to the model surface and hence is a significant feature (29). Other potential targets for fighting this resistance are those enzymes involved in cell wall biosynthesis (54). By preventing the expression of the vancomycin-resistant enzymes, the relay of responding signals can be averted. The VanR-VanS system can be seen as the next step in chemotherapeutic intervention and using it as a target to overcome resistance (46).

There is a longer evolutionary process in which various regulatory and structural genes are assembled in a modular fashion. This may be due to the complexity of gene organization and differences in gene order and the degree of similarity (41). There are diverse measures that assist against antibiotic resistance, these are cross-resistance and co-resistance. Other measures that are employed are the design of new *in vitro* resistance detection techniques of new drugs, as well as the development of sensitive techniques for epidemiology (29). Vigilant detection of resistant bacteria provides an essential basis for infection control measures and antimicrobial inspection systems (98). However, true effectiveness of prevention and treatment of these infections can only be achieved with the comprehensive understanding of the mechanisms of vancomycin resistance (96).

1.8. Aim of the Study

The main objectives of this study were (i) to screen the *S. milleri* strains: P213; P35 and B200 and the Enterococcal strains: *E. faecalis* 123; *E. faecalis* 126 and *E. faecium* for the presence of vancomycin-resistance genes by means of conventional susceptibility tests; (ii) to confirm these results with more advanced DNA-based techniques; (iii) to compare the genotypes of these strains with their phenotypes and (iv) to study the transcription of the resistant genes at the different stages of peptidoglycan synthesis by examining the cell wall precursors under different antibiotic conditions, viz., vancomycin and bacitracin.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Bacterial Strains and Growth Conditions

The strains used in this study are listed in Table 2.1. Long term storage of cultures were stored at -70°C in tryptone soy broth (TSB) (Biolab) supplemented with 30% glycerol. Working cultures were subcultured fortnightly on tryptone soy agar (Biolab) plates containing 1.4% agar per litre and stored at 4°C. Overnight starter cultures were grown in 10 ml TSB at 37°C in 5% CO_2 atmosphere.

Table 2.1. Strains and their source used during this study

Strain	Strain name	Source
1	*Streptococcus milleri* B200	Pietermaritzburg Pathology Lab
2	*Streptococcus milleri* P213	Pietermaritzburg Pathology Lab
3	*Streptococcus milleri* P35	Pietermaritzburg Pathology Lab
4	*Enterococcus faecalis* 123	Pietermaritzburg Pathology Lab
5	*Enterococcus faecalis* 126	Pietermaritzburg Pathology Lab
6	*Enterococcus faecium*	Pietermaritzburg Pathology Lab
7	*Enterococcus faecium* BM4147*	Wits Medical School Pathology Lab
8	*Enterococcus faecalis* ATCC**	Wits Medical School Pathology Lab

* *Enterococcus faecium* BM4147 was used as a VanA control

** *Enterococcus faecalis* ATCC was used as a VanB control

2.2. Micro - Titre Plate Dilution Assay

Overnight starter cultures for each strain were grown in 10 ml TSB at 37°C in 5% CO_2 atmosphere. Using cation-adjusted Mueller-Hinton broth by micro-broth dilution method, vancomycin (Sigma) susceptibility was tested at different concentrations ranging from 0 µg/ml to 160 µg/ml. Each well contained various levels of vancomycin and a 1% inoculum of strain in a final volume of 200 µl. A positive control was used in which no antibiotic was added and was inoculated with 1% culture and the negative control had no antibiotic and was not inoculated. The 96 well micro-titre plate was incubated at 37°C in 5% CO_2 atmosphere for 18 hours. The MIC for each isolate was determined at the lowest concentration at which an isolate did not grow.

2.3. Disk - Diffusion Assay

MIC's were determined by disk-diffusion assay. A 1% inoculum was transferred to 10 ml sloppy agar, which is a nominally nutrient media with half the agar concentration of that used in pour plates or spread plates. The sloppy agar was overlayed on TSA plates. Sterile Whatman antibiotic assay disks, to which different concentrations of 0.5; 1; 2; 3; 4; 5 and 10 μg/ml of the antibiotic, vancomycin, had been added, were then applied to the surface of the agar. Plates were incubated for 24 hours at 37°C in 5% CO_2 atmosphere. The plates were then checked for growth and zones of inhibition around paper disks.

2.4. Lytic Curves

Overnight starter cultures of all strains listed in Table 2.1., were grown in 10ml TSB and incubated at 37°C in an atmosphere of 5% CO_2. The cells were harvested by centrifugation at 8000 x g for 5 minutes at 4°C. The bacterial pellets were washed approximately four times in 50 mM phosphate buffer, pH 7.5. Cells were pelleted and resuspended in either 1 ml of 50 mM phosphate buffer or in 1ml 10 μg/ml of vancomycin and incubated at 37°C. The optical density (OD) at 600 nm were measured at hourly intervals and lytic curves constructed. A bar graph of the regression slope was constructed by working out the regression curves from the lytic curves using the mathematical formula.

2.5. Genomic Isolation

Intact genomic DNA for all strains were isolated using the NucleoSpin® C+T (Machery-Nagel) Kit according to manufacturer's instructions. Using the support protocol for gram-positive bacteria, 1 ml samples were prepared by centrifuging for 10 minutes at 12 000 x g from an overnight starter culture. The supernatant was discarded and this step repeated three times. The pellet was then resuspended in 180 μl of buffer T1. Proteinase K (30 mg/ml) was then added and the samples were vortexed vigorously. Samples were incubated at 56°C for 3 hours until complete lysis was obtained. This step was followed by the addition of 200 μl buffer B3 to each sample, which were then vortexed and incubated at 70°C for 10 minutes. Absolute alcohol was added to each of the samples and vortexed. For each sample, one NucleoSpin® Tissue column was placed into a 2 ml collecting tube. The sample was applied to the column and centrifuged for 1 minute at 12 000 x g, the

flow-through was discarded and the column placed back into the collecting tube. The column was washed with 500 µl buffer BW and centrifuged for 1 minute at 12 000 x g. The flow-through was discarded. A second wash was performed by adding 600 µl buffer B5 and centrifuged for 1 minute at 12 000 x g and discarding the flow-through. The silica membrane was dried and residual ethanol removed by centrifuging the column for 2 minutes at 12 000 x g. DNA was eluted by placing the NucleoSpin® Tissue column into a 1.5 ml microcentrifuge tube and adding 100 µl prewarmed elution buffer BE (70°C) at room temperature for 1 minute. Tubes were centrifuged for 1 minute at 12 000 x g and DNA collected in the collecting tube. Samples were stored at -20°C until required.

2.5.1. Quantitation of DNA

The genomic DNA obtained was quantified to measure the quality of the DNA using the GeneQuant pro RNA/DNA calculator (Pharmacia). DNA samples were diluted a 100 times in BE buffer. Absorbance measurements were determined at A_{260} and A_{280}. This was used to calculate the A_{260}/A_{280} ratio of the DNA sample.

2.5.2. Detection and Analysis of Extracted DNA by Agarose Gel Electrophoresis

Extracted DNA was analysed via agarose gel electrophoresis on a 0.8% (w/v) agarose gel, prepared in 1 x TAE (4.846 g of Tris, 0.41 g of anhydrous sodium acetate, and 0.372 g EDTA, pH 7.8) buffer. The gel was stained with 2.5 µl of ethidium bromide stock solution (10 mg/ml) to give a final concentration of 0.5µg/ml of ethidium bromide. The DNA samples (10 µl) were added to 2 µl of loading dye and then loaded into separate wells. Electrophoresis was initially carried out at a 100 volts for 5 minutes and then adjusted to 80 volts for 90 minutes at maximum current. The presence of DNA was verified using a UV transilluminator.

2.6. Polymerase Chain Reaction (PCR)

2.6.1. PCR Primers

Oligonucleotide primers were synthesized according to Miele *et al.*, 1995 (Table 2.2.) and Dukta-Malen *et al.*, 1995 (Table 2.3.) at the University of Cape Town, Department of Biochemistry. The sequences and properties of the primers are listed in Table 2.2.

Table 2.2. Properties and nucleotide sequences of PCR primers for *vanA*, *vanB* and *vanC* associated genes according to Miele *et al.*, 1995

Primer pair	Nucleotide sequence 5' to 3'	T_a	Size of PCR product)(bp)
$VanA_1$	ATGAATAGAATAAAAGTTGCAATAC	62	1,029
$VanA_2$	CCCCTTTAACGCTAATACGAT	62	
$VanB_1$	CCCGAATTTCAAATGATTGAAAA	59	457
$VanB_2$	CGCCATCCTCCTGCAAAA	59	
$VanC_1$	GCTGAAATATGAAGTAATGACCA	58	811
$VanC_2$	CGGCATGGTGTTGATTTCGTT	58	

T_a = annealing temperature

Table 2.3. Properties and nucleotide sequences of PCR primers for *vanA*, *vanB* and *vanC* associated genes according to Dukta-Malen *et al.*, 1995

Primer pair	Nucleotide Sequence 5' to 3'	Size of PCR product (bp)
$VanA_1$	GGGAAAACGACAATTGC	732
$VanA_2$	GTACAATGCGGCCGTTA	
$VanB_1$	ATGGGAAGCCGATAGTC	635
$VanB_2$	GATTTCGTTCCTCGACC	
$VanC_1$	GGTATCAAGGAAACCTC	822
$VanC_2$	CTTCCGCCATCATAGCT	

2.6.2. DNA Amplification by PCR

Isolated genomic DNA of each strain was used as a template. *Taq* Polymerase (Roche) was used as the amplifying enzyme. The PCR reaction mixtures were constituted as outlined in Table 2.4.

Table 2.4. Reaction mixtures used for the PCR amplification of DNA target sequences of v*anA*, v*anB* and v*anC*

Reagent	Initial conc.	Required conc.	Volume (µl)
Template DNA		0.5 µg	2
Sterile distilled water			15.45
10 X PCR reaction buffer with $MgCl_2$	10 x	1 x	2.5
dNTP	10 mM	500 µM	1.25
Primer (Forward)	7.5 µM	0.5 µM	1.7
Primer (Reverse)	7.5 µM	0.5 µM	1.7
Taq	5 U/µl	2 U	0.4
Total			25

A negative control was also prepared together with the other reaction mixtures in which the template DNA was substituted with sterile distilled water. PCR amplifications were carried out in an automated thermal cycler (Perkin-Elmer Applied Biosystem GeneAmp PCR system 9700, Norwalk, USA). The PCR parameters for primers in Table 2 consisted of an initial denaturation at 94°C for 3 minutes; this was followed by 30 cycles of DNA denaturation at 94°C for 30 seconds; primer annealing at the appropriate temperature (Table 2.2) for each set of primers for 2 minutes; and DNA extension at 72°C for 2 minutes. After the last cycle, a final extension at 72°C for 6 minutes was performed and the reaction mixtures were stored at 4°C.

The PCR parameters for primers in Table 2.3. consisted of an initial denaturation at 94°C for 2 minutes; this was followed by 30 cycles of DNA denaturation at 94°C for 1 minute; primer annealing at 54°C for each set of primers for 1 minute; and DNA extension at 72°C for 1 minute. After the last cycle, a final extension at 72°C for 5 minutes was performed and the reaction mixtures were stored at 4°C.

The PCR products were analysed by electrophoresis in 1 x TAE buffer on a 1.5% (w/v) agarose gel. The gel was stained with 10 mg/ml stock solution of ethidium bromide to a

final concentration of 0.5 μg/ml. Molecular weight marker III (Roche) was used as a standard marker to confirm the approximate size of the amplified DNA.

2.7. Southern Blot/Hybridisation

2.7.1. Transfer of DNA

The VacuGene XL Protocol No. 1 (Pharmacia) was used to transfer DNA onto nitrocellulose membrane of 110 x 70 mm in size. The nitrocellulose membrane was pre-treated prior to transfer with sterile distilled water for 1 minute and 20 x SSC for 10 minutes. Genomic DNA of the bacterial strains was resolved by electrophoresis on a 0.8% agarose gel. The gel was subjected to depurination, denaturation and neutralization for 20 minutes each on a VacuGene XL apparatus. DNA from the gel was transferred onto the nitrocellulose membrane with transfer solution (20 x SSC) for 60 minutes. Following transfer the wells were clearly marked, and the gel removed. The nitrocellulose membrane was washed in 2 x SSC for 5 minutes to wash away debris. The filter was then air dried for 30 minutes, baked at 80°C for 2 hours and stored at room temperature until required.

2.7.2. Oligonucleotide Tailing With DIG-dUTP dATP

The Roche 3' end tailing kit was used to label oligonucleotide probes. The probe used was the primers of vanA_1 , vanB_1 and vanC_1 , respectively. The tailing reaction was constituted by adding the following solutions to a sterile microcentrifuge tube: (i) 4 μl of 5 x reaction buffer (1 M Potassium cacodylate, 0.125 M Tris-Cl, 125 mg/ml BSA pH 6.6); (ii) 4 μl of 25mM $CoCl_2$; (iii) 1 μl DIG-dUTP solution; (iv) 1 μl (50 U) terminal transferase; (v) 0.2 μl probe (100 pmoles) and 9.8 μl sterile distilled water. The solutions were mixed and incubated at 37°C for 30 minutes, thereafter placed on ice. A 100 μl Hybridization solution (6 x SSC, 0.5% SDS and 5 x Denhardt's solution) was added to the tailing reaction and stored at -20°C for use in hybridization experiments.

2.7.3. Pre-Hybridization and Hybridization

The blotted membrane was placed in a hybridization tube containing 10 ml of pre-hybridization solution and pre-hybridized for 3 hours at 37°C. The probe was then added to

hybridization solution and mixed. The pre-hybridization solution was decanted and the probe/hybridization solution was added to the tube. The membrane was hybridized overnight at 37°C. After hybridization the membrane was washed twice in 2 x SSC, 0.1% SDS for 15 minutes each and then twice in 0.1 x SSC, 0.1% SDS, for 15 minutes each. The membrane was allowed to air dry and stored for the detection step.

2.7.4. DIG Nucleic Acid Detection

Detection was performed using the DIG-detection Kit (Roche) according to manufacturer's instruction. The membrane was washed in buffer 1 (0.1 M Tris-Cl, pH 7.5; 0.15 M NaCl) for 1 minute. The membrane was blocked using freshly prepared Buffer 2 (1% skim milk powder in 100 ml Buffer 1) at room temperature for 30 minutes with continuous shaking. After blocking, the membrane was washed briefly in Buffer 1. Anti-digoxigenin-AP conjugate was diluted into Buffer 2 to a final concentration of 150 mU/ml and the membrane was incubated in 20 ml of this solution for 30 minutes. The membrane was thereafter washed in Buffer 1 twice for 15 minutes, to remove any unbound antibody conjugate. This was then equilibrated in 20 ml of Buffer 3 (0.1 M Tris-Cl, pH 9.5; 0.1 M NaCl; 50 mM $MgCl_2$) for 2 minutes. Freshly prepared colour substrate solution (200 µl NBT/BCIP solution in 10 ml Buffer 3) was made and the membrane incubated in colour substrate solution in the dark. A colour precipitate develops and when desired bands were detected, the membrane was washed in 1 x TE, pH 8.0, to stop the reaction.

2.8. Analysis of Peptidoglycan Precursors

Extraction and analysis of peptidoglycan precursors was performed as described by Baptista *et al.*, 1997. A 1% inoculation of the respective bacterial strain in TSB supplemented with 0.5% yeast extract was grown to the mid exponential phase (OD_{600} = 1), 20 µg/ml vancomycin of 2 x the MIC for each of the uncharacterized strains were used to accumulate peptidoglycan precursors at the transglycosylation and transpeptidation stage of biosynthesis. Bacitracin (100 µg/ml) was also used to arrest peptidoglycan synthesis at the transpeptidation stage. Incubation was continued for 30 minutes and bacterial cells were harvested by centrifugation at 11 000 x g for 10 minutes at 4°C, and treated with 7% trichloroacetic acid (TCA) for 15 minutes at 0°C in a final volume of 2 ml.

The extract was centrifuged at 46 000 x g for 10 minutes at 4°C, and the supernatant fraction containing the cytoplasmic peptidoglycan was collected.

TCA was neutralized by the addition of 0.11 g solid sodium bicarbonate, and salt was removed from the extract by gel filtration on a Sephadex G10 (Sigma) column (20 x 1.5 cm). The elute was monitored at 252 nm and cell wall precursors eluted immediately after the void volume of the column. Fractions (100 µl) containing the cell wall precursors were analyzed by high-performance liquid chromatography (HPLC) and applied to a C-18 reverse-phase column of 250mm by 4.6 mm (ODS-Hypersil, 5 µM; Thermo Hypersil-Keystone). 0.05 M ammonium acetate (pH 5.03) was used as the mobile phase at a flow rate of 0.2 ml/min with the application of a methanol gradient (0 - 25%) in the same buffer from 5 to 45 minutes. The elution times for the precursors were approximately: UDP-MurNAc-tripeptide, 7.7 minutes; UDP-MurNAc-tetrapeptide, 16 minutes; UDP-MurNAc-pentapeptide, 25 minutes and UDP-MurNAc-pentadepsipeptide, 42 minutes. The relative proportions of these four precursors were determined from the integrated peak areas, and the results were expressed as percentages.

2.9. Mass Spectrometry

Analysis of peptidoglycan precursor peaks collected during HPLC from the various precursors of various strains were analysed by mass spectrometry on an API III quadruple mass spectrometer equipped with an IonSpray source (Sciex, Thornhill, Canada), at the University of Stellenbosch (South Africa).

2.10. DNA Sequencing

Automated DNA sequencing utilizes fluorescent tracers instead of radioisotopes to detect the DNA, thereby eliminating or significantly reducing the use of radioactive materials in some research laboratories.

2.10.1. Big Dye Terminator v3.0 Ready Reaction Cycle Sequencing

The first step of the DNA sequencing procedure involved a PCR Cycle using the GeneAmp PCR System 2700. The following reaction components used for PCR were added to a sterile PCR tube as follows: (i) 400 ng of DNA; (ii) 5 x Big Dye terminator v3.1 buffer (3 µl); (iii)

7.5μM primer; (iv) Big Dye Terminator v3.1 cycle sequencing ready reaction mix-100 (2 μl) and sterile distilled water (12.01 μl) to give a total volume of 20 μl reaction. The PCR profile used was as follows: 96°C for 10 seconds, 55°C for 30 seconds, 60°C for 4 seconds and for 35 cycles. The reaction tube was immediately removed and samples used for purification of extension product.

2.10.2. Purification of Extension Products

For each sequencing reaction a sterile 1.5 ml microcentrifuge tube with the DNA extraction number, PCR primers used, and date of sequencing reaction is labelled. To each tube 50 μl of 100% ethanol was added, 2 μl of 125 mM EDTA (Ethylene diamine trifluro acetate) and 2 μl of 3 M NaAc (sodium acetate, pH 4.6-5.2). The entire 20 μl of the DNA sequencing reaction was added to the tube and vortexed gently for a few seconds. The tubes were placed at 4°C for 20 minutes to precipitate sequencing reaction extension products. The tubes were placed in a microcentrifuge and spun for 20 minutes at maximum speed. The supernatant was carefully removed, and the pellet washed by resuspending the pellet with 120 μl of 100% ethanol by pipetting up and down. This was vortexed briefly. Samples were centrifuged for 15 minutes at maximum speed and all traces of supernatant removed. The pellet was washed using 100% ethanol. Samples were dried in a laminar flow hood for 20 minutes.

2.10.3. Sequencing of Extension Products

The resulting sequencing reaction was loaded onto a 96 well optical reaction plate and placed into a Genetic Analyzer 3100 (Applied Biosystems). The reaction was run for 4 hours and the raw data was analyzed using Bio Edit.

CHAPTER THREE

RESULTS AND DISCUSSION

The increasing amount of the reported incidences of outbreaks of vancomycin resistance has highlighted that there is a call for laboratories to efficiently and accurately detect the various types of resistance (27, 38). The first line of defence against the spread of vancomycin resistant organisms, is the laboratory (27). Simple and accurate phenotypic screening methods need to be in place together with confirmatory tests to identify resistance. These tests will entail the elucidation of the molecular and biochemical mechanisms of resistance (96). However, the detection of some vancomycin-resistant organisms in the laboratory still prove to be difficult since those with lower levels of resistance are less easily detectable compared to that of high level resistance which are more readily detected (106).

3.1. Micro – Titre Plate Dilution Assay

In vitro susceptibility tests are performed on those micro-organisms that are suspected to be responsible for diseases and that may have resistance to antimicrobial agents (40). Standard susceptibility testing protocols are able to detect resistance from moderate to high concentrations of vancomycin (50). The traditional method of determining the MIC of strains is agar or broth dilution. The National Committee for Clinical Laboratory Standards (NCCLS) has recognized broth micro-dilution in cation-adjusted Mueller-Hinton broth and some studies have used it as the reference method instead of agar dilution (27, 40, 106). Mueller-Hinton is the medium of choice since it allows for good growth and is a low antagonist (40).

Broth dilutions monitor the micro-organisms capability to produce visible growth on a series containing dilutions of the antibiotic. The MIC of the micro-organism to an antibiotic is the lowest concentration of the antibiotic which prevents the appearance of visible growth within a specified time (40).

The typical vancomycin MICs for various species of Enterococci established by the NCCLS are > 128 μg/ml for isolates that typically contain the *vanA* gene and are therefore classified as the VanA phenotype. The VanB phenotype would have an MIC of 16 – 64 μg/ml of vancomycin whilst the VanC phenotype would typically produce vancomycin MICs of 2 – 16 μg/ml (27).

As determined by micro-titre broth dilution and in accordance to the NCCLS, the following MICs to vancomycin for the strains tested are represented by the bar chart in Figure 3.1. All the strains, *S. milleri* P213, *S. milleri* P35, *S. milleri* B200, *E. faecalis* 123 and 126, *E. faecium,* and the VanA control appear to have MICs above 64 μg/ml and hence are considered to be of the VanA phenotype. The VanB control was correctly identified as a VanB phenotype since it had an MIC of 61 μg/m.

The high MIC value obtained for all strains, including that of the VanB control could be because of the amount of the peptidoglycan precursor, D-Ala-D-Lac present. Hence these bacterial strains are resistant to vancomycin at such a high value because of the high proportion of these depsipeptides.

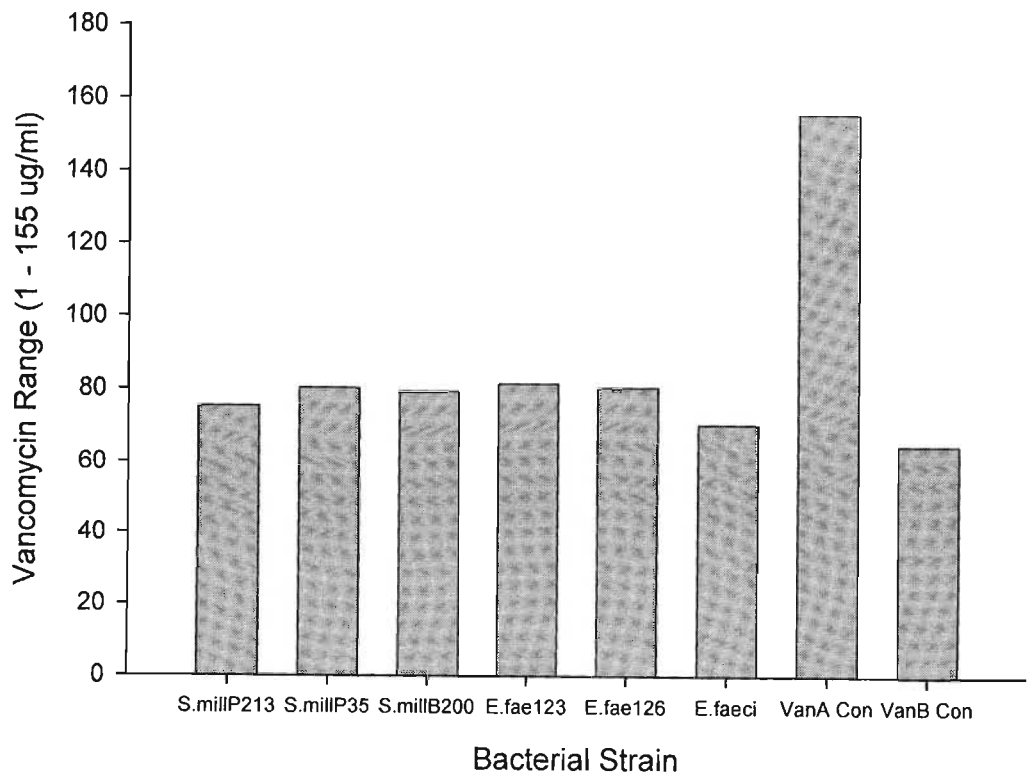

Figure 3.1. Bar chart showing the MIC's of each strain via micro-tire plate dilution assay.

3.2. Disk - Diffusion Assay

The vancomycin resistance level was confirmed by disk-diffusion assay since it has been effective in detecting intrinsically resistant gram-positive organisms, especially those that are highly resistant (106). Agar screening for the detection of the resistance phenotypes by means of disk-diffusion and agar dilution seem to be the most dependable and straightforward method (27). The accuracy of this method is improved by incubating the plate for 24 hours and reading the plate using strong transmitted light (50).

The MIC was the lowest concentration of antibiotic which did not allow for growth (75). Upon examination of plates under transmitted light, circular zones of inhibition around the disk could be seen. The antibiotic was responsible for the appearance of this zone and the diameter of each zone was measured. The zones of all the strains varied between 8 – 14 mm in size. In 1991, the NCCLS introduced its zone diameter and MIC testing interpretive approved standards for susceptibility testing. The interpretive standards for susceptible and resistant Enterococcal strains are zone diameters of ≥ 17 mm and that of ≤ 14 mm respectively (106). Hence, these strains were found to be resistant to vancomycin.

The MIC's of each strain by the disk-diffusion method are found in Table 3.1. This table also indicates their phenotype according to their corresponding MIC value and NCCLS susceptibility testing standards. This phenotypic susceptibility test identified strains 1- 6 as VanC phenotypes whereas in the previous test by micro-dilution these strains were characterized as VanA phenotypes. However, even though the MIC of the VanA control was lower than that of micro-dilution, it is still correctly identified as a VanA phenotype since its value is equal to 64μg/ml, which is characteristic of the VanA class resistance. The VanB phenotype also showed a lower MIC value compared to that in broth micro-dilution, however this value still lies between 16 – 64 μg/ml and is still therefore correctly classified as a VanB phenotype. Since the control strains were accurately identified, the MIC values of the other bacterial strains could be recognized as that of the proposed VanC phenotype. It is thought that the balance between normal and abnormal peptidoglycan synthesis is responsible for the level of resistance expressed. Hence this low MIC obtained for the strains can be explained by the amount D-Ala-D-Ser present (27).

The reason for the lower MIC values obtained during the disk-diffusion susceptibility test could be accounted for the fact that these strains are allowed to grow for 24 hours on agar, whereas, in broth micro-dilution they are grown in cation adjusted Mueller-Hinton broth. Mueller-Hinton broth facilitates the fastidious growth of these organisms and hence the rate of growth is much higher in 18 hours than that of agar, even though it was incubated for 24 hours. Agar plates require sufficient incubation time for the expression of resistant determinants in order for a substantial amount of growth to be visible (96). Another reason is that even though a 1% inoculum was used for both tests the final volumes in which they were incubated were different. For the micro-titre plate the MICs were assessed in a smaller volume compared to an agar plate.

The discrepancy in MIC obtained from the various susceptibility tests could also be attributed to the heterogenous population of cells in a bacterial strain. This factor accounts for the difference and irreproducibility of these quantitative methods of detection (96). There are other methods that aid in the accurate detection of these genes, a valuable technique used is that of PCR (38).

Table 3.1. The MIC values of vancomycin against each strain obtained via disk-diffusion

No	Strain	MIC (μg/ml)	Phenotype
1	*Streptococcus milleri* B200	5- 10	VanC
2	*Streptococcus milleri* P213	5 – 10	VanC
3	*Streptococcus milleri* P35	5 – 10	VanC
4	*Enterococcus faecalis* 123	5 – 10	VanC
5	*Enterococcus faecalis* 126	5 – 10	VanC
6	*Enterococcus faecium*	5 – 10	VanC
7	*Enterococcus faecium* BM4147	> 64	VanA
8	*Enterococcus faecalis* ATCC	32	VanB

3.3. Lytic Curves

This method was used to determine the sensitivity of the strains to vancomycin, which is a cell wall synthesis inhibitor. Vancomycin is also thought to be a secondary autolytic-inducing antimicrobial agent in clinical settings (89). Autolytic enzymes are responsible for the bactericidal activity of those antibiotics that interrupt cell wall synthesis (67). The antibiotic then triggers these enzymes to digest the cell wall exoskeleton. Autolysis of all strains was also observed in phosphate buffer, pH 7.5. This was used as the untreated growth control since it is a nutrient free substrate. Data was represented as a percentage of the absorbance at 600nm relative to that at time zero for each sample. The rate of autolysis was measured by the decrease in the percentage optical density at 600nm. A decrease in the percentage OD_{600}, shows an increase in autolytic activity.

Autolytic-induced lysis of *S. milleri* P213 is depicted in Figure 3.2. The rate of autolysis for this strain appeared to be much higher in phosphate buffer than under induced conditions with vancomycin. There was a much more rapid decrease in the percentage OD under uninduced conditions. This strain seemed to avoid the lysis-inducing effect of vancomycin at an early stage. Hence vancomycin in this case had low autolytic capabilities. Therefore there must be a resistant mechanism present, which allows for the growth of the strain in the presence of the antibiotic. This organism may be avoiding cell death by modifying the drug's peptide target to a depsipeptide such as D-Ala-D-Lac instead of the normal D-Ala-D-Ala and allows peptidoglycan polymerisation to occur in the presence of the antibiotic (64, 65). The presence of vancomycin is required by some bacterial cells that are resistant in order to grow and depend on the alternate D-Ala-D-Lac pathway since these cells do not have the normal D-Ala-D-Ala pathways. However, the D-Ala-D-Lac pathways are only activated in the presence of this antibiotic (14). The rate of autolysis was also much higher in phosphate buffer than in vancomycin for *E. faecalis* 123 shown in Figure 3.6. In Figure 3.7., *E. faecalis* 126 exhibited an increase in autolysis in phosphate buffer, though the rate was very similar under induced conditions. These patterns indicate that lysis induced by vancomycin does not appear to have a negative effect on cell growth or lysis hence the normal autolysis-signalling pathway remains unperturbed.

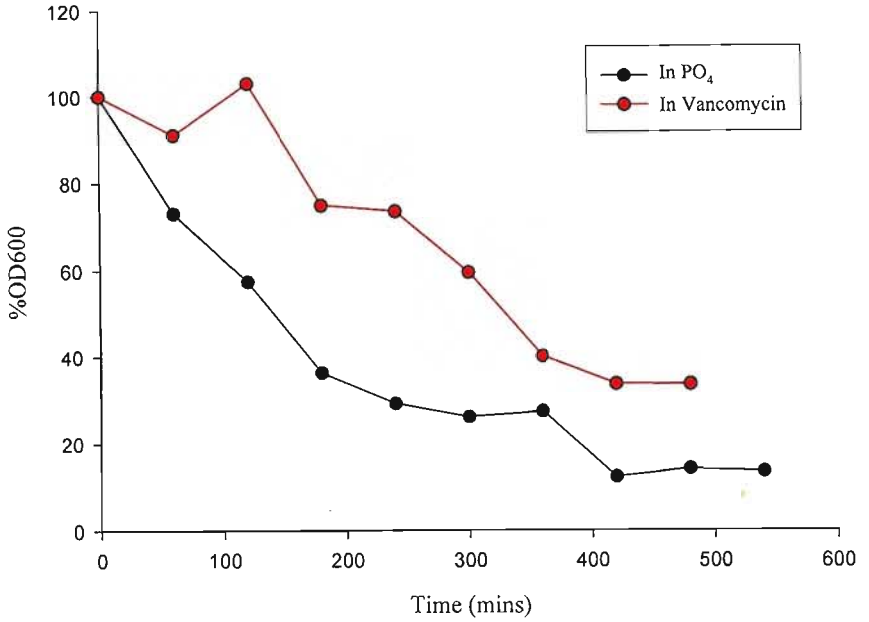

Figure 3.2. Vancomycin induced autolysis assay of *S. milleri* P213. Strain in phosphate buffer served as a control of autolysis.

Figure 3.3. shows autolytic-induced lysis of *S. milleri* B200. Initially a rapid decrease in OD was seen for cells under uninduced lysis, however, a higher rate of autolysis was observed for *S. milleri* B200 under induced conditions in the presence of vancomycin. This indicates that eventually vancomycin does induce lysis of these cells. Therefore, after a while cells are much more susceptible to the action of this antibiotic even though initially it displayed some sort of resistance to it. Other strains such as *E. faecium*, Figure 3.5.; the VanA control, Figure 3.8.; and the VanB control, Figure 3.9., showed similar kinetics of autolysis. None of these strains seem to afford any protection to the vancomycin challenge.

However, all strains, if they are resistant, as confirmed by the MICs obtained from disk-diffusion should show patterns of autolysis whereby autolysis occur at a higher rate in vancomycin than under normal conditions.

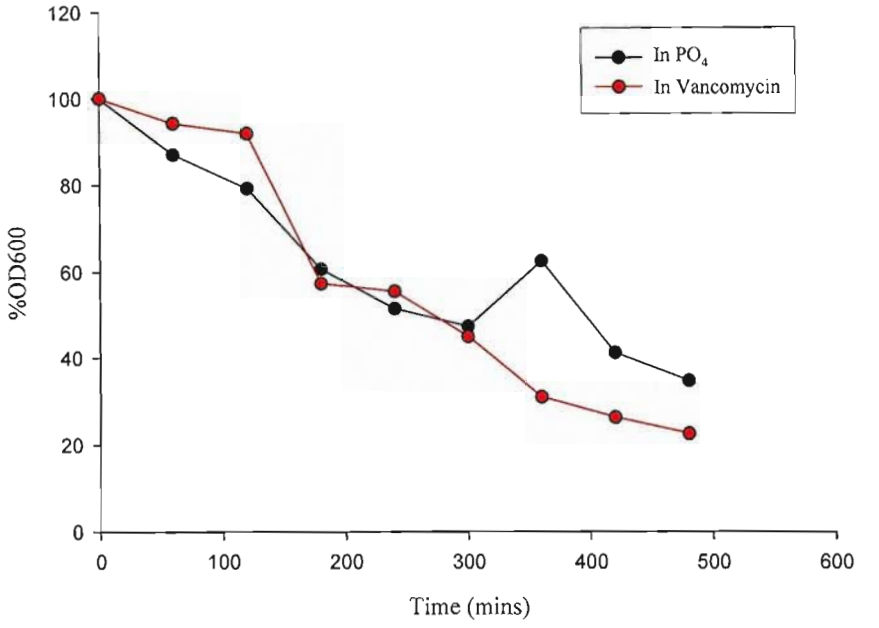

Figure 3.3. Vancomycin induced autolysis assay of *S. milleri* B200. Strain in phosphate buffer served as a control of autolysis.

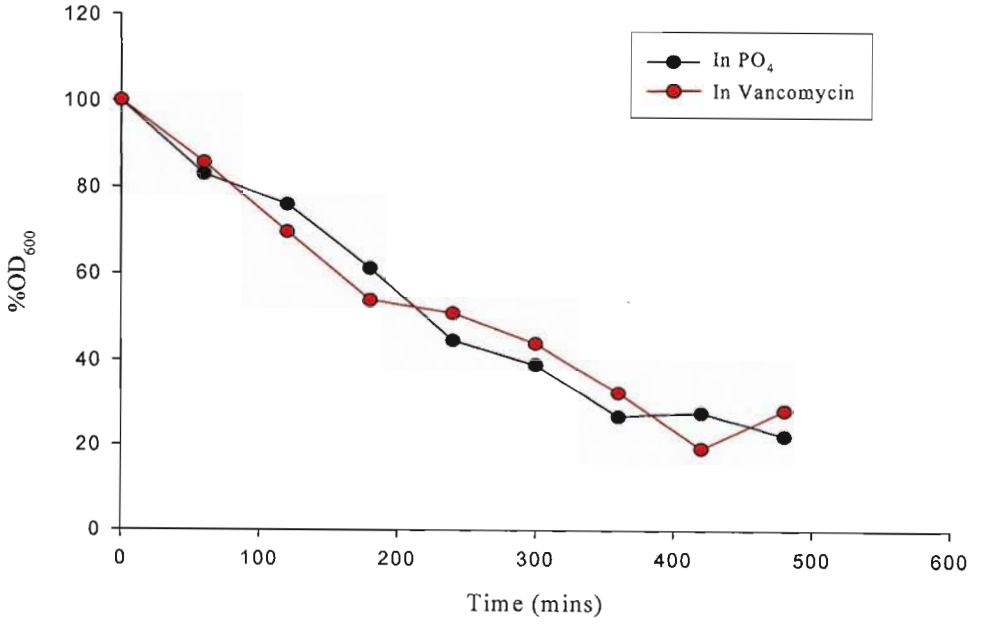

Figure 3.4. Vancomycin induced autolysis assay of *S. milleri* P35. Strain in phosphate buffer served as a control of autolysis.

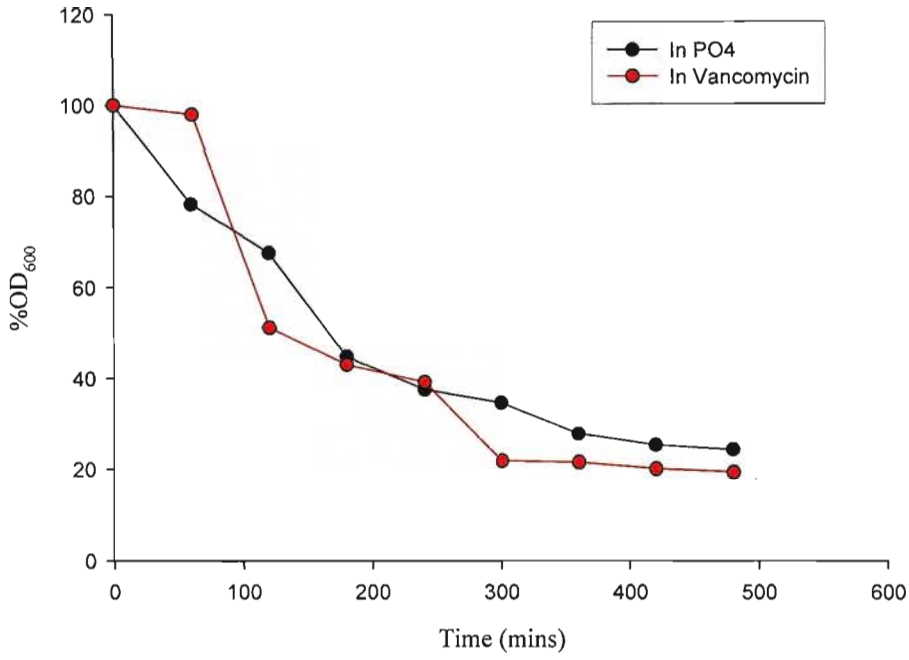

Figure 3.5. Vancomycin induced autolysis assay of *E. faecium*. Strain in phosphate buffer served as a control of autolysis.

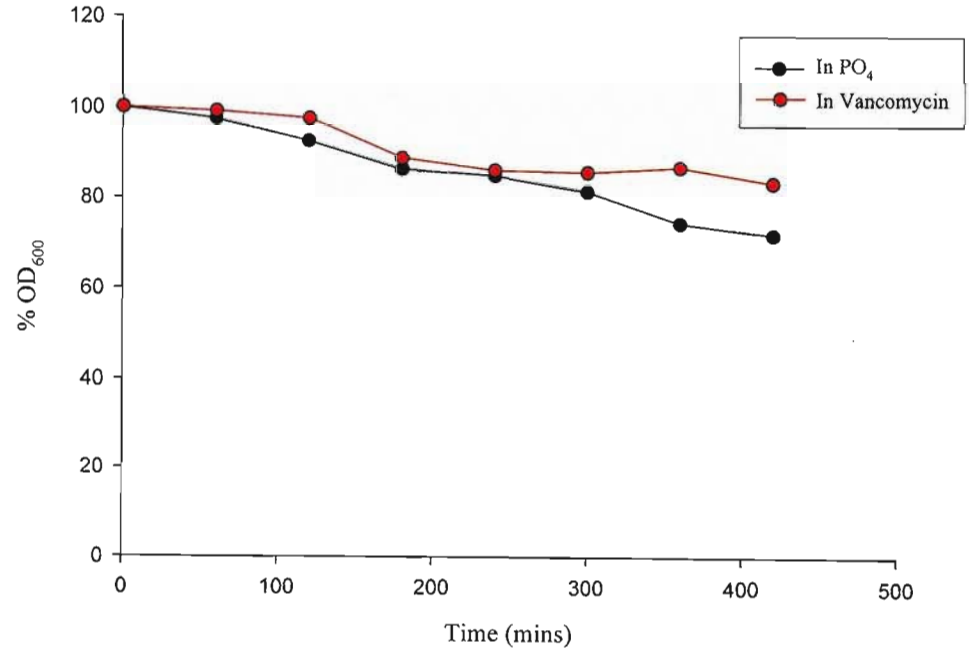

Figure 3.6. Vancomycin induced autolysis assay of *E. faecalis* 123. Strain in phosphate buffer served as a control of autolysis.

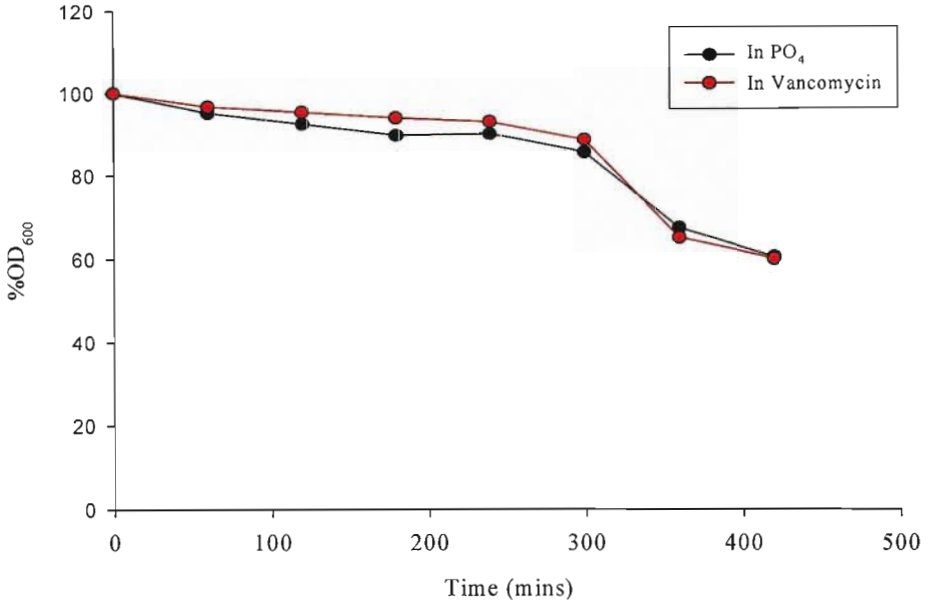

Figure 3.7. Vancomycin induced autolysis assay of *E. faecalis* 126. Strain in phosphate buffer served as a control of autolysis.

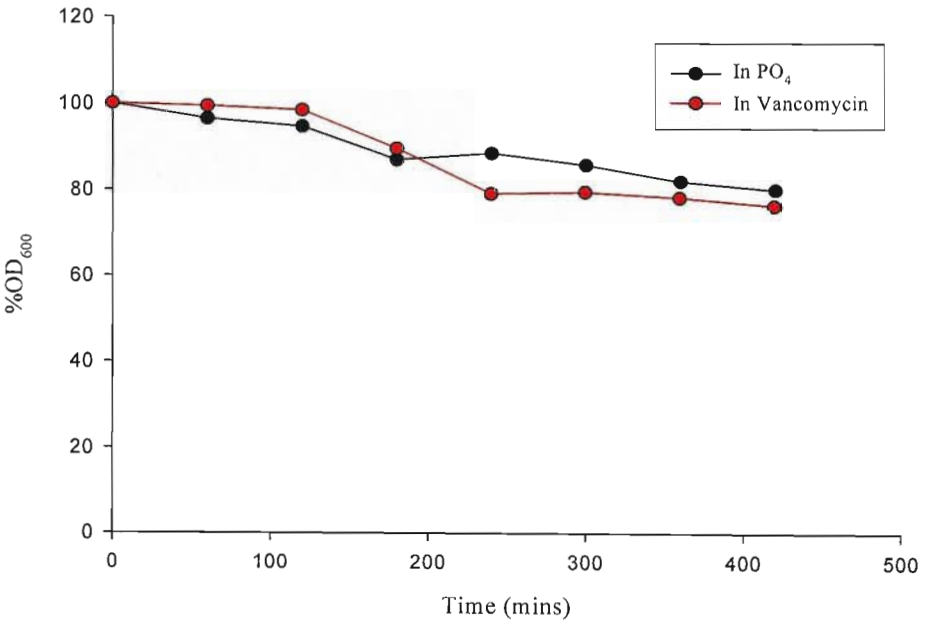

Figure 3.8. Vancomycin induced autolysis assay of VanA Control. Strain in phosphate buffer served as a control of autolysis.

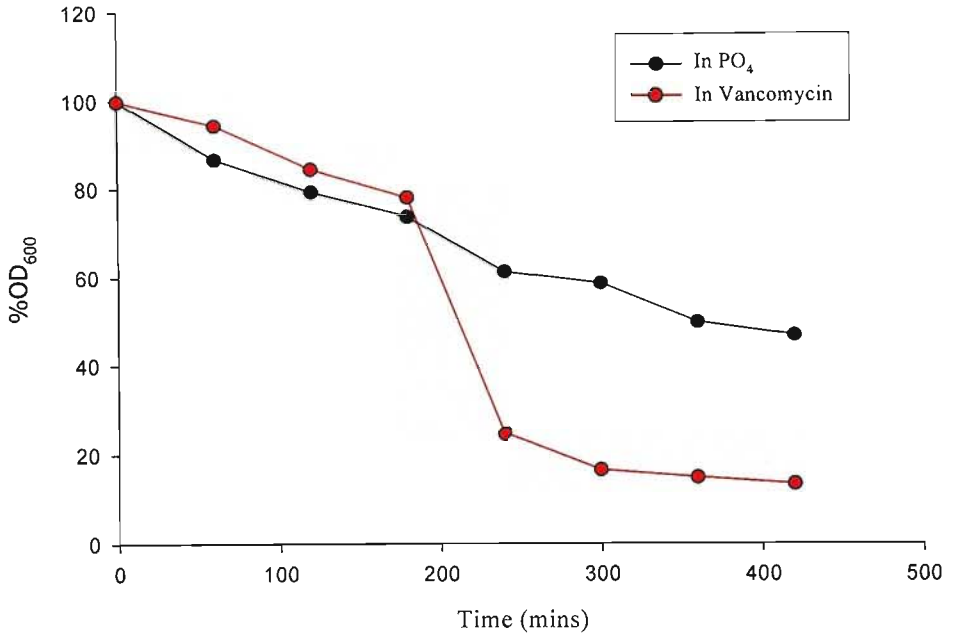

Figure 3.9. Vancomycin induced autolysis assay of VanB Control. Strain in phosphate buffer served as a control of autolysis.

A regression curve (Figure 3.10.) was then obtained for all strains to compare the autolysis under normal and induced conditions. An increased regression slope is indicative of a higher rate of autolytic activity. Only *S. milleri* P213 and *E. faecalis* 123 seemed to have higher autolysis under normal conditions than under vancomycin-induced conditions. *S. milleri* P35 and *E. faecalis* 126 showed the exact same rate of autolysis, under both normal and induced conditions. This implies that vancomycin did not have any effect on these cells because it did not affect the normal autolytic pathway. However, vancomycin did induce lysis in *S. milleri* B200, *E. faecium*, the VanA and VanB control, respectively.

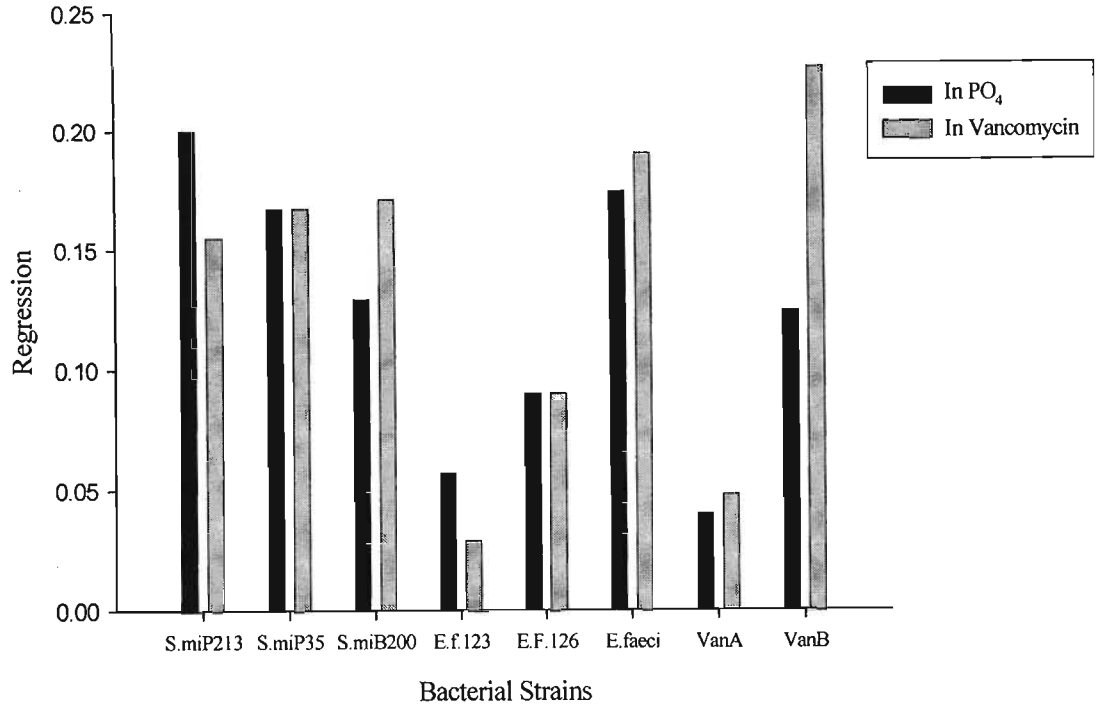

Figure 3.10. Regression slopes indicating the rate of autolysis for all strains under normal conditions in phosphate buffer and under autolytic-induced conditions in 10 μg/ml vancomycin.

3.4. Genomic DNA Isolation

DNA isolated for each bacterial strain using the NucleoSpin® Kit was run on a 0.8 % (w/v) agarose gel to verify that chromosomal DNA, was, in fact present, and to ensure that there was no RNA contamination. Figure 3.11. shows that DNA was successfully isolated and that no RNA contamination occurred during the procedure. The bright bands observed in this figure indicate that the concentration of the DNA obtained was high. This quality of the DNA obtained was confirmed by the absorbance readings in which the A_{260}/A_{280} ratio was between 1.8 and 2, which indicated that the DNA obtained were of pure content. The DNA isolated was then used as a template in the subsequent PCR reactions.

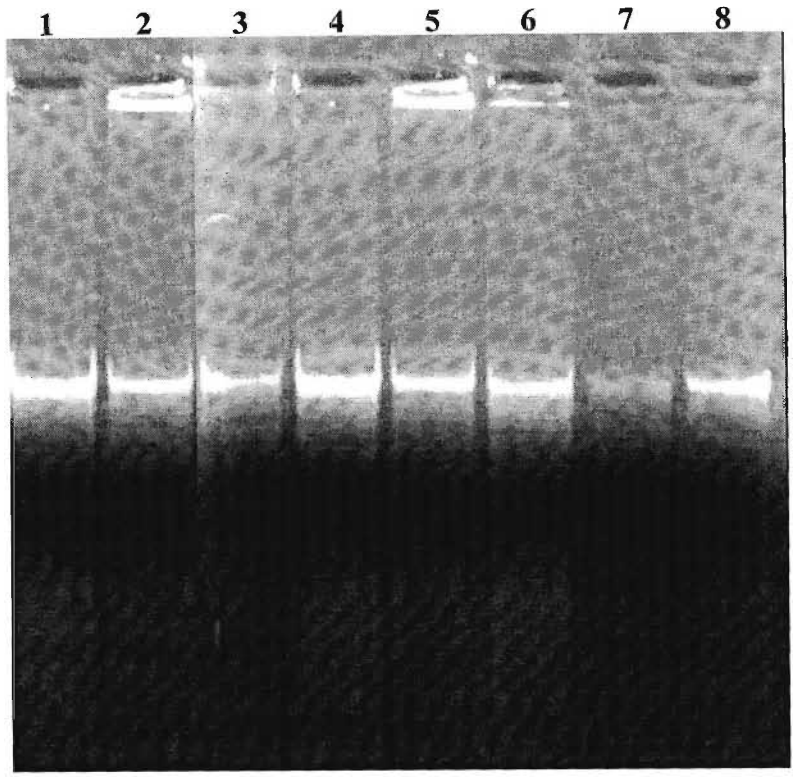

Figure 3.11. An agarose gel of 0.8% (w/v) showing DNA isolated. Lane 1: *S. milleri* P213; Lane 2: *S. milleri* P35; Lane 3: *S. milleri* B200; Lane 4: *E. faecalis* 123; Lane 5: *E. faecalis* 126; Lane 6: *E. faecium*; Lane 7: VanA Control; Lane 8: VanB control.

3.5. Polymerase Chain Reaction (PCR)

Because of the rapidly increasing progress of technology, techniques such as PCR have been used in various applications, including in the medical field for diagnosis of certain infectious agents. This molecular technique can now be used in the detection of vancomycin-resistance genes, either that, of acquired or intrinsic. It is a more rapid and sensitive technique compared to that of phenotypic identification by traditional susceptibility testing or DNA-DNA hybridization. It is also more readily available and is especially used more so to detect low-levels of vancomycin resistance (38, 45, 106). PCR is a much more reliable method since it can be applied directly to clinical samples and can also be used on purified DNA (70).

In this study, this advanced molecular biology tool was used parallel to phenotypic susceptibility tests.

3.5.1. Using Primers From Miele *et al.*, 1995

These primer sets were initially used to detect the vancomycin resistant genes for the different phenotypes. To establish the specificities of these primers that would reliably correlate the genotypes of vancomycin resistance with their respective phenotypes, a BLAST search was performed on the oligonucleotide sequences. These sequences were homologous to published nucleotides sequences.

In Figure 3.12., PCR products were obtained using the VanA primer pair. These results show that there is amplification in lanes 1 - 5. This primer pair targeting the *vanA* gene cluster yielded products of approximately the appropriate size of 1029 bp, hence these strains seem to have the *vanA* genes and are of this phenotype. This correlates with the MIC values obtained by micro-titre plate dilution. There also seems to be multiple amplicons between 1375 and ≤ 564 bp. Hence, this PCR reaction needed to be optimised. Negative controls, in lanes 6 and 7, without *Taq* polymerase and without DNA template, respectively, were also included in the PCR reaction to check for false positive results. No bands were seen in these lanes, therefore no amplification occurred. This implies that the amplification obtained in lanes 1 - 5 were not false positives. This reaction was then repeated and again multiple amplification occurred.

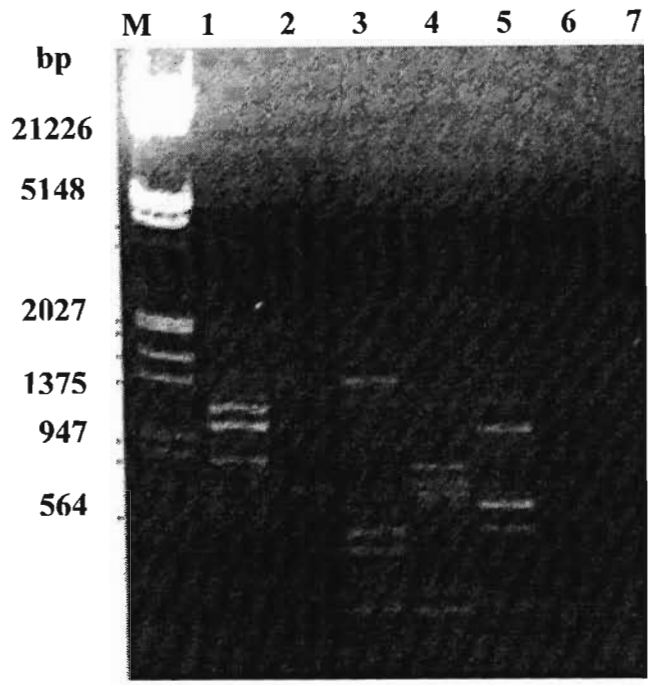

Figure 3.12. Image of PCR Products amplified with Primer VanA. Lane M: Molecular Weight Marker III; Lane 1: *S. milleri* P213; Lane2: *S. milleri* B200; Lane3: *S. milleri* P35; Lane 4: *E. faecalis* 123; Lane 5: *E. faecalis* 126; Lane 6: Negative Control (No *Taq*); Lane 7: Negative Control (No DNA).

PCR optimisation entails increasing the stringency of the reaction. This could be done either by varying the magnesium chloride concentration or the annealing temperature. In Figure 3.13. optimisation was done by using a magnesium concentration of 1.5 mM, whilst all other reagents and variables were kept constant. In this reaction, VanA and VanB controls were also included since these strains are PCR analysed strains. These were also used to assess the ability of these primers to identify vancomycin resistance in the strains used in this study. These positive controls are critical in a PCR reaction in order to check for false negative results (98).

In this figure, multiple amplification was again observed for every strain. The PCR products obtained were estimated to be between 900 – 400 bp. The expected size,

however, was supposed to be that of 1029bp. Lane 7 was the VanA control. Even though amplification occurred for this control, it was also of the incorrect size.

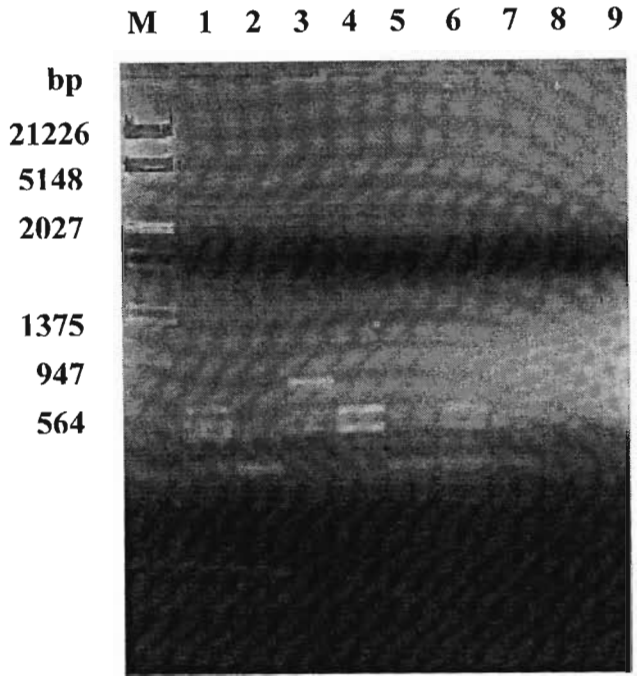

Figure 3.13. PCR products obtained with Primer VanA at an [$MgCl_2$] of 1.5 mM. Lane: M, Molecular Weight Marker III; Lane 1: *S. milleri* P213; Lane 2: *S. milleri* B200; Lane 3: *S. milleri* P35; Lane 4: *E. faecalis* 123; Lane 5: *E. faecalis* 126; Lane 6: *E.faecium*; Lane 7: VanA Control; Lane 8: VanB Control; Lane 9: Negative Control (No DNA).

The DNA template of each strain was used for amplification with the VanB primer. Figure 3.14. shows clearly amplification in each lane. Amplification obtained was of the correct size since the expected product at 457 bp was seen. However, other bands can also be seen. This suggests that the reaction was not entirely specific and the magnesium chloride concentration and annealing temperature needed to be adjusted to more stringent conditions. No bands can be seen for the negative controls, which indicates no random DNA was amplified and none of the buffers contained any contamination. Even though products of the correct sizes were observed, this did not correlate with the MIC values obtained in both, micro-titre plate dilution or disk-diffusion.

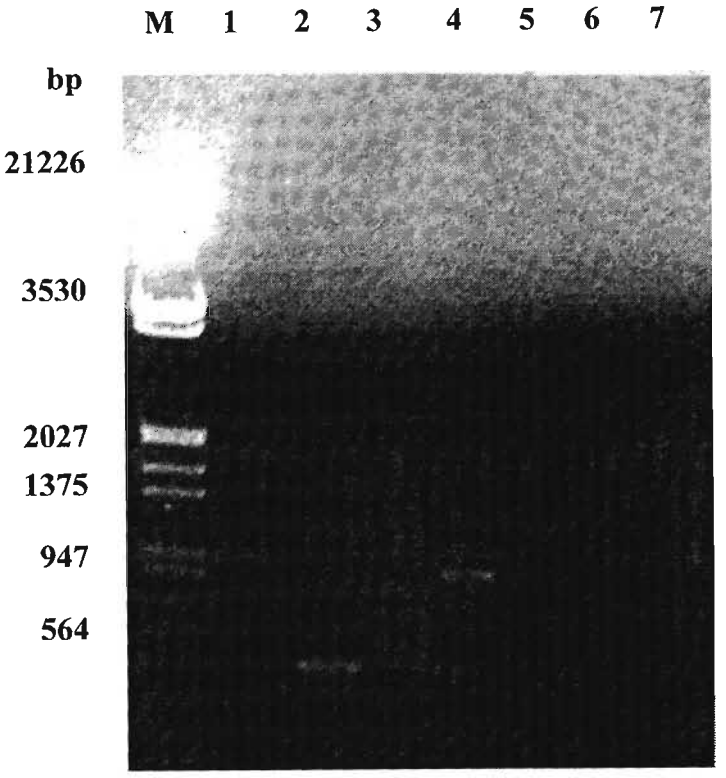

Figure 3.14. Gel imaging showing isolates amplified with Primer VanB. Lane: M, Molecular Weight Marker III; Lane 1: *S. milleri* P213; Lane 2: *S. milleri* B200; Lane 3: *S. milleri* P35; Lane 4: *E. faecalis* 123; Lane 5: *E. faecalis* 126; Lane 6: Negative Control (No Taq); Lane 7: Negative Control (No DNA).

Another PCR reaction was also performed in which the DNA of each strain was amplified with the VanC primers. Amplification was only observed in lanes 1 and 2, for *S. milleri* P213 and B200, respectively. The expected size of the PCR product was 811 bp. In Figure 3.15., one of the multiple bands in lane 2 corresponded to that size. *S. milleri* B200 could perhaps be of the VanC phenotype, however, this strain also had amplification with the other two primer sets. The results obtained from this PCR reaction, and those performed under stringent, or optimised conditions appeared to have varying results. Even though controls were added to the PCR reactions, the phenotype of these strains still remained unclear.

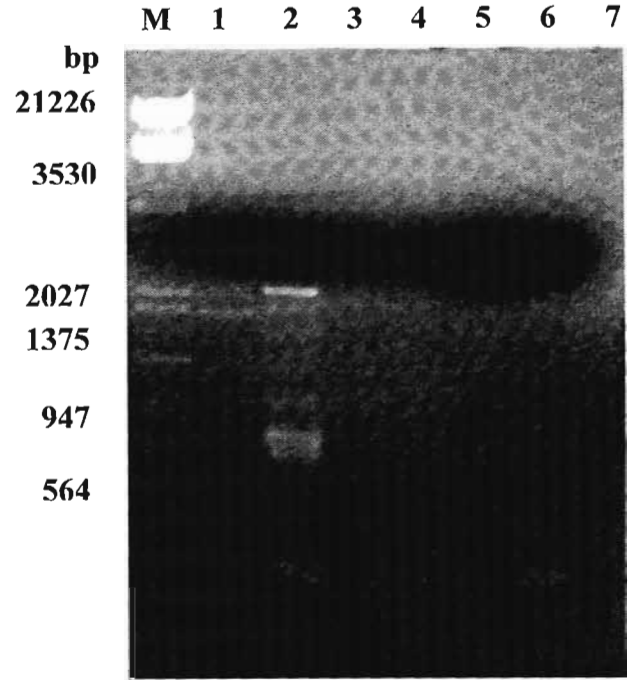

Figure 3.15. Image of DNA amplified with Primer VanC. Lane: M, Molecular Weight Marker III; Lane 1: *S. milleri* P213; Lane 2: *S. milleri* B200; Lane 3 *S. milleri* P35; Lane 4: *E. faecalis* 123; Lane5: *E. faecalis* 126; Lane 6: Negative Control (No Taq); Lane 7: Negative Control (No DNA).

Since no definite conclusions could be made as to which resistant phenotype each bacterial strain represents, sequencing was performed on those amplicons. The amplified products obtained via PCR of *S. milleri* B200 and P213, amplified with Primer VanA respectively, were then used for DNA sequencing.

3.6. Sequencing of PCR Products Generated By Amplification With Miele *et al.*, 1995 Primers

The specificity of the amplicons obtained from PCR was confirmed by DNA sequencing. The sequencing results obtained are shown in Figures 3.16. and 3.17. for *S. milleri* B200 and Figures 3.18. – 3.20. for *S. milleri* P213. The sequences obtained in these figures were subjected to a BLAST search. *S. milleri* B200 was found to be homologous to a

pGEM vector system, whilst *S. milleri* P213 showed homology to a Mus musculus BAC clone. These results were found to be totally unrelated to any of the vancomycin resistance genes tested, since these do not contain vancomycin resistance genes.

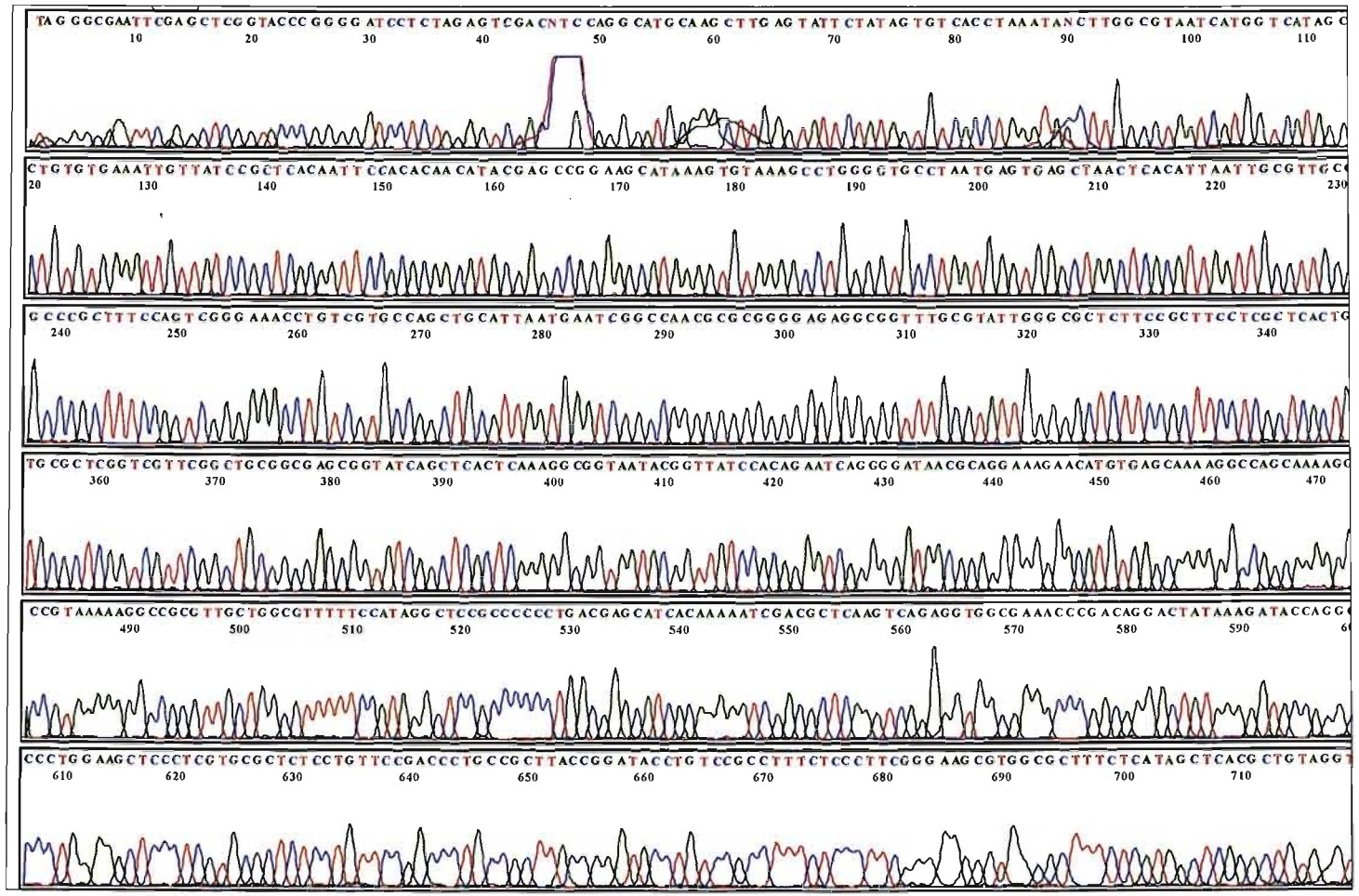

Figure 3.16. DNA sequencing results of *S. milleri* B200 amplified with Primer VanA from Miele *et al.*, 1995.

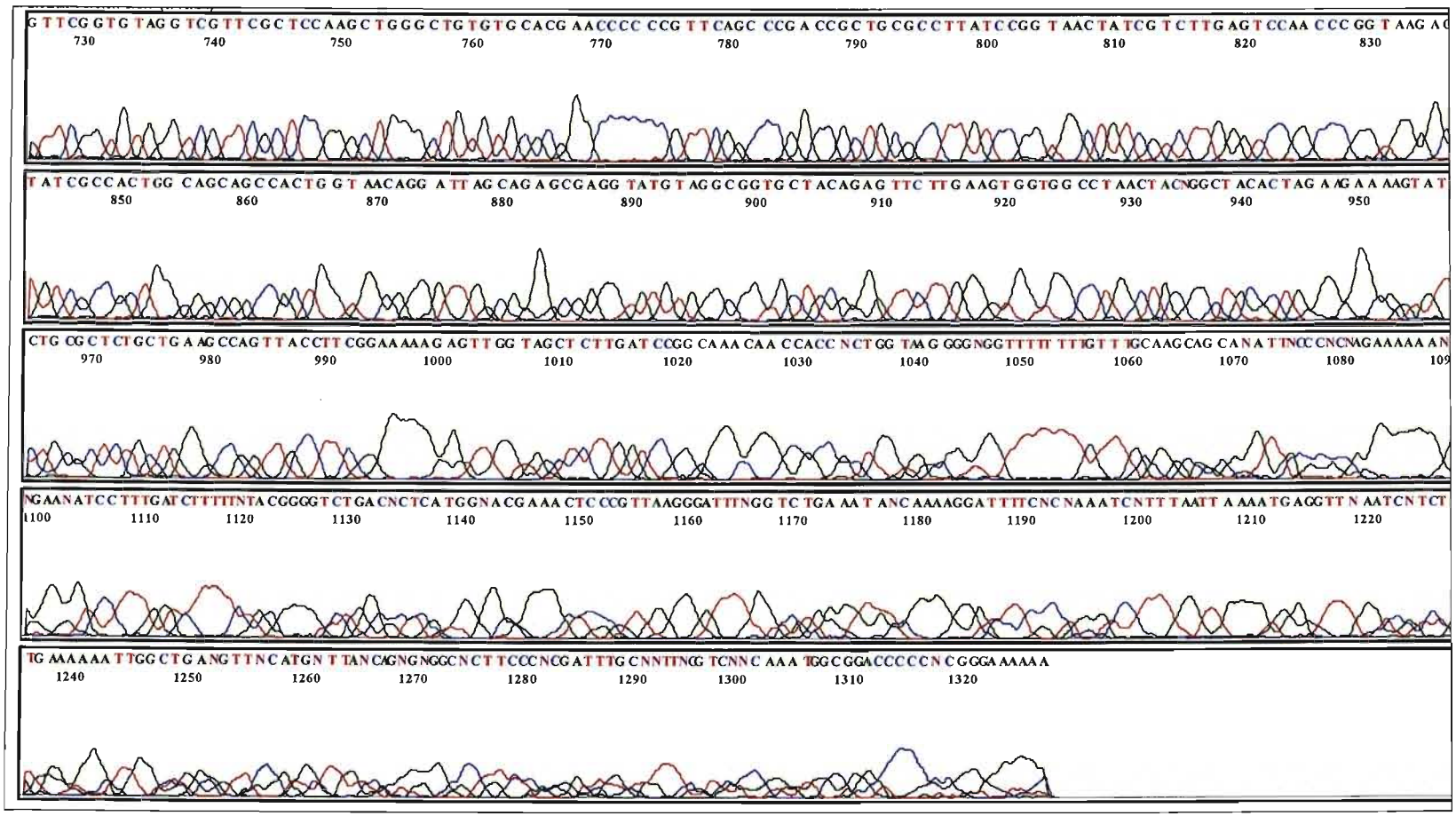

Figure 3.17. DNA sequencing results of *S. milleri* B200 amplified with Primer VanA from Miele *et al.*, 1995.

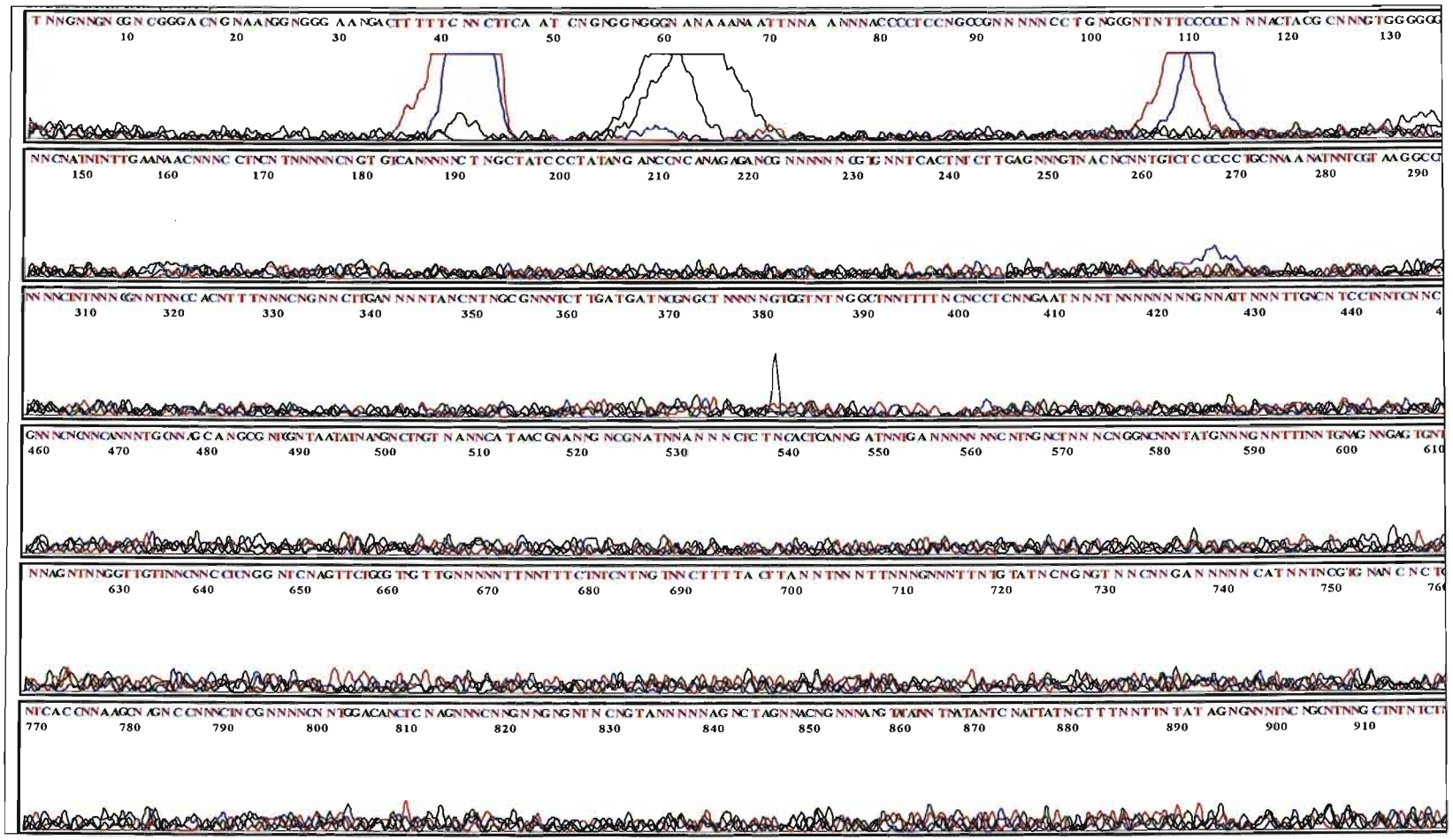

Figure 3.18. DNA sequencing results of *S. milleri* P213 amplified with Primer VanA using Miele *et al.*, 1995 Primers.

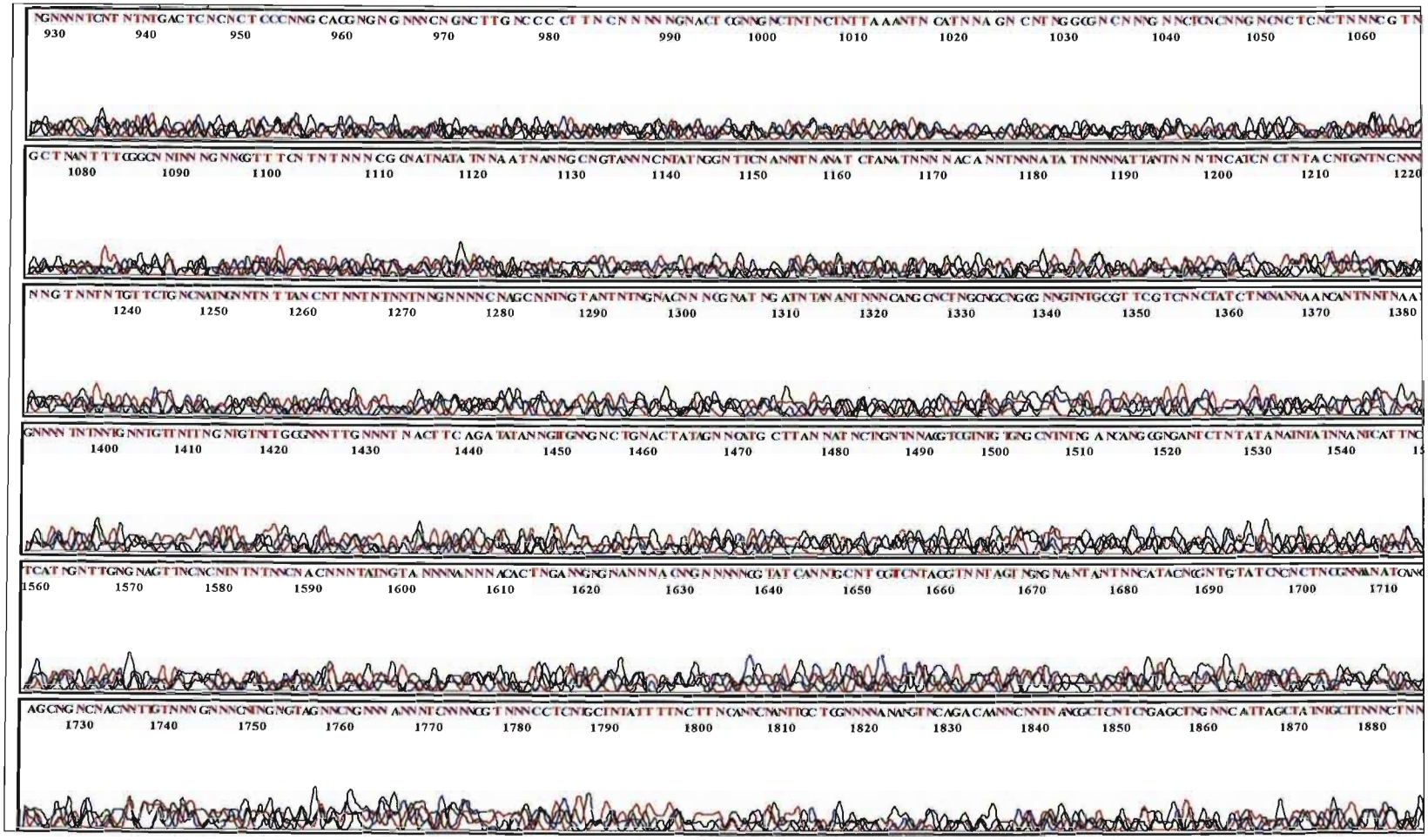

Figure 3.19. DNA sequencing results of *S. milleri* P213 amplified with Primer VanA using Miele *et al.*, 1995 Primers.

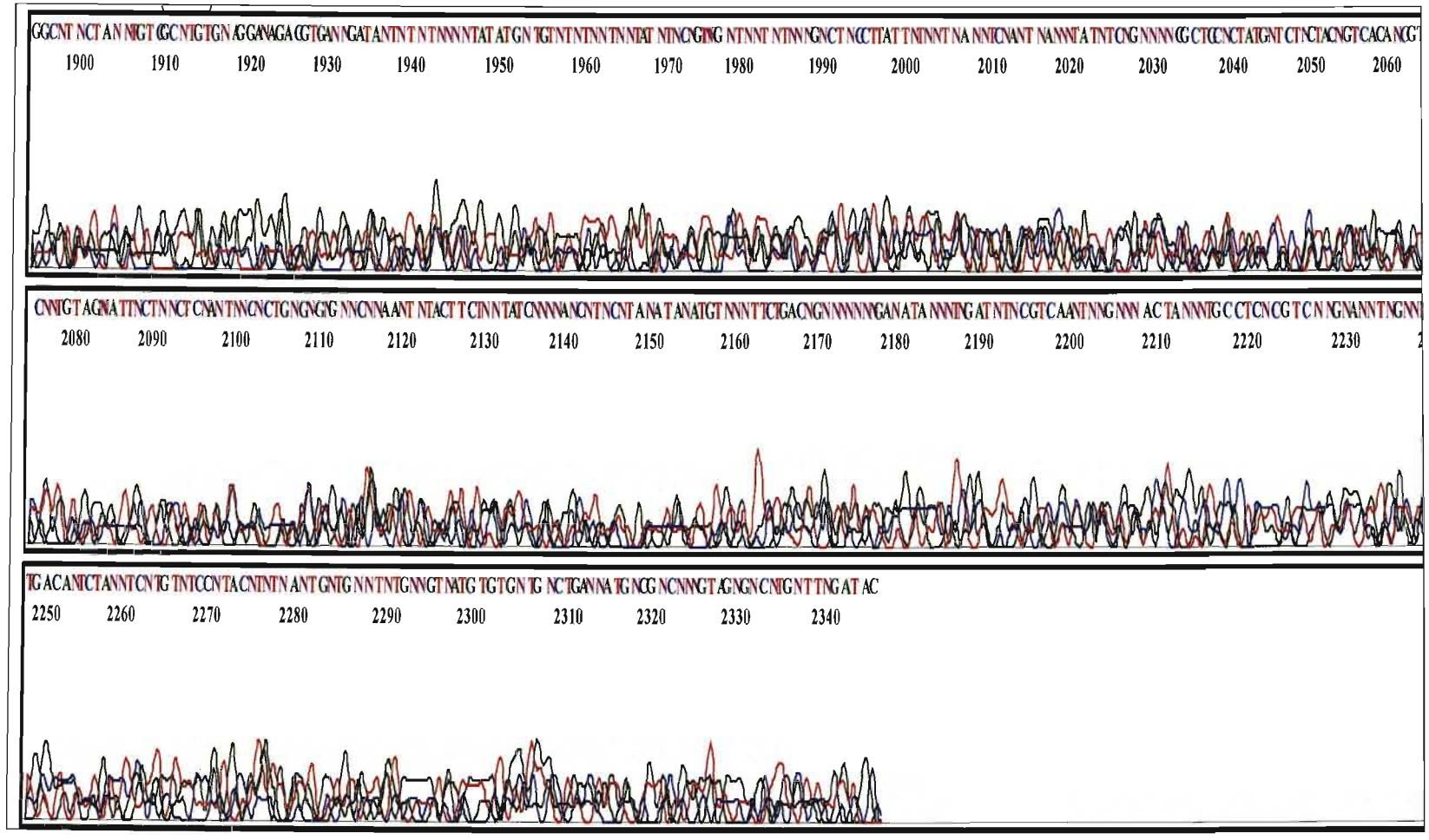

Figure 3.20. DNA sequencing results of *S. milleri* P213 amplified with Primer VanA using Miele *et al.*, 1995 Primers.

3.7. Southern Blot Hybridization Using Miele *et al*, 1995 Primers as Probes

Southern blotting is another approach using probes for the vancomycin genes, which is being applied for the determination and detection of vancomycin phenotypes. Seeing as how the PCR reactions yielded uncertain results and the sequencing produced much unrelated matches, the primer sets were then used as probes for southern blot hybridisation. Southern blotting is performed to confirm whether the gene sequences for vancomycin resistance is present in these strains. The reason why the primers for PCR are used as probes is to check whether they are compatible with the DNA since no conclusive results were obtained from neither the different PCR reactions nor the sequencing results. Another reason could be that the gene sequences could be present in these strains but were not detected.

Figure 3.21. is the southern blot obtained using primer VanA as a probe. From this figure only the DIG-labelled Molecular Weight Marker could be seen. This indicates that the detection worked; however, no other bands were seen. This implies that the probes did not hybridise to the DNA sequence, hence these primers are not specific enough to detect whether vancomycin resistant genes are present. The VanA primer as a probe did not even detect the vancomycin resistant gene sequence in the VanA control, which is already confirmed as a vancomycin resistant phenotype. pJF 5.5 was used as a negative control since it is a recombinant vector and does not code for vancomycin resistance. This control did not appear on the blot as expected.

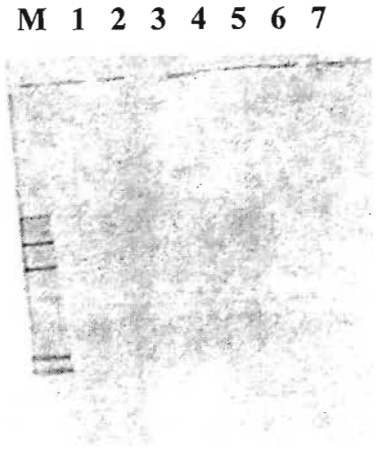

Figure 3.21. Southern blot hybridisation of PCR amplicons using Primer VanA (Miele *et al.*, 1995) as a probe. Lane M: Dig-labelled Molecular Weight Marker; Lane 1: *S. milleri* P213; *S. milleri* B200; Lane 3: *E. faecalis* 123; Lane 4: *E. faecalis* 126; Lane 5: *E. faecium*; Lane 6: VanA Control; Lane 7: Negative control (pJF 5.5 DNA).

Primer VanB was also used as a probe in another southern blot/hybridisation experiment. The results obtained may be seen in Figure 3.22. Again, in this blot only the DIG-labelled Molecular Weight Marker was detected. However, no other bands can be seen, hence nothing else was detected, not even the VanB control which contains the gene sequence for the VanB resistant phenotype. Hence, this primer was also not specific enough to detect the presence of vancomycin resistance. The negative control used in this blot was also pJF 5.5, which does not contain genes for VanB type resistance. No false negatives were observed.

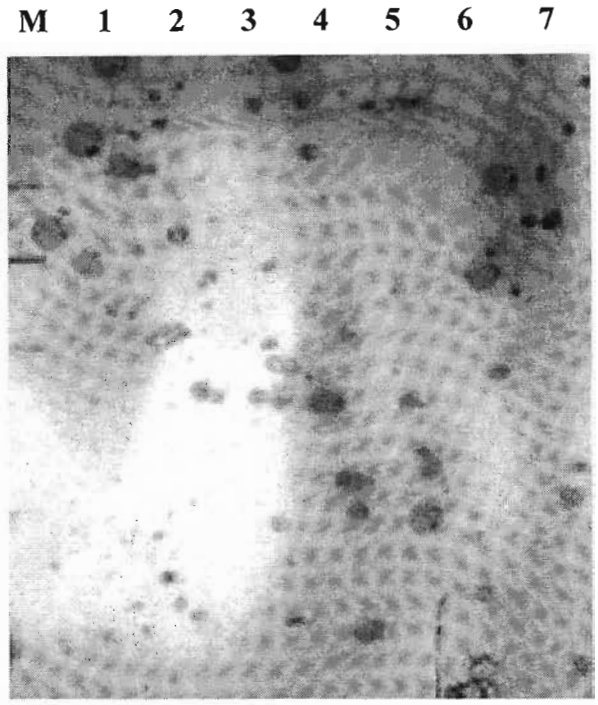

Figure 3.22. Southern blot using Primer VanB (Miele *et al.*, 1995) as a probe. Lane M: Dig-labelled Molecular Weight Marker; Lane 1: *S. milleri* P213; *S. milleri* B200; Lane 3: *E. faecalis* 123; Lane 4: *E. faecalis* 126; Lane 5: *E. faecium*; Lane 6: VanB Control; Lane 7: Negative control (pJF 5.5 DNA).

Figure 3.23 is a southern blot/hybridisation result of all strains using the VanC set of primers as a probe. From this figure, it can be seen that only the DIG-labelled Molecular Weight Marker was detected. No other bands were observed in any of the lanes. This suggests that none of the strains, including the negative control, pJF 5.5, does not contain the VanC sequence.

The southern blot/hybridisation experiments performed using each of the primer pairs as probes did not yield any results. Hence, the results obtained using this method, together with PCR and sequencing, were not conclusive enough to determine the phenotype of each of the strains. This also meant that the primers used were not specific enough to detect the various vancomycin resistant phenotypes.

Another reason why these primers might not be specific enough was that the properties of the oligonucleotide sequences were based on that of their ligases.

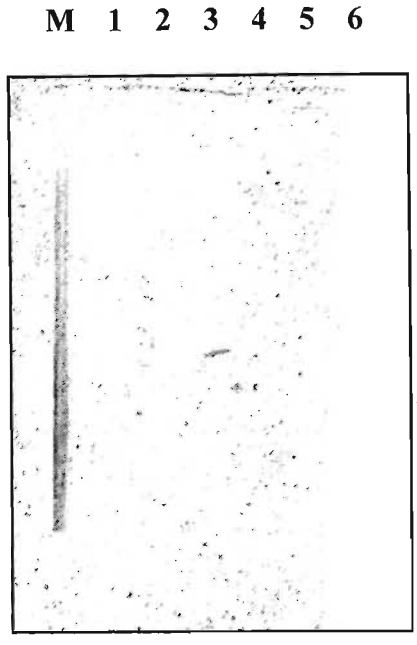

Figure 3.23. Southern blot using Primer VanC (Miele *et al.*, 1995) as a probe. Lane M: Dig-labelled Molecular Weight Marker; Lane 1: *S. milleri* P213; *S. milleri* B200; Lane 3: *E. faecalis* 123; Lane 4: *E. faecalis* 126; Lane 5: *E. faecium*; Lane 6: Negative control (pJF 5.5 DNA).

3.8. PCR Using Primers from Dukta-Malen *et al.*, 1995

In designing primers it is vital to test their specificities and to define the optimal conditions for amplification (70). The Dukta-Malen *et al.*, primers were based on the specific detection of genes encoding D-alanine : D-alanine (D-Ala : D-Ala) ligases and related glycopeptide resistant enzymes, since they are responsible for synthesizing D-Ala-D-Lac, which give rise to the VanA and B-type resistance. Ligases that produce D-Ala-D-Ser are responsible for the VanC-1 resistance phenotype (38). The primer sets used were all of similar size and GC content to prevent variations in annealing temperatures.

PCR was performed with DNA from every strain as a template. In Figure 3.24., the VanA primer pair was used. This primer pair was targeting the *vanA* gene cluster and yielded a product of the appropriate size of 732 bp for only the VanA control DNA in lane 1. This

product was correctly amplified, hence the rest of the results did not show false negatives. This suggests that the other bacterial strains do not contain the VanA resistant phenotypes.

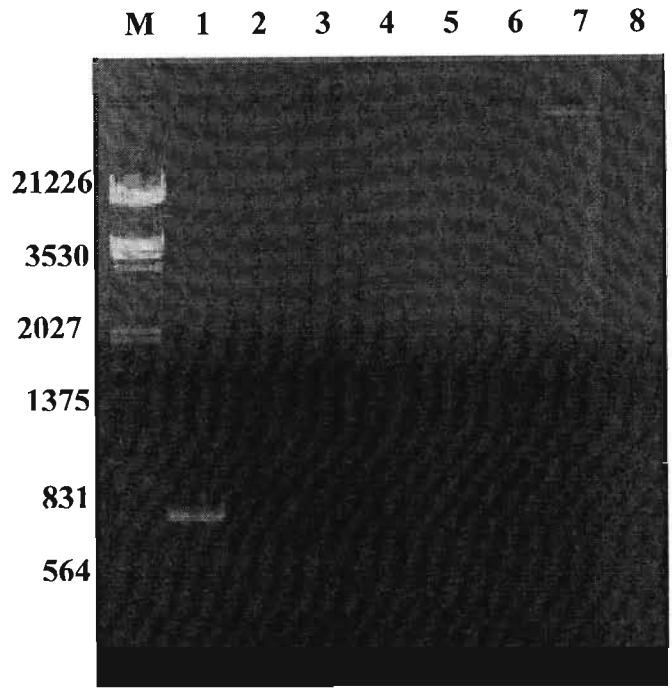

Figure 3.24. Agarose gel [1.5% (w/v)] showing PCR amplification with Primer VanA (Dukta-Malen *et al.*, 1995). Lane M: Molecular Weight Marker III; Lane 1: VanA Control DNA; Lane 2: *S.milleri* P213; Lane 3: *S.milleri* P35; Lane 4: *S.milleri* B200; Lane 5: *E. faecalis* 123; Lane 6: *E. faecalis* 126; Lane 7: *E. faecium*; Lane 8: Negative Control (No template DNA).

In the following PCR experiment, primer VanB was used for amplification. Figure 3.25. illustrates the results obtained. A single band of an estimated size of 635 bp can be seen in lane 1. This is the only amplificon that was produced. The VanB control DNA of the correct size. Hence, no amplification in the other lanes, prove there are no false negatives. This then implies that none of the bacterial strains are of the VanB phenotype.

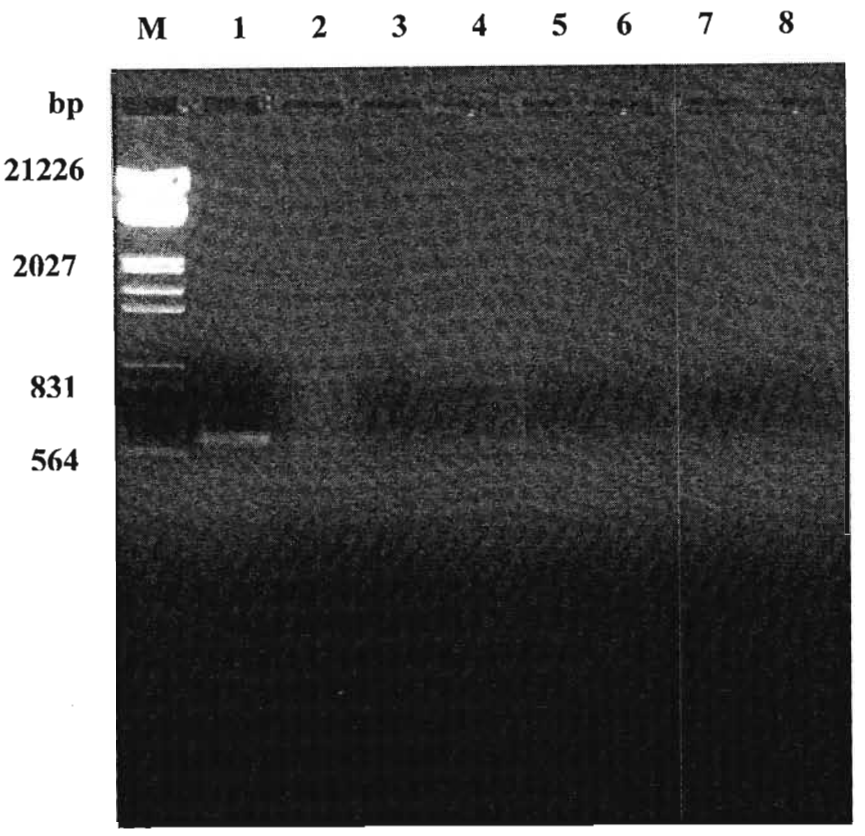

Figure 3.25. Agarose gel [1.5% (w/v)] showing PCR amplification with Primer VanB (Dukta-Malen *et al.*, 1995). Lane M: Molecular Weight Marker III; Lane 1: VanB Control DNA; Lane 2: *S.milleri* P213; Lane 3: *S.milleri* P35; Lane 4: *S.milleri* B200; Lane 5: *E. faecalis* 123; Lane 6: *E. faecalis* 126; Lane 7: *E. faecium*; Lane 8: Negative Control (No template DNA).

In the following PCR experiment, bacterial DNA was amplified with primer VanC in Figure 3.26. In lanes 1 – 6, bands of the same size can be seen. The approximate size of these products is 822 bp correctly targeting the *vanC* gene cluster. This indicates that *S. milleri* P213, *S. milleri* P35, *S. milleri* B200, *E. faecalis* 123, *E. faecalis* 126 and *E. faecium* are all of the VanC phenotype. This correlates with the MIC values obtained from disk-diffusion assay. This phenotype is more commonly found in *E. gallinarum*. The negative control did not appear, this implies that the bands obtained in lanes 1 – 6 did not show false positive results.

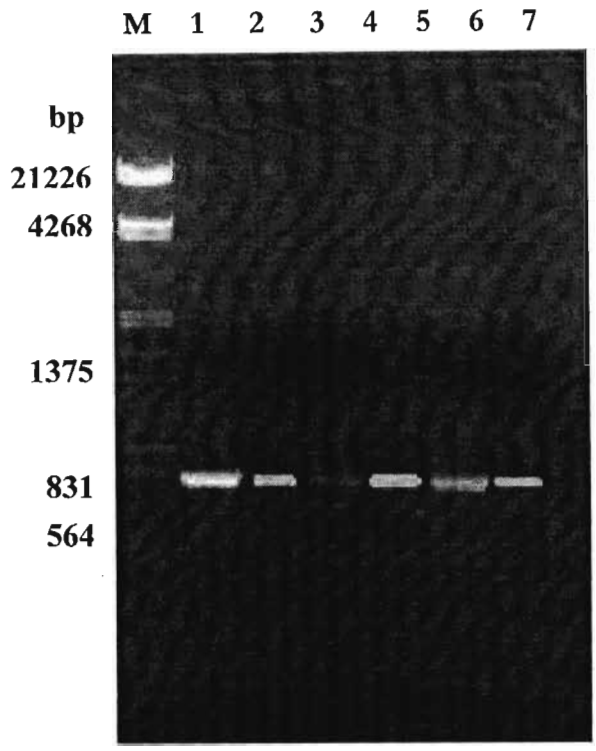

Figure 3.26. Agarose gel [1.5% (w/v)] showing PCR amplification with Primer VanC (Dukta-Malen *et al.*,1995). Lane M: Molecular Weight Marker III; Lane 1: *milleri* P213; Lane 2: *S.milleri* P35; Lane 3: *S.milleri* B200; Lane 4: *E. faecalis* 123; Lane 5: *E. faecalis* 126; Lane 6: *E. faecium*; Lane 7: Negative Control (No template DNA).

3.9. Sequencing of PCR Products Generated By Amplification with Dukta-Malen *et al.*, 1995 Primers

In order to verify whether the products obtained by PCR were the correct ones, sequencing was performed on these products. From the sequencing results in Figure 3.27 and 3.28, the VanA product obtained from the VanA control DNA amplified with the VanA primers were found to be homologous to *E. faecium* BM4147. This confirms that this is indeed the well-characterized glycopeptide Enterococci belonging to the VanA phenotype.

The VanB amplicons attained from the VanB control DNA amplified with the VanB primer pair by PCR was also sequenced. The sequence can be seen in Figure 3.29. This nucleotide sequence was found to be a hundred percent homologous to an *E. faecalis*

Tn1549 transposon which validates that this is the well-characterized vancomycin-resistant Enterococcal strain belonging to the VanB phenotype.

E. faecalis 126 amplified with the VanC primers were also sequenced. The results can be seen in Figures 3.29 and 3.30. These sequences were found to be ninety-six percent homologous to the *E. gallinarum* VanC vancomycin-resistant gene cluster. This confirms that products obtained from PCR in Figure 3.26 were accurate and one can infer that the other strains, *S. milleri* P213, *S. milleri* P35, *S. milleri* B200, *E. faecalis* 123 and *E. faecium* were also correctly identified as VanC phenotypes.

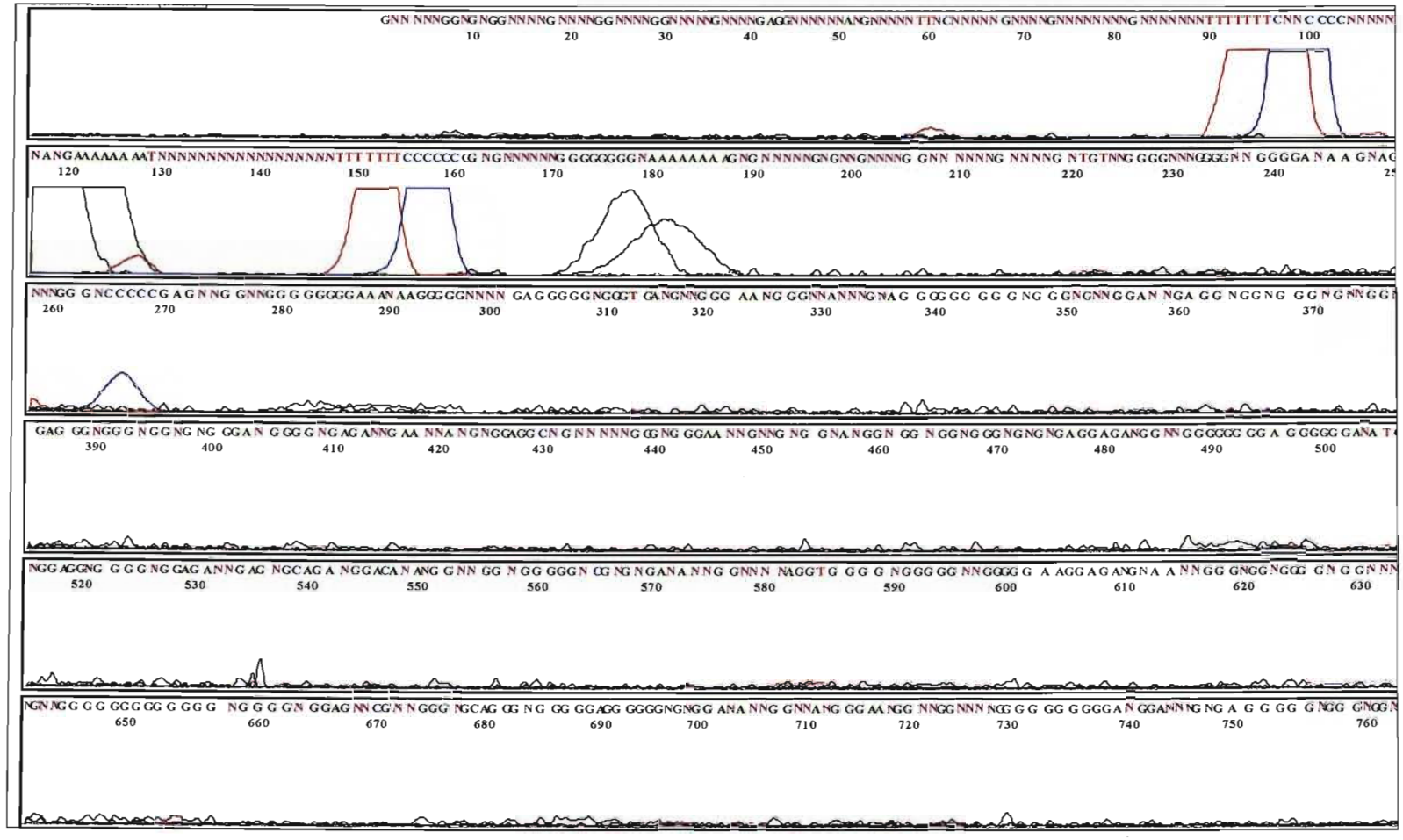

Figure 3.27. DNA sequencing results of VanA control DNA amplified with Primer VanA using Dukta-Malen *et al.*, 1995 Primers.

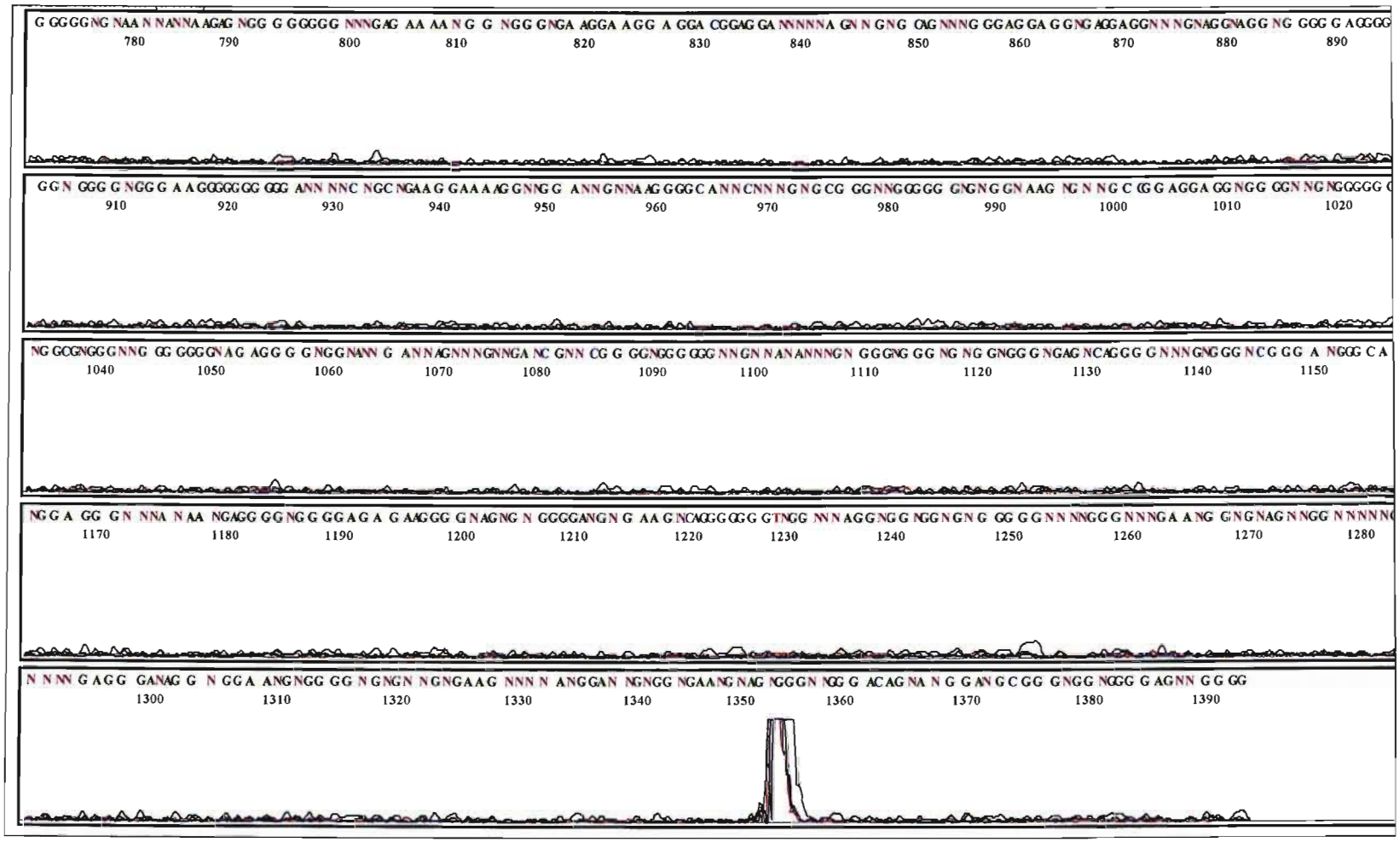

Figure 3.28. DNA sequencing results of VanA control DNA amplified with Primer VanA using Dukta-Malen *et al.*, 1995 Primers.

```
1   atgaatagaa taaaagtcgc aatcatcttc ggcggttgct cggaggaaca tgatgtgtcg
61  gtaaaatccg caatagaaat tgctgcgaac attaatactg aaaaattcga tccgcactac
121 atcggaatta caaaaaacgg cgtatggaag ctatgcaaga agccatgtac ggaatgggaa
181 gccgacagtc tccccgccat actctccccg gataggaaaa cgcatgggct gcttgtcatg
241 aaagaaagcg aatacgaaac acggcgtatt gatgtggctt tcccggtttt gcatggcaaa
301 tgcggggagg atggtgcgat acagggtctg tttgaattgt ctggtatccc ctatgtaggc
361 tgcgatattc aaagctccgc agcttgcatg gacaaatcac tggcctacat tcttacaaaa
421 aatgcgggca tcgccgttcc cgaatttcaa atgattgata aaggtgacaa gccggaggcg
481 ggtgcgctta cctaccctgt ctttgtgaag ccggcacggt caggttcgtc ctttggcgta
541 accaaagtaa acggtacgga agaacttaac gctgcgatag aagcggcagg acaatatgat
601 ggaaaaatct taattgagca agcgatttcg ggctgtgagg tcgggtgtgc ggtcatgggg
661 aacgaggatg
```

Figure 3.29. Nucleotide sequence of the VanB amplicon obtained from the PCR of the VanB control amplified with VanB primers from Dukta-Malen *et al.*, 1995.

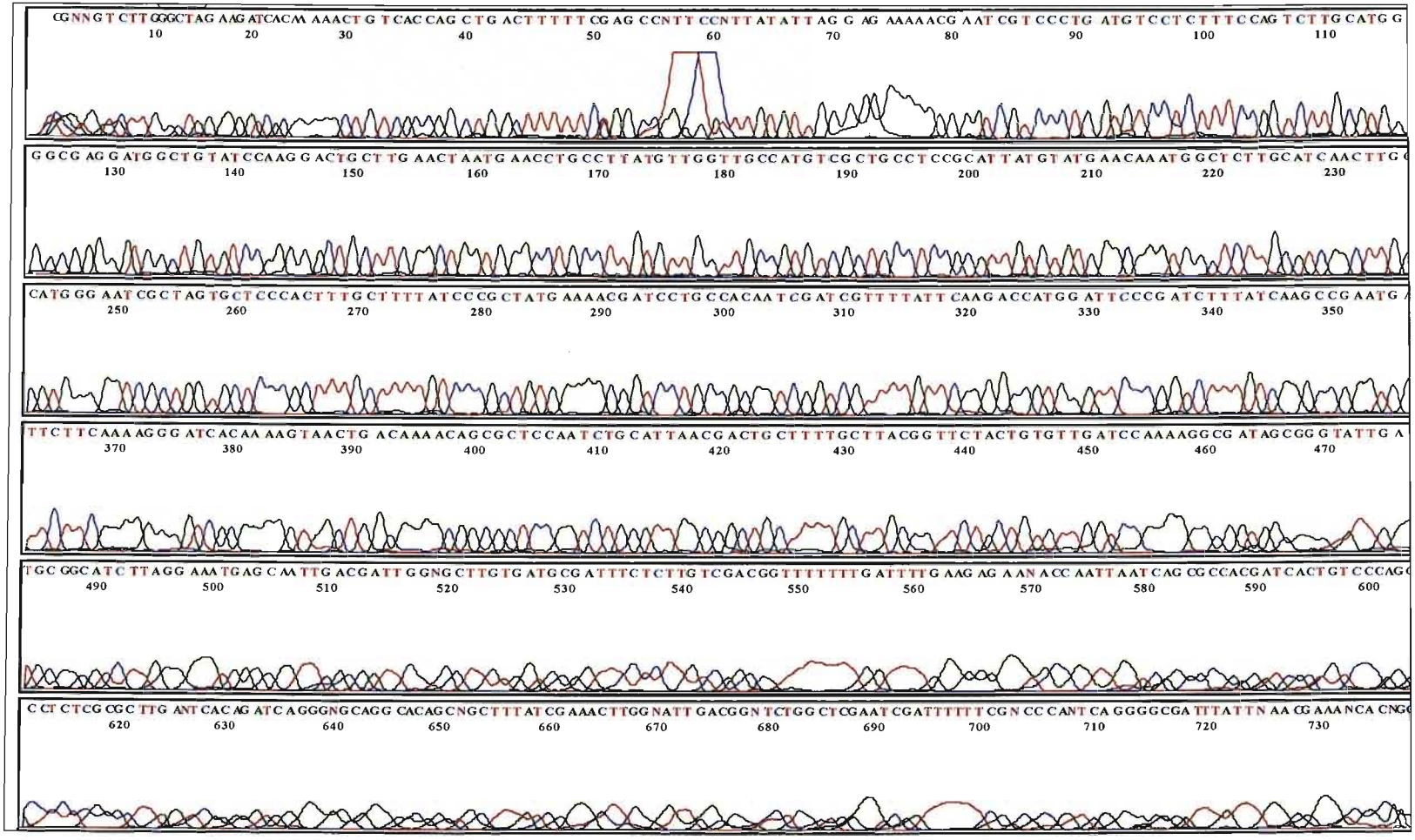

Figure 3.30. DNA sequencing results of *E. faecalis* 126 amplified with Primer VanC using Dukta-Malen *et al.*, 1995 Primers.

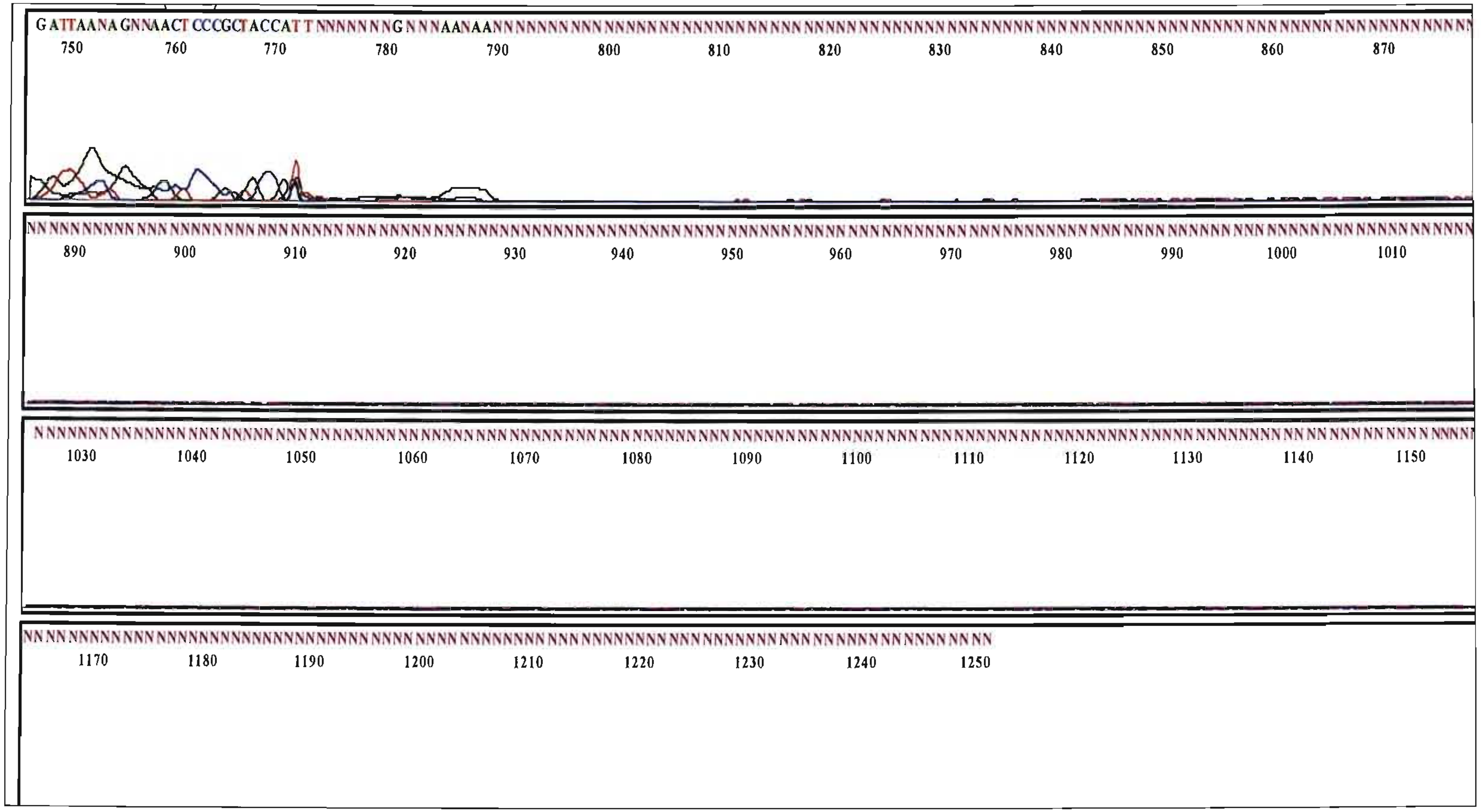

Figure 3.31. DNA sequencing results of *E. faecalis* 126 amplified with Primer VanC using Dukta-Malen *et al.*, 1995 Primers.

3.10. Southern Blotting Using Dukta-Malen *et al.*, 1995 Primers as Probes

Southern blot/hybridisation was performed on bacterial strains, using the primers obtained from Dukta-Malen *et al.*, 1995 as probes to confirm the results obtained by PCR analysis and sequence analysis.

The blot in Figure 3.32. used primer VanA as a probe. In this picture one can see the DIG-labelled Molecular Weight Marker in lane M. This indicates that the detection worked. This blot was successful since the positive control, which was the VanA control DNA had appeared. Hence, only the VanA sequence was detected using the VanA primer as the probe. These results correlate with that obtained in PCR that the other bacterial strains are not of the VanA phenotype. This also correlates with the MIC values obtained via disk diffusion. However, in lane 8 the negative control, pJF 5.5 was also detected.

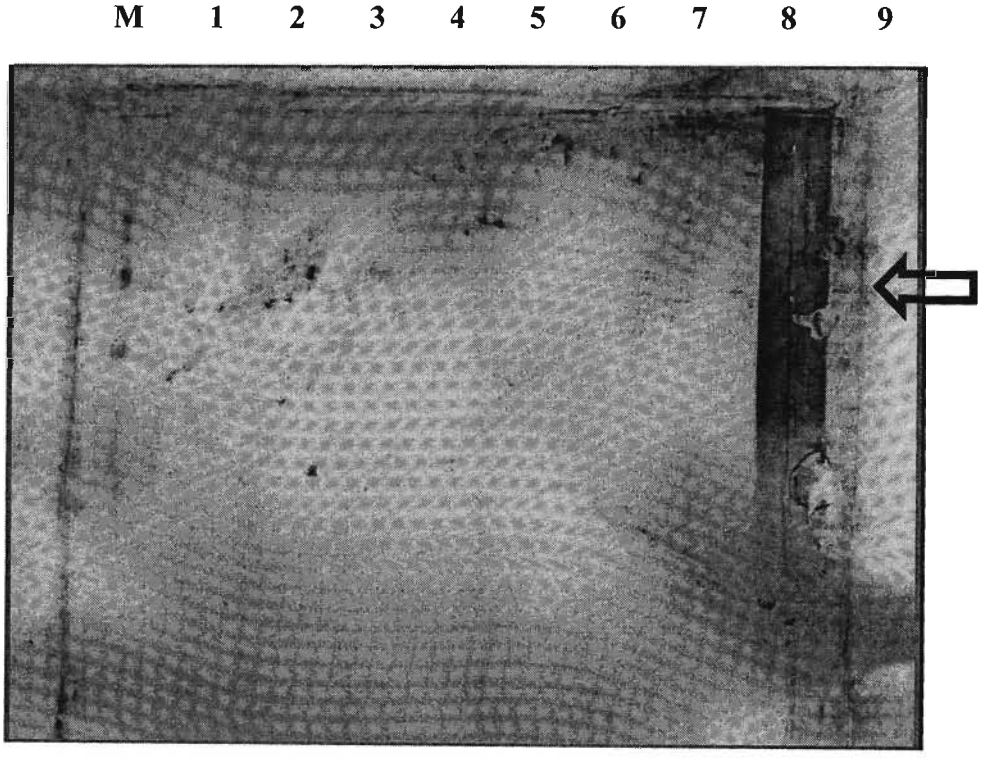

Figure 3.32. Southern blot using Primer VanA (Dukta-Malen *et al.*, 1995) as a probe. Lane M: Dig-labelled Molecular Weight Marker; Lane 1: *S. milleri* P213; Lane 2: *S. milleri* P35; Lane 3: *S.milleri* B200; Lane 4: *E. faecalis* 123; Lane 5: *E. faecalis* 126; Lane 6: *E. faecium*; Lane 7: VanB Control DNA; Lane 8: Negative Control (pJF 5.5 DNA); Lane 9: VanA Control.

The VanB primer was also used as a probe for southern blot/hybridisation. The results obtained can be clearly seen in Figure 3.33. The DIG-labelled Molecular Weight Marker can be seen. This indicates that the detection was successful. In lane 9, the VanB control, which was the positive control, was detected. This suggests that the absence of bands in lanes 1 - 8 were not false negatives. Hence, these bacterial strains are not of the VanB type vancomycin resistance. This is in concordance with results obtained in PCR and sequencing.

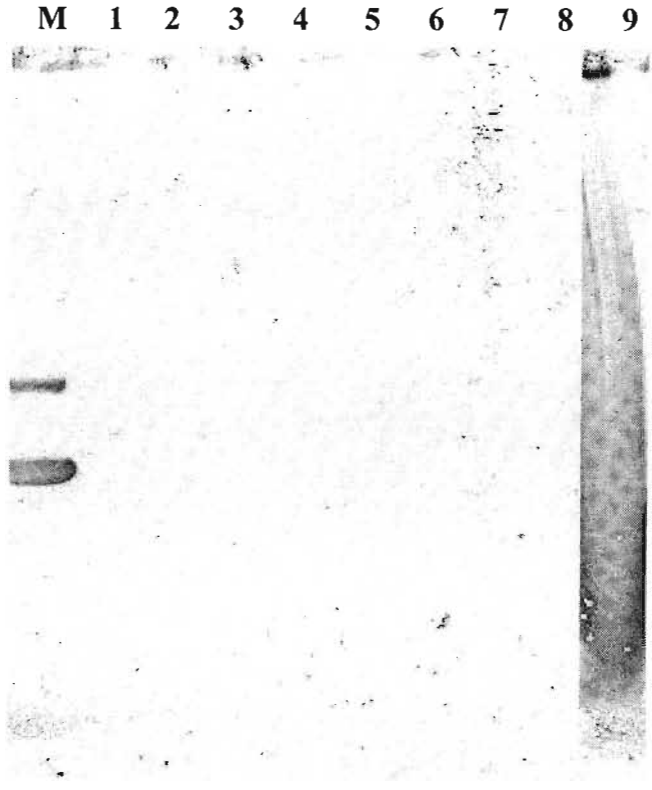

Figure 3.33. Southern blot/hybridisation using Primer VanB (Dukta-Malen *et al.*, 1995) as a probe. Lane M: Dig-labelled Molecular Weight Marker; Lane 1: *S. milleri* P213; Lane 2: *S. milleri* P35; Lane 3: *S.milleri* B200; Lane 4: *E. faecalis* 123; Lane 5: *E. faecalis* 126; Lane 6: *E. faecium*; Lane 7: VanA Control DNA; Lane 8: Negative Control (pJF 5.5 DNA); Lane 9: VanB Control.

Southern blot/hybridisation was performed again on all bacterial strains, using the VanC primers as a probe. From the blot in Figure 3.34, the DIG-labelled Molecular Weight Marker, as well as other bands can be seen in lanes 1 – 6. This indicates that the detection was successful. The other bands confirm the presence of the VanC sequence in the bacterial strains used. This correlates to the PCR and sequencing performed with the same set of primers. Hence, these strains are definitely of the VanC phenotype. The negative control, pJF 5.5 in lane 7, did not appear. This confirms that the results are not false positives. Under highly stringent conditions, each probe hybridised to the isolates of the same class.

The phenotypes can also be confirmed by examining the peptidoglycan precursors produced under induced condition by High Performance Liquid Chromatography (HPLC).

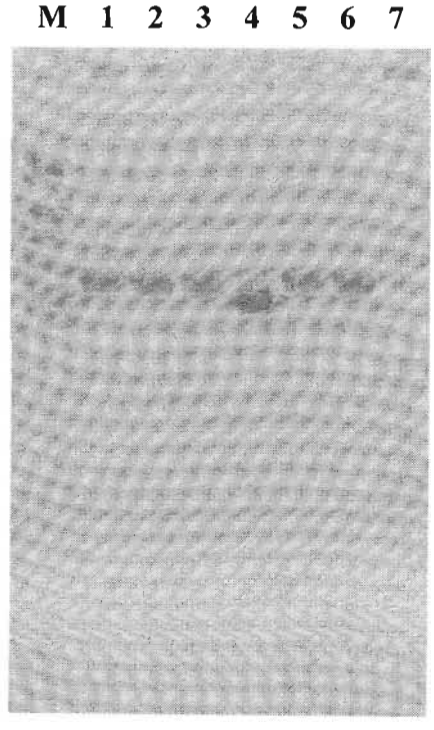

Figure 3.34. Southern Blot using the VanC primers (Dukta-Malen *et al.*, 1995) as probes. Lane M: Dig-labelled Molecular Weight Marker; Lane 1: *S. milleri* P213; Lane 2: *S. milleri* P35; Lane 3: *S.milleri* B200; Lane 4: *E. faecalis* 123; Lane 5: *E.faecalis* 126; Lane 6: *E. faecium*; Lane 7: Negative Control (pJF 5.5 DNA).

3.11. Analysis of Peptidoglycan Precursors

Samples of the fraction containing the cell wall precursors of each bacterial strain were analysed by HPLC. This method is based on the separation of suspension into components using liquid chromatography over a short period of time at a high pressure. For reverse-phase chromatography, separation is based on the hydrophobic interaction between the solute molecules in the mobile phase and the immobilized hydrophobic ligand in the stationary phase. The C-18 column binds peptides tighter than proteins and thereby getting rid of 'junk' proteins. This technique is a means of measuring the decrease in the amount of substrate and the increase in the amount of product (83). The approximate elution times for the precursors were: UDP-MurNAc-tripeptide, 7.7min; UDP-MurNAc-tetrapeptide, 16min; UDP-MurNAc-pentapeptide, 25min; UDP-MurNAc-pentadepsipeptide, 42min according to the method by Baptista *et al.*, 1997.

There are various steps that take place inside and outside the cell membrane during the synthesis of peptidoglycan. Transglycosylation is the process whereby the carbohydrates, N- acetyl glucosamine (GlcNAc) and the N- acetyl muramic acid (MurNAc) subunits are put together to form the glycan chains. Transpeptidation is the peptide cross-linking between these chains. This step involves linking the sugar portion of the peptidoglycan subunit to the glycan backbone of the existing cell wall polymer. D-Alanine is enzymatically removed from the end of the pre-existing peptide side chain. This allows for the peptide side chain to be cross-linked to the recently synthesized peptidoglycan subunit. Both these steps of peptidoglycan biosynthesis occur just outside the membrane.

3.11.1. HPLC Profiles of Precursors Arrested With 20 μg/ml Vancomycin

Cytoplasmic peptidoglycan precursors were extracted after strains were grown in the presence of vancomycin to induce the transcription of the resistant genes. Vancomycin inhibits both transglycosylation as well as the transpeptidation steps of peptidoglycan synthesis (34, 35). The action of this glycopeptide is restricted to these steps since the disaccharide-pentapeptide unit is formed in the bacterial cytoplasm and only thereafter transported across the cytoplasmic membrane. In susceptible bacterial strains, vancomycin exposure leads to the accumulation of the lipid-associated disaccharide pentapeptide (lipid intermediate II) in the membrane. This gives rise to the inhibition of the transglycosylation step in which the emergent peptidoglycan polymer is extended by a single disaccharide-pentapeptide at the reducing end. The bottom surface of vancomycin makes five hydrogen bonds to the D-Ala-D-Ala amino acids at the end of the peptide cross-bridges, thus sterically inhibiting transpeptidation. This antibiotic also prevents these residues from being easily reachable to the active site of the transpeptidases because of its high affinity. Peptide cross-linking therefore cannot occur, thus resulting in cell lysis. Hence, vancomycin only affects the late stage reactions in peptidoglycan synthesis. One of the effects is that vancomycin resistance is not being expressed and only the normal UDP-MurNAc-pentapeptide precursor is produced (2, 19). The increasing concentration of D-Ala-D-Ala corresponds to the increasing inactivation of vancomycin (2).

In vancomycin resistant strains, such as Enterococci, a major portion of the lipid intermediate II molecules have the peptidoglycan precursor terminating in D-Ala-D-Lac instead of the normal D-Ala-D-Ala. This implies that transglycosylation is resistant to vancomycin since this antibiotic can now form only four hydrogen bonds with this terminus. This causes the binding affinity to decrease by a thousand fold (51). Transglycosylation is therefore not repressed except at a high concentration of the antibiotic (1).

The HPLC chromatogram in Figure 3.35 shows the results of the chemical analysis of the cell wall precursor pool of *S. milleri* P213 grown in the presence of vancomycin at twice its MIC value, i.e., 20 μg/ml, as determined by the disk-diffusion assay. The major component of the precursor pool in this strain was UDP-MurNAc-pentapeptide at approximately 20 minutes. At 14 minutes, a lower amount of UDP-MurNAc-tetrapeptide compared to that of UDP-MurNAc-pentapeptide was present.

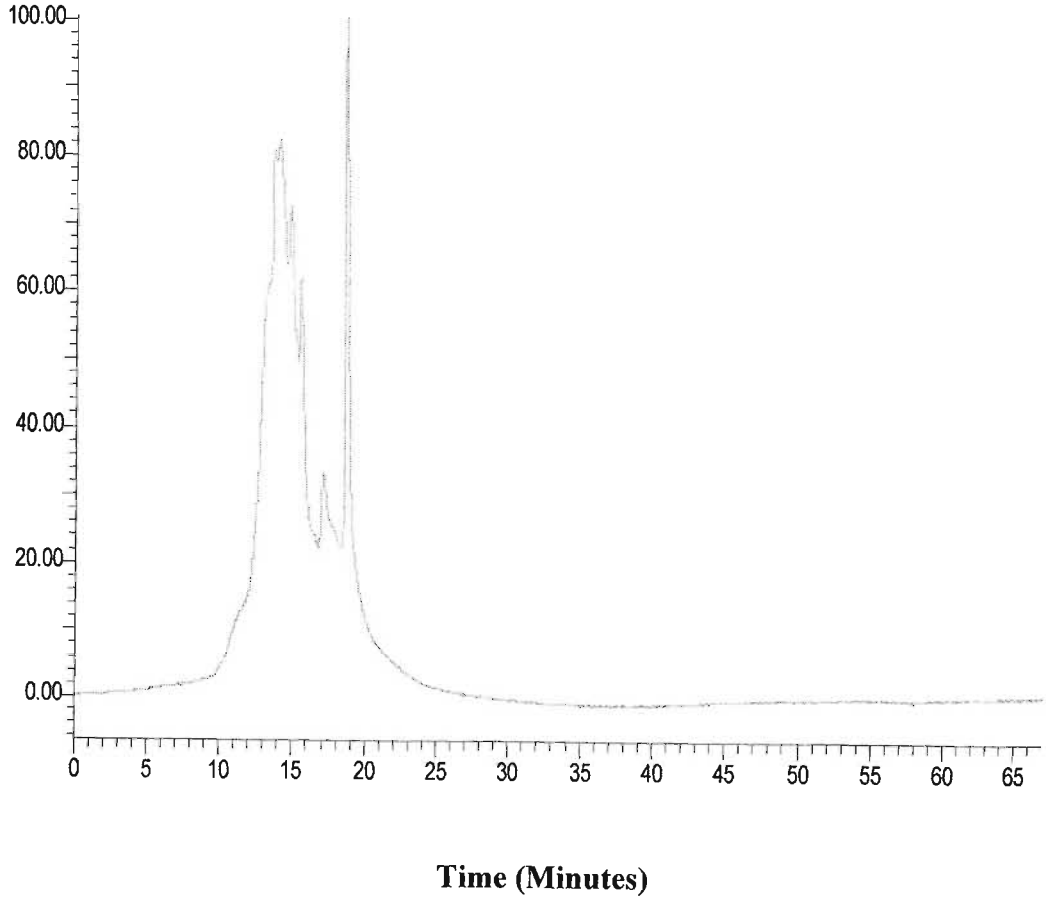

Figure 3.35. HPLC analysis of peptidoglycan precursors from *S. milleri* P213 arrested with 20 μg/ml vancomycin at OD_{252}.

The separation of peptidoglycan precursors of *S. milleri* P35 by HPLC can be seen in the chromatogram in Figure 3.36. From this figure, a high proportion of UDP-MurNAc-tetrapeptide can be seen at 17 minutes. At 39 minutes a moderate amount of UDP-MurNAc-pentadepsipeptide was present.

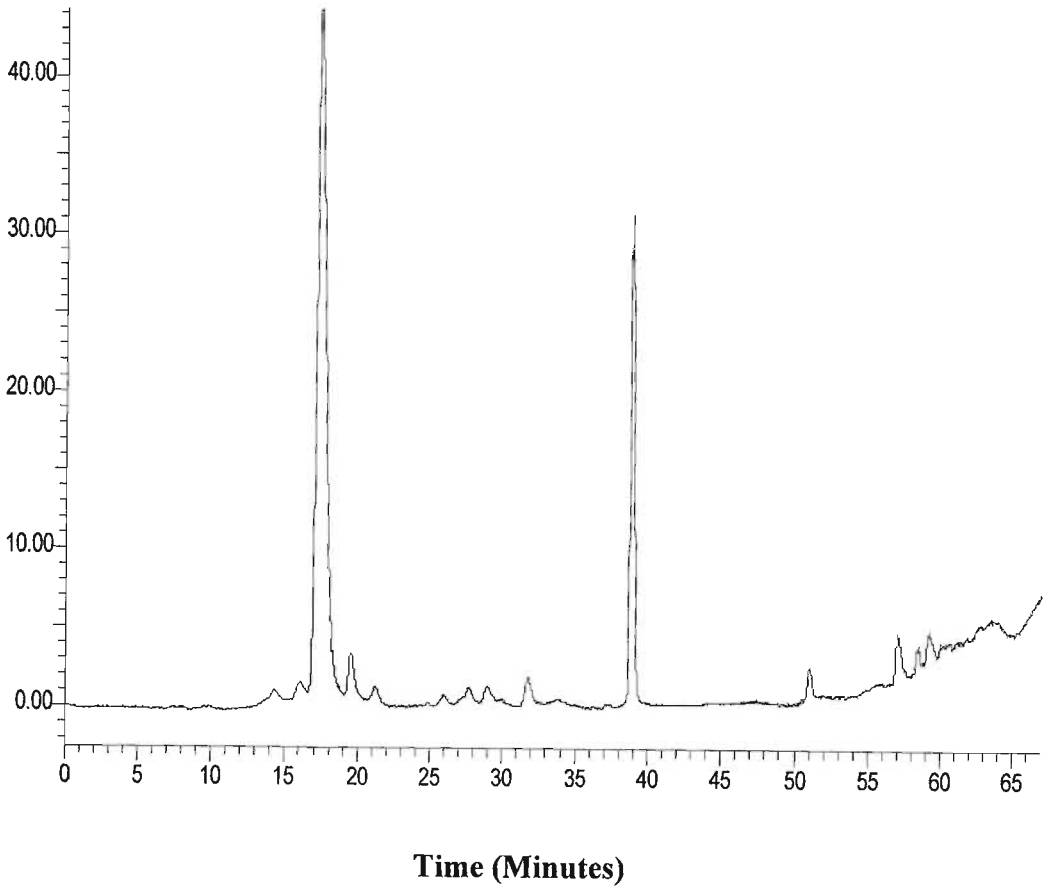

Figure 3.36. HPLC analysis of peptidoglycan precursors from *S. milleri* P35 arrested with 20 μg/ml vancomycin at OD_{252}.

Cell wall peptidoglycan precursors of *S. milleri* B200 separated by HPLC is represented by the chromatogram in Figure 3.37. Peptidoglycan precursors were arrested with 20 μg/ml of vancomycin. In this figure, a small amount of UDP-MurNAc-tripeptide at 11minutes was present, followed by a high proportion of UDP-MurNAc-tetrapeptide at 14 minutes. The moderate peak at approximately 20 minutes is representative of the peptidoglycan precursor pool, UDP-MurNAc-pentapeptide.

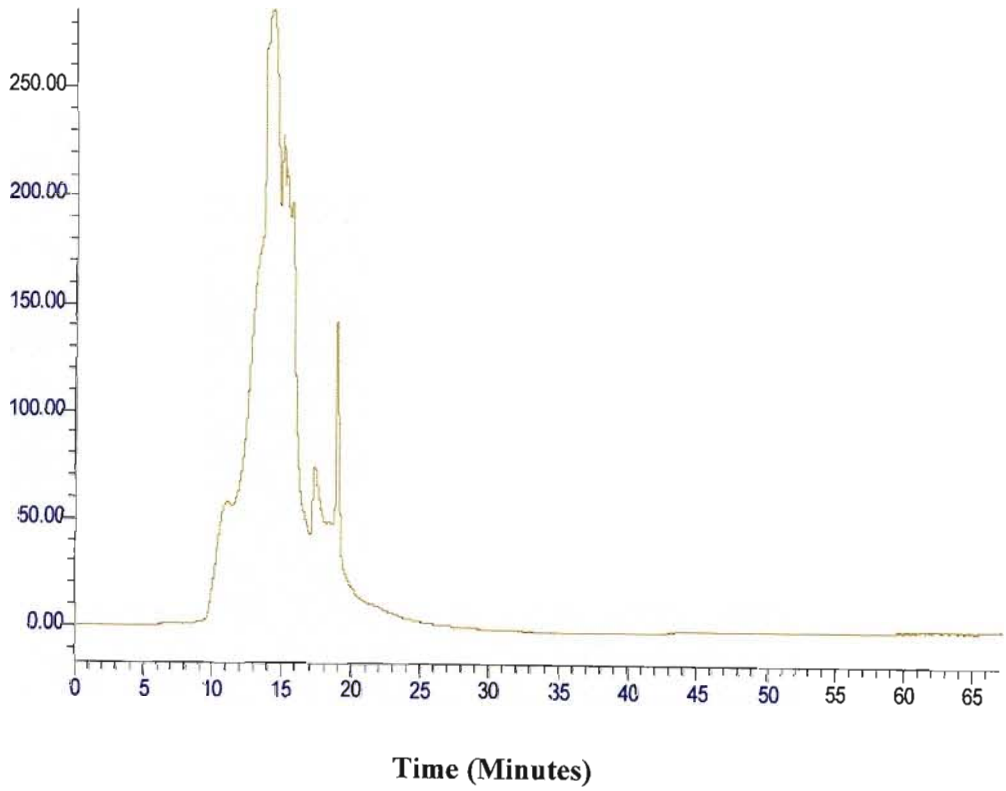

Figure 3.37. HPLC analysis of peptidoglycan precursors from *S. milleri* B200 arrested with 20 μg/ml vancomycin at OD_{252}.

Figure 3.38 represents the separation of the precursors of *E. faecalis* 123 induced with vancomycin at a concentration of twice its MIC. Only a high amount of UDP-MurNAc-pentadepsipeptide was seen at roughly 39 minutes.

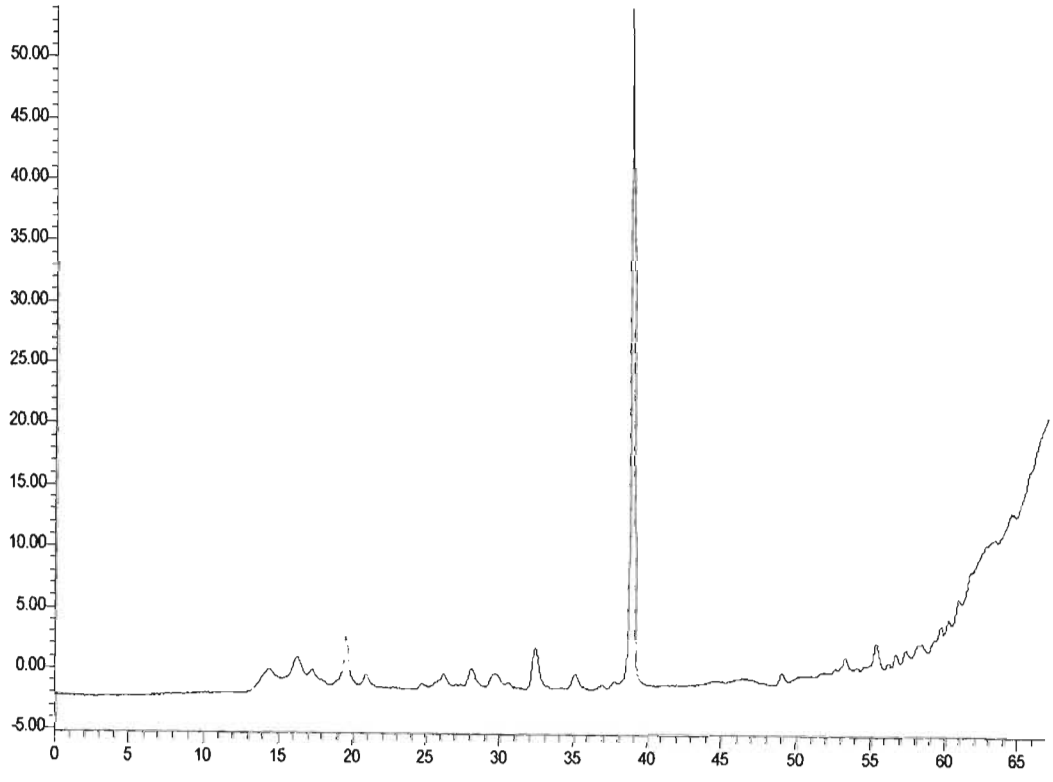

Figure 3.38. HPLC analysis of peptidoglycan precursors from *E. faecalis* 123 arrested with 20 μg/ml vancomycin at OD_{252}.

HPLC analysis of peptidoglycan precursors from *E. faecalis* 126 arrested with 20 μg/ml vancomycin at OD_{252} is illustrated in Figure 3.39. *E. faecalis* 126 contained a moderate amount of UDP-MurNAc-tripeptide as can be seen by the peak between 14 – 16 minutes. A high proportion of UDP-MurNAc-pentapeptide was seen at approximately 20 minutes.

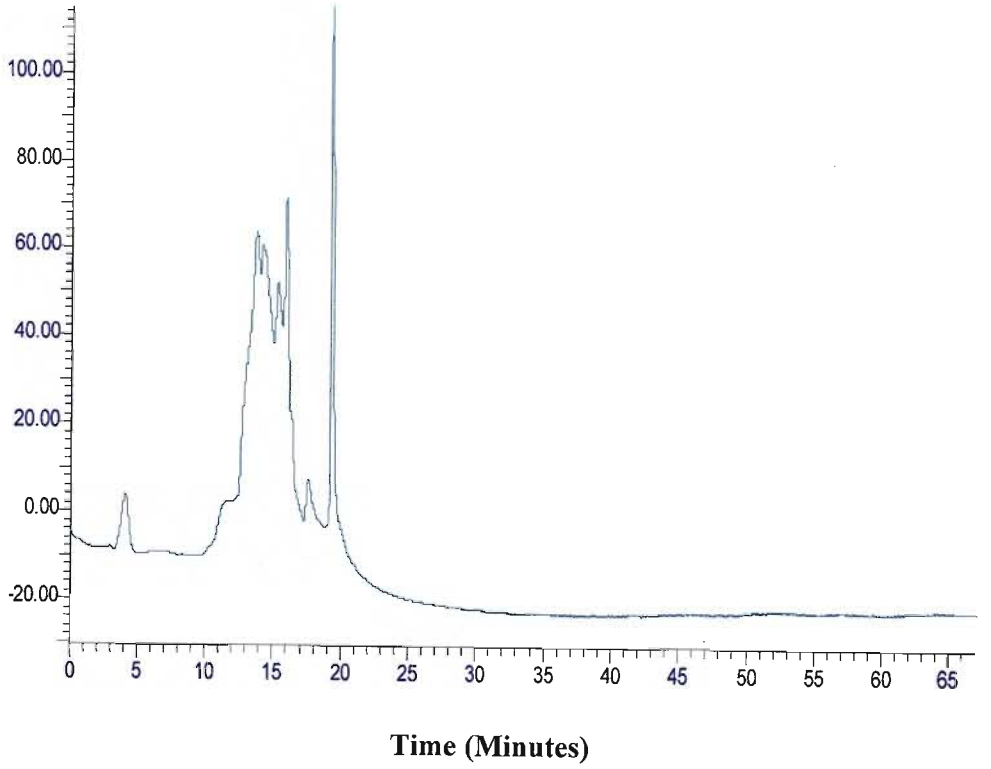

Figure 3.39. HPLC analysis of peptidoglycan precursors from *E. faecalis* 126 arrested with 20 μg/ml vancomycin at OD_{252}.

The HPLC chromatogram in Figure 3.40. represents the separation of the precursors found in the *E. faecium* bacterial strain. In this chromatogram, the first peak at 10minutes is representative of the UDP-MurNAc-tripeptide precursor. UDP-MurNAc-tetrapeptide was seen at a high proportion at 15minutes, followed by a moderate amount of UDP-MurNAc-pentapeptide at approximately 19minutes.

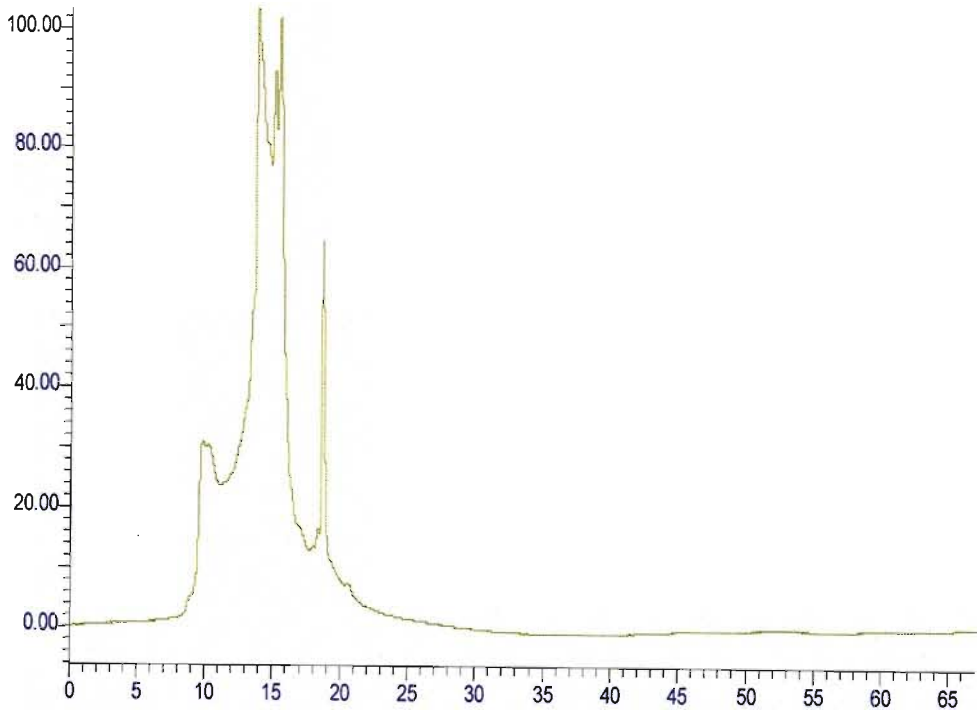

Figure 3.40. HPLC analysis of peptidoglycan precursors from *E. faecium* arrested with 20 μg/ml vancomycin at OD_{252}.

The relative proportion of peptidoglycan precursor of the VanA control separated by HPLC is illustrtated by Figure 3.41. In this chromatogram, a large proportion of UDP-MurNAc-tetrapeptide can be inferred by the peak at approximately 17 minutes. The moderate peak at 39 minutes represents the UDP-MurNAc-pentadepsipeptide precursor.

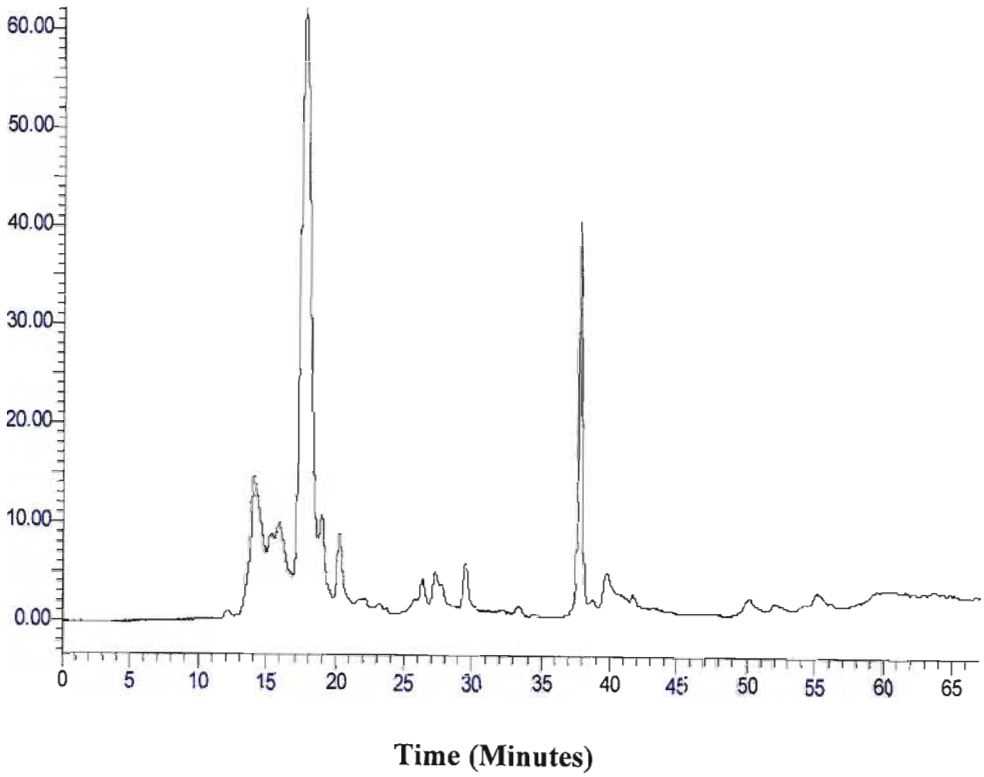

Figure 3.41. HPLC analysis of peptidoglycan precursors from VanA control arrested with 20 μg/ml vancomycin at OD_{252}.

HPLC analysis of peptidoglycan precursors from VanB control arrested with 20 μg/ml vancomycin can be seen in Figure 3.42. Peak 1 at 15minutes was of the UDP-MurNAc-tetrapeptide precursor, whilst a small amount of UDP-MurNAc-pentapeptide is present.

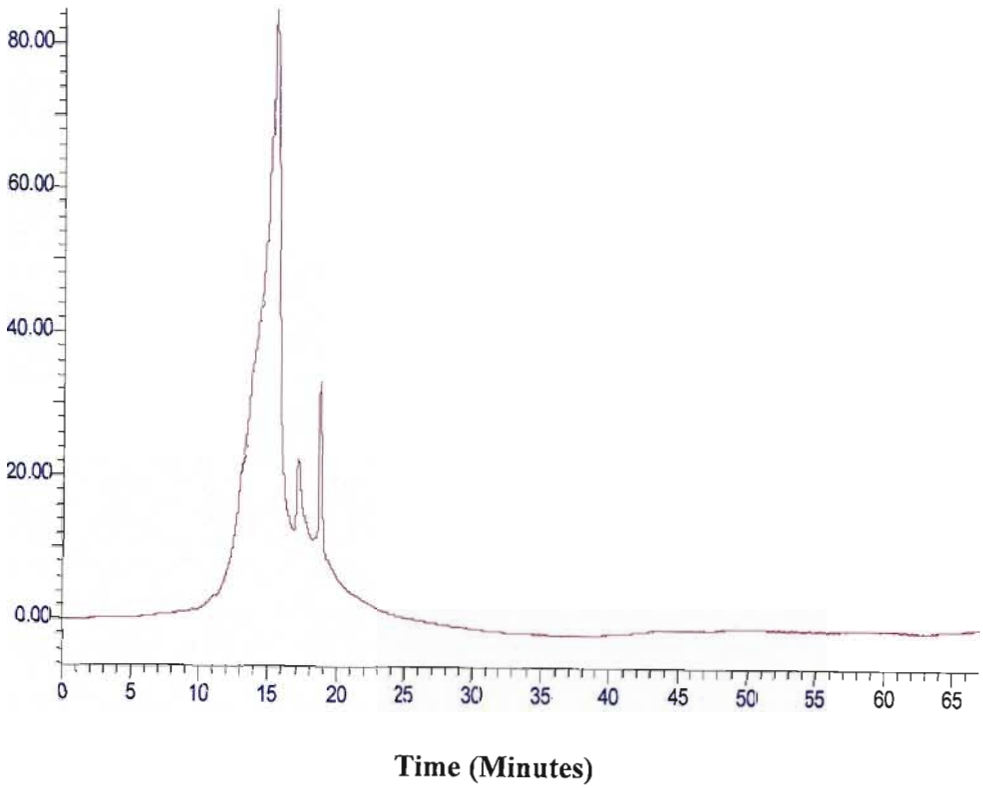

Figure 3.42. HPLC analysis of peptidoglycan precursors from VanB control arrested with 20 μg/ml vancomycin at OD_{252}.

In summary (Table 3.2), only *S. milleri* B200 and *E. faecium* showed a relatively small amount of UDP-MurNAc-tripeptide present. All strains, except for *E. faecalis* 123 contained the cytoplasmic peptidoglycan precursor, UDP-MurNAc-tetrapeptide in large proportions. UDP-MurNAc-pentapeptide was seen for most of the strains in moderate amounts, except in *E. faecalis* 123 and the VanA control. However, *S. milleri* P35, *E. faecalis* 123 and the VanA control seemed to have the precursor, UDP-MurNAc-pentadesipeptide.

Table 3.2. Summary of the peptidoglycan precursors accumulated analysed by HPLC

Strain	Tri-peptide (7.7min)	Tetra-peptide (16min)	Penta-peptide (25min)	Pentadepsi-peptide (42min)
In Vancomycin:				
S. milleri P213		XX	XXX	
S. milleri P35		XXX		XX
S. milleri B200	X	XXX	XX	
E. faecalis 123				XXX
E. faecalis 126		XX	XXX	
E. faecium	X	XXX	XX	
VanA Control		XXX		XX
VanB Control		XXX	X	
In Bacitracin:				
S. milleri P213		X	XX	
S. milleri P35		XXX	XX	
S. milleri B200		XXX	XX	
E. faecalis 123	X	XX	XXX	
E. faecalis 126				XXX
E. faecium		XXX	XX	
VanA Control		XXX	X	
VanB Control		XXX	XX	

X – shows a small amount of precursors accumulated

XX – shows a moderate proportion of precursors accumulated

XXX – shows a relatively large amount of precursors accumulated

De Jonge *et al.*, 1996, found in their studies that UDP-MurNAc-tetrapeptide was produced abundantly and identified as the major dimer. The reason this occurs is because vancomycin obstructs the integration of pentapeptides (D-Ala-D-Ala) and the most appropriate acceptors available in the transpeptidation reaction are tetrapeptides (6, 33). Now that the tetrapeptide is the appropriate acceptor, depsipeptide precursors can be used as donors in the transpeptidation reaction (33). The terminal residue of the donor result in the peptidoglycan deficient lactate residues leading to the formation of tetrapeptides from the pentapeptides (33). UDP-MurNAc-tetrapeptide-D-Lac has a lower affinity for vancomycin and its accumulation when peptidoglycan synthesis is inhibited by vancomycin implies that the active site is inside the cytoplasm (19). Billot-Klein *et al.*, 1996, found in their studies that after the induction of vancomycin resistance, a lingering amount of normal UDP-MurNAc- pentapeptide was present during the synthesis of the new UDP-MurNAc-tetrapeptide phenotype (19).

The presence of tetrapeptides can also be attributed to VanY hydrolysing cytoplasmic peptidoglycan precursors and does not act as an intermediate in precursor synthesis (6, 33). The precursor can then be incorporated into the cell wall and acts as an acceptor in transpeptidation reactions. This allows for the sufficient cross-linking for cell wall integrity. This is afforded only if there are adequate D-Ala-D-Lac ending precursors to act as donors. This step is necessary for resistance since it involves the removal of D-Ala-D-Ala ending precursors (6). D-lactate is incorporated into the UDP-muramyl precursor as D-Alanyl-D-Lactate, forming a depsipeptide, which is now resistant to vancomycin binding (52). This substitution in peptidoglycan production prevents binding of vancomycin to the cell wall components and allows the polymerisation of peptidoglycan in the presence of the antibiotic (5). Strains that are now resistant rely completely on production of peptidoglycan precursors containing D-Ala-D-Lac for growth (35). The presence of UDP-MurNAc-tetrapeptide and altered peptidoglycan cross bridges contribute to resistance (19).

In resistant strains, the D, D carboxyesterase activity is responsible for enzymatically excising the lactate residue from the stem peptide. This is possible because the resistant strain has the *vanY* gene. This gene is implicated in the removal of the D-Alanine residue from the D-Ala-D-Ala pentapeptide precursor. It is also proposed to have a highly selective hydrolytic activity for the depsipeptide bonds. D-Alanine infiltrates the

cytoplasm and fuels the synthesis of the pentapeptide. This is done by VanY, which is a D, D-carboxypeptidase. This enzyme contributes to vancomycin resistance by hydrolysing D-Ala or D-Lac of peptidoglycan precursors (6). However, this enzyme is not sufficient for resistance. VanY can remove pentapeptide at the expense of the formation of some tetrapeptide from pentadepsipeptide thereby increasing the level of resistance (5, 6). The production of pentapeptide together with pentadepsipeptide leads to susceptibility to the antibiotic (6). In resistant strains, the pentapeptide is more efficient at competing for the binding of vancomycin (19). In vancomycin-induced cells, a portion of the cytoplasmic membrane reduces the access of vancomycin to the pentapeptide. The D-Ala-D-Ala moiety of the pentapeptide prevents binding of vancomycin by preventing binding of the induced protein to the pentapeptide leading to the ensuing alterations and this is the most probable mechanism of vancomycin resistance. The pentapeptide binding sites appears to be similar to enzymes that bind D-Ala-D-Ala. These enzymes are those that are utilized in the metabolism of the dipeptide, or the pentapeptide, or in the polymerisation of the disaccharide-pentapeptide. The task of these proteins resembles those of a carboxypeptidase or of a transpeptidase. The transformed cell shape of vancomycin-induced strains implies that the normal synthesis of peptidoglycan by the cell wall enzymes does not occur. Normal synthesis is prevented because of the proteins binding to the customized pentapeptide (2).

Since these strains are of the VanC phenotype, the VanC ligase favours the production of a pentapeptide. The pentapeptide terminates in D-Ala-D-Ser and weakens the binding of the antibiotic to the new pentapeptide. VanT, which is a serine racemase, VanC D-Ala : D-Ser ligase and $VanXY_C$ is responsible for the synthesis of D-serine by eliminating the D-Ala terminating peptidoglycan precursor (27). This last enzyme, $VanXY_C$ has a very low dipeptidase activity against D-Ala-D-Ser and has no activity against UDP-MurNAc-pentapeptide terminating in D-Ser. The elimination of D-Ala is catalyzed by this single protein, $VanXY_C$ in order to purge 'susceptible' precursors. However, the accumulation of tetrapeptides indicates that the elimination of D-Ala-D-Ala is not complete (4).

Cells that express the *vanA* gene complex usually have UDP-MurNAc-pentapeptide in the wall precursor pool. This precursor does not inhibit the D-Ala : D-Ala ligase activity since VanA cannot hamper the binding of vancomycin to its target. This occurs because an alternative cell wall biosynthetic pathway is activated. The vancomycin-sensitive step is

avoided, by producing an abnormal cell wall precursor in which the dipeptide D-Ala-D-Ala is substituted by D-Ala-D-Lac, which is vancomycin-insensitive (94). VanA and VanB facilitate the synthesis of pentadepsipeptides ending in D-Ala-D-Lac since they are structurally similar (12, 27). Vancomycin resistance of both the VanA and VanB control are due to the assembly of these peptidoglycan precursors that bind to vancomycin with reduced affinity. Arthur *et al.*, 1992, propose that in resistant cells, the pathway for the peptidoglycan precursor production is by depsipeptide synthesis (10).

3.11.2. HPLC Profiles of Precursors Arrested With 100 μg/ml Bacitracin

Bacitracin was used to amplify the cell wall precursors from each of the bacterial strains. It is an antibiotic produced by strains of *Bacillus licheniformis* and is a mixture of high molecular weight polypeptides, which acts bactericidally (82, 97). This antibiotic is an inhibitor of the lipid phosphatase in which the *N*-acetylmuramyl peptide is transferred from UDP to a lipid carrier (82). It is then modified to form a complete emerging peptidoglycan subunit. This process occurs on the inner surface of the cytoplasmic membrane and ends with the translocation of the terminated subunit to the exterior of the cytoplasmic membrane. Hence, bacitracin inhibits the dephosphorylation reaction and in the absence of monophosphorylated carrier peptidoglycan synthesis stops. Normally the peptidoglycan subunit is passed across the cytoplasmic membrane attached to the undecaprenol diphosphate. After the growing peptidoglycan monomer leaves the carrier on reaching the cell wall, the undecaprenol diphosphate is dephosphorylated to its monophosphate form (91). This reaction is essential for restoration of the lipid carrier required for the cyclic synthesis of peptidoglycan (97). Therefore, bacitracin is also thought to induce resistance since one of its main targets is subsequent the transglycosylation step which polymerises the intermediates into peptidoglycan (14). Therefore, inhibition of peptidoglycan polymerisation is critical for induction (12).

Figure 3.43. represents the cytoplasmic peptidoglycan precursor of *S. milleri* P213 arrested with 100 μg/ml. The relatively small peak at 15 minutes is that of UDP-MurNAc-tetrapeptide. At 27 minutes, a fairly moderate amount of UDP-MurNAc-pentapeptide was seen.

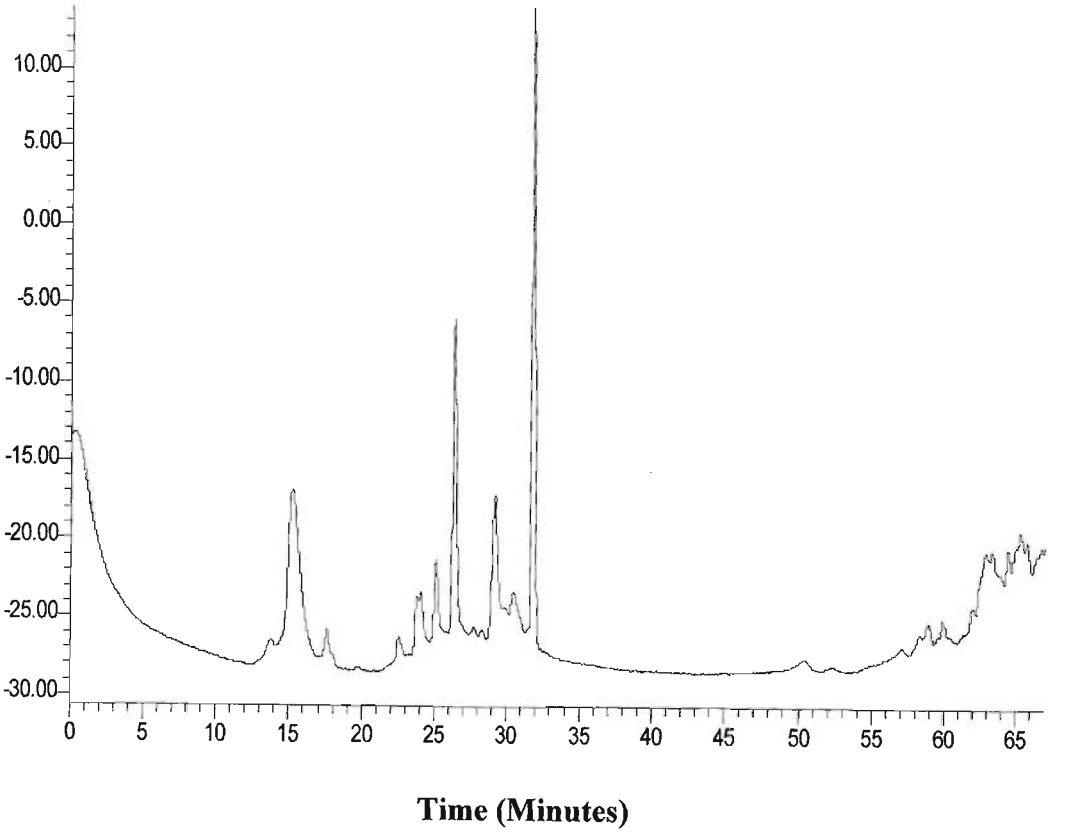

Figure 3.43. HPLC analysis of peptidoglycan precursors from *S. milleri* P213 arrested with 100 μg/ml bacitracin at OD_{252}.

Analysis of the peptidoglycan precursor of *S. milleri* P35 by HPLC can be seen in Figure 3.44. A high proportion of UDP-MurNAc-tetrapeptide was present at 15 minutes, whilst a moderate amount of UDP-MurNAc-pentapeptide was seen at 20 minutes.

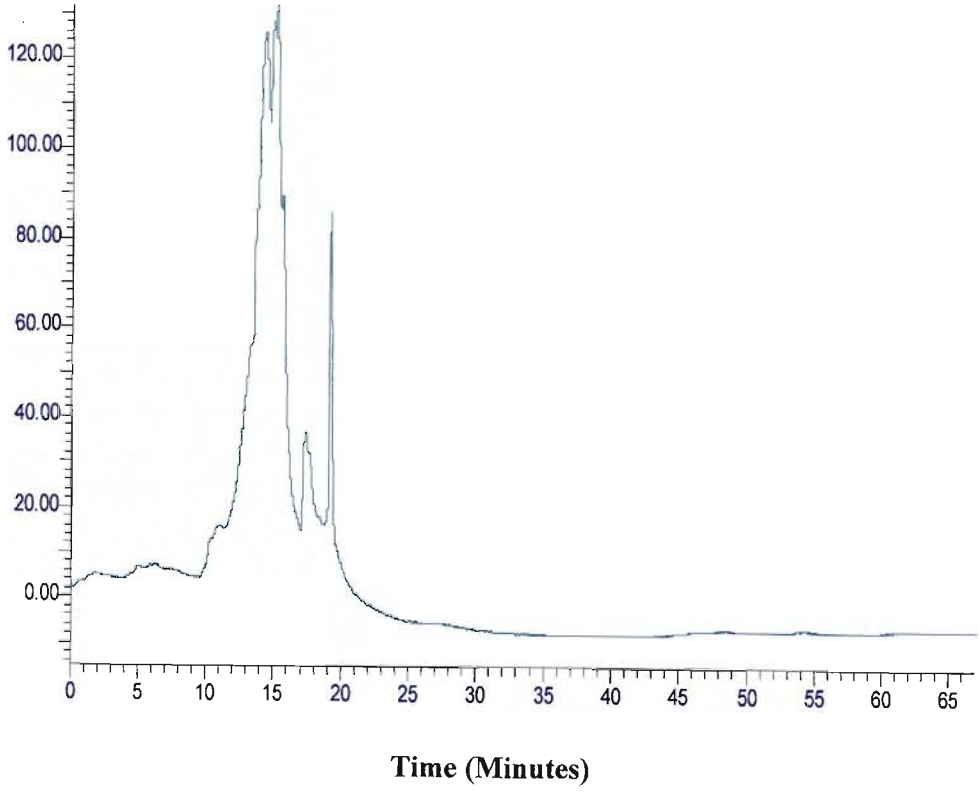

Figure 3.44. HPLC analysis of peptidoglycan precursors from *S. milleri* P35 arrested with 100 μg/ml bacitracin at OD_{252} .

Peptidoglycan precursor of *S. milleri* B200 was extracted and analysed in Figure 3.45. The chromatogram showed a high amount of UDP-MurNAc-tetrapeptide eluted at 15 minutes followed by a moderate proportion of UDP-MurNAc-pentapeptide at 20 minutes.

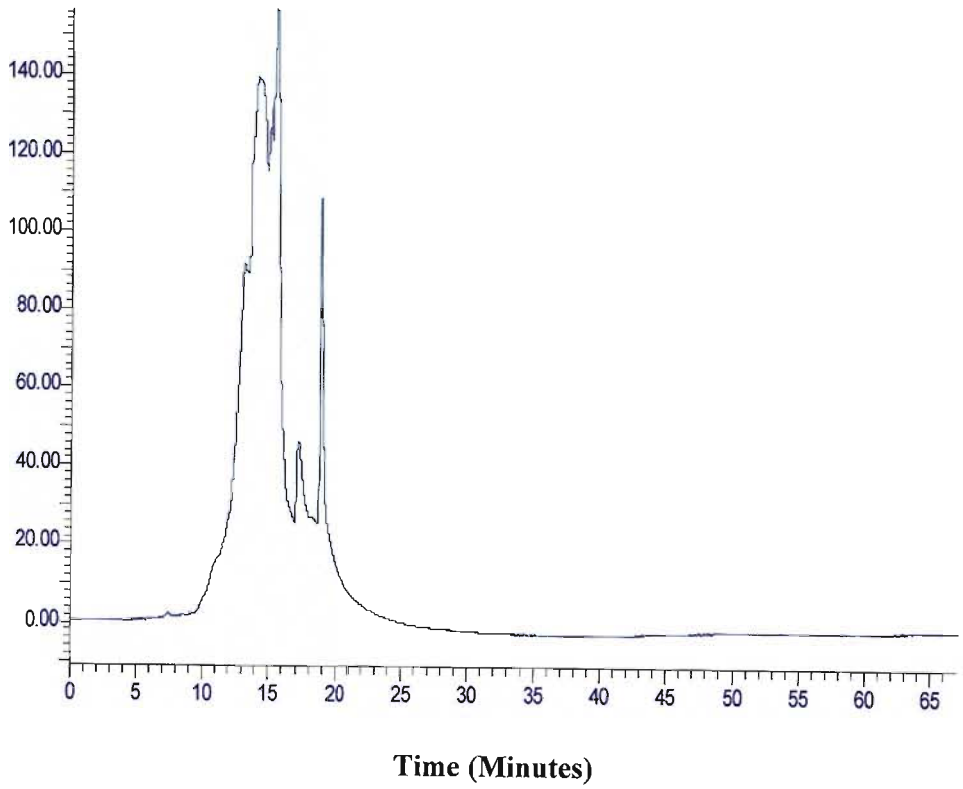

Figure 3.45. HPLC analysis of peptidoglycan precursors from *S. milleri* B200 arrested with 100 μg/ml bacitracin at OD_{252}.

HPLC analysis of peptidoglycan precursors from *E. faecalis* 123 arrested with 100 μg/ml bacitracin at OD_{252} is represented by the chromatogram in Figure 3.46. A relatively small amount of UDP-MurNAc-tripeptide was present compared to the moderate amount of UDP-MurNAc-tetrapeptide at 15 minutes. The high peak at 20 minutes showed the high proportion of UDP-MurNAc-pentapeptide present.

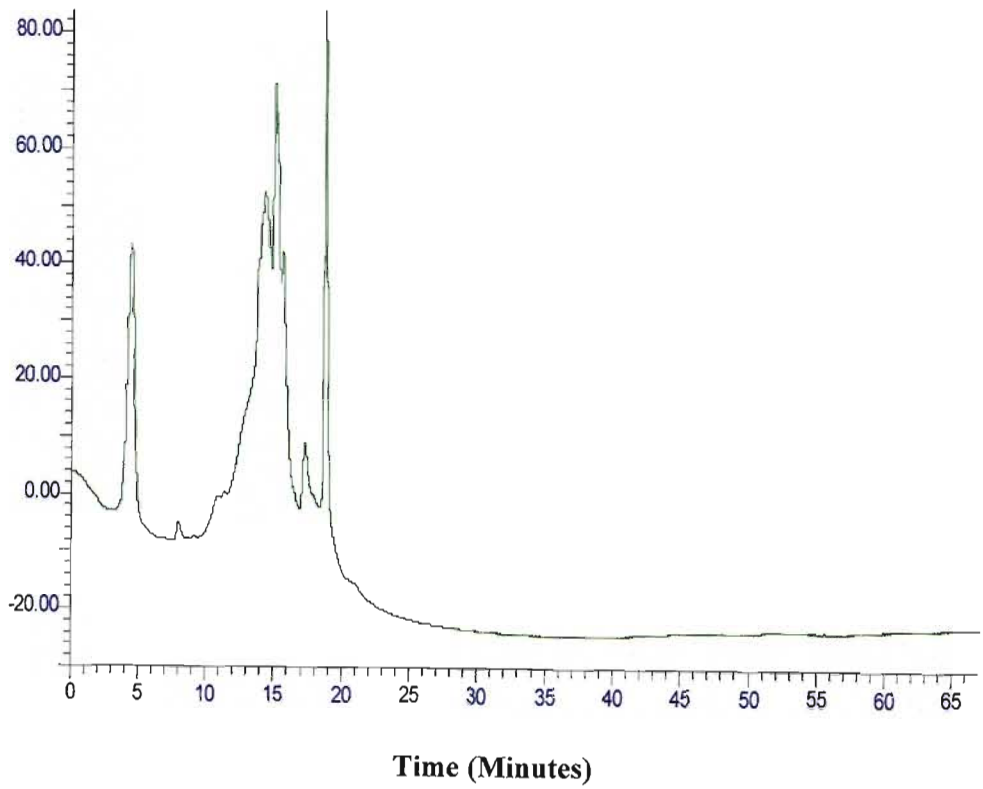

Figure 3.46. HPLC analysis of peptidoglycan precursors from *E. faecalis* 123 arrested with 100 μg/ml bacitracin at OD_{252}.

The chromatogram in Figure 3.47. shows the HPLC analysis of the cytoplasmic peptidoglycan precursor of *E. faecalis* 126 extracted with bacitracin. A relatively high peak showed a large amount of UDP-MurNAc-pentadepsipeptide was seen being eluted at 40 minutes.

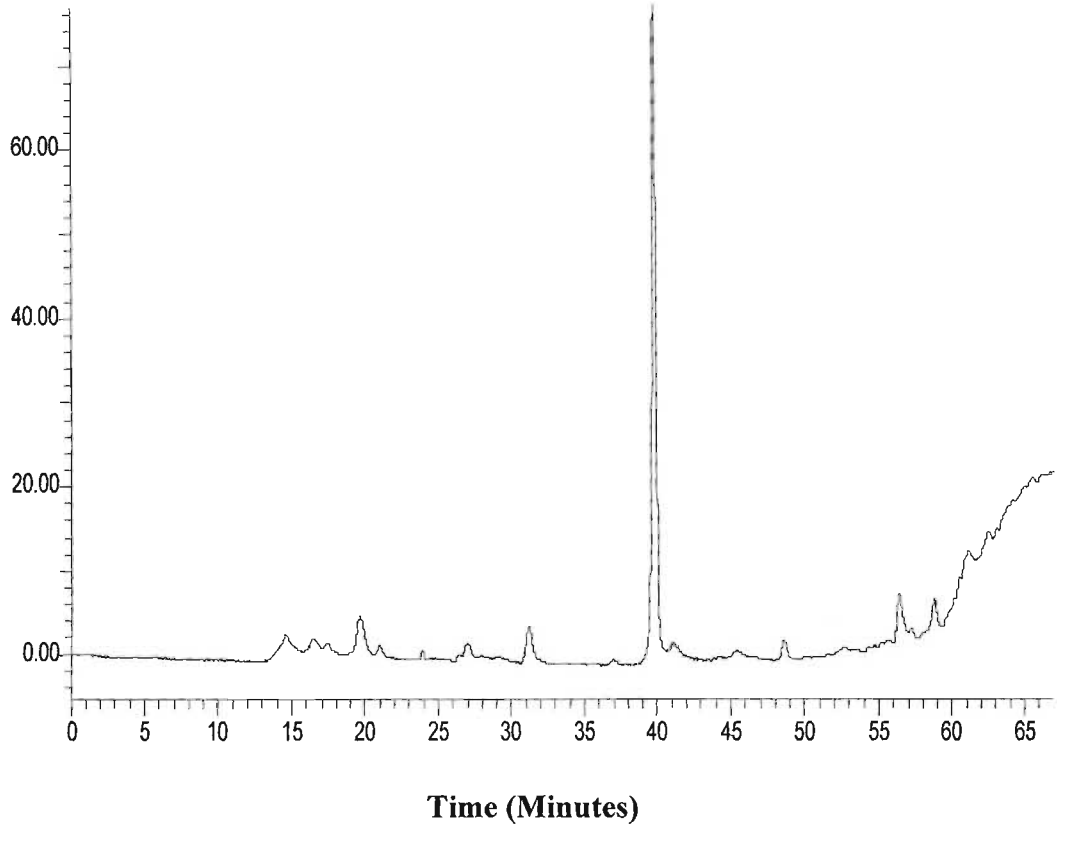

Figure 3.47. HPLC analysis of peptidoglycan precursors from *E. faecalis* 126 arrested with 100 μg/ml bacitracin at OD_{252}.

HPLC separation of cytoplasmic peptidoglycan precursor of *E. faecium* is shown in Figure 3.48. This figure shows a high amount of UDP-MurNAc-tetrapeptide present at 16 minutes and moderate proportion of UDP-MurNAc-pentapeptide was also seen at 20 minutes.

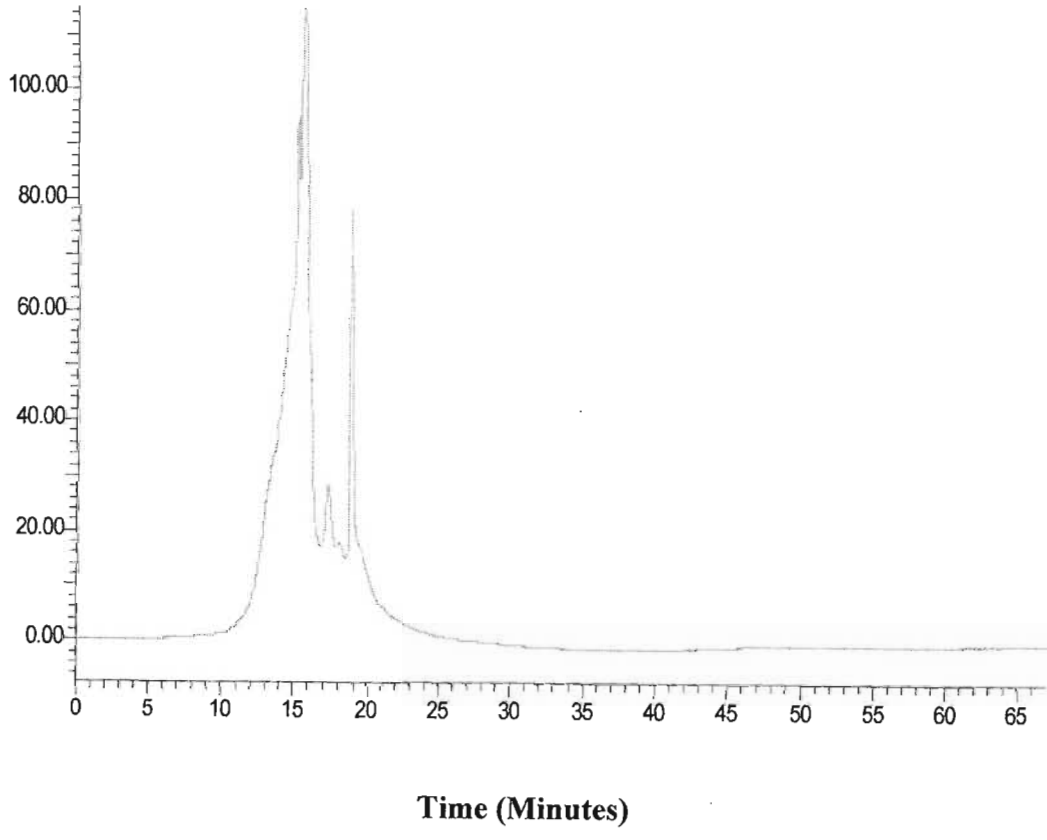

Figure 3.48. HPLC analysis of peptidoglycan precursors from *E. faecium* arrested with 100 μg/ml bacitracin at OD_{252}.

The cytoplasmic peptidoglycan precursor of the VanA control is analysed by HPLC as can be seen in Figure 3.49. This chromatogram showed a high amount of UDP-MurNAc-tetrapeptide eluted at 15 minutes. This chromatogram also illustrates a relatively small proportion of UDP-MurNAc-pentapeptide present as can be seen in the peak at 20 minutes.

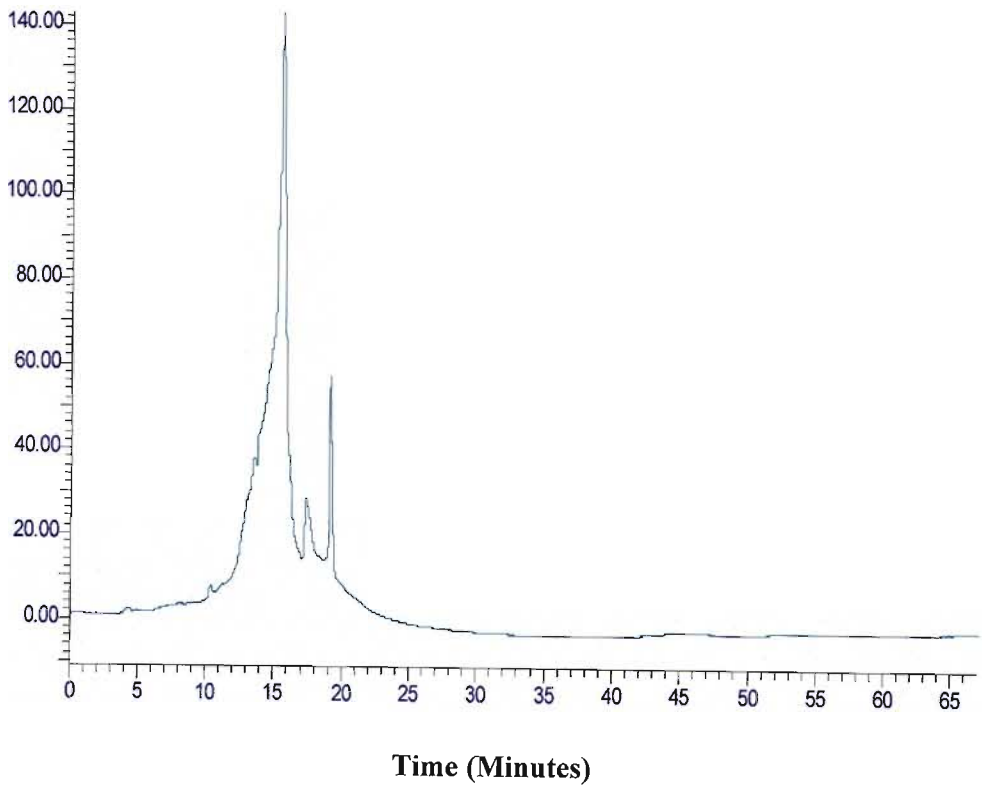

Figure 3.49. HPLC analysis of peptidoglycan precursors from VanA control arrested with 100 μg/ml bacitracin at OD_{252}.

Two peaks can be seen in the HPLC chromatogram in Figure 3.50. This figure is that of the cytoplasmic peptidoglycan precursor of the VanB control extracted with bacitracin. The first peak at 15 minutes represents UDP-MurNAc-tetrapeptide found in a relatively large proportion compared to the small amount of UDP-MurNAc-pentapeptide present at 20 minutes.

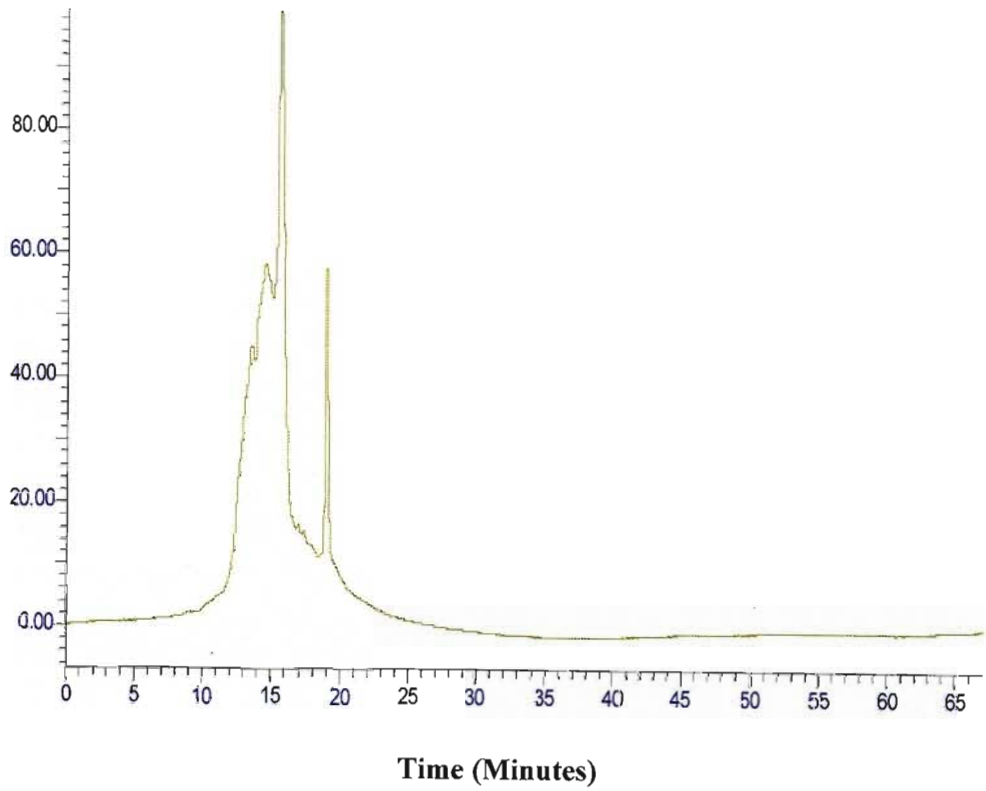

Figure 3.50. HPLC analysis of peptidoglycan precursors from VanB control arrested with 100 μg/ml bacitracin at OD_{252}.

A summary of the results obtained can be seen in Table 3.2. Analyses of the cytoplasmic peptidoglycan precursors arrested with bacitracin showed that UDP-MurNAc-tripeptide was present only in the bacterial strain, *E. faecalis* 123. Most of the strains except *E. faecalis* 126 had the precursor UDP-MurNAc-tetrapeptide. This precursor was found in relatively large amounts. UDP-MurNAc-pentapeptide was found in moderate amounts in all strains except in *E. faecalis* 126. Whilst *E. faecalis* 126 was the only strain to contain the UDP-MurNAc-pentadepsipeptide precursor.

Bacitracin is thought to lead to the accumulation of cytoplasmic peptidoglycan precursors, UDP-MurNAc-pentapeptides since it follows transglycosylation. As expected, all the strains accrued this precursor, except for *E. faecalis* 126. However, the presence of UDP-MurNAc-tetrapeptide should not have occurred. The presence of tetrapeptides implies that the eradication of D-Ala-D-Ala was partial. The elimination of the D-Ala-D-Ala precursor is vital for resistance since the attachment of the antibiotics to the lipid intermediate II at the outer surface of the membrane is expected to remove the lipid carrier. This prevents the incorporation of the D-Ala-D-Lac precursor into peptidoglycan (6). The lack of pentadepsipeptides being detected suggests that D-Ala-D-Ala was not incorporated into the peptidoglycan. If this precursor is not incorporated into the growing peptidoglycan, then the cell must surely be resistant to the antibiotic, vancomycin, since under normal conditions, a cell that is susceptible to the action of vancomycin would have the normal D-Ala-D-Ala precursor. This again confirms that these strains are resistant strains.

The trends obtained for precursors with the arrest by bacitracin are very similar to that accumulated by vancomycin, in that a large amount of tetrapeptides were present. This was followed by a moderate amount of UDP-MurNAc-pentapeptides in each strain, except in *E. faecalis* 126. By synthesizing the peptidoglycan precursor, UDP-MurNAc-pentapeptide, these resistant strains avoid the inhibition of cell wall assembly by vancomycin and bacitracin. It has been suggested that the accumulation of cell wall precursors may be the signal that prompt the expression of resistant genes in these strains.

The precursors obtained from HPLC analysis are not conclusive enough based only on their retention times. Consequently mass spectrometry was performed to confirm these precursors.

3.12. Analysis of Mass Spectrometry Data

Peaks of the precursors following HPLC were collected at the appropriate retention times. Mass spectrometry was performed on these samples in order to determine the molecular masses to reaffirm the appropriate precursors.

A summary of the molecular masses obtained is shown in Table 3.3. The molecular masses of the normal precursors are the mass calculated, in the table. The mass observed is the molecular mass obtained by mass spectrometry with a difference of one m/z. By comparing the mass observed and those calculated they are closely related. This confirms that the peaks obtained during HPLC analysis at the various retention times were in fact the correctly identified precursors.

Table 3.3. Molecular masses of the peptidoglycan precursors isolated from the bacterial strains

Peaks	Mass Observed	Mass Calculated
Tri - peptide	1284 +/- 1 m/z	1286.16
Tetra – peptide	1376 +/- 1 m/z	1375.16
Penta – peptide	1462 +/- 1 m/z	1464.16
Penta – depsipeptide	1481 +/- 1 m/z	1480.16

CHAPTER FOUR

CONCLUSION AND FUTURE PROSPECTS

To effectively harness and contain the spread of the prevalence of vancomycin resistance in strains such as Streptococci, Enterococci and *Staphylococcus aureus*, high-throughput screening methods need to be in place. The current conventional screening methods are not efficient enough to detect the presence of resistant determinants because of the increasing changes in susceptibility patterns of pathogenic organisms. Molecular assays are now being applied for the detection of resistance genes and prove to be more superior and advanced in identifying the presence of the resistant genes in bacterial strains. However, molecular based detection requires the correct tools to provide the answers. A key factor used in these assays is the appropriate sequence of oligonucleotides, used as primers in PCR, as probes in southern blot/hybridisation and also for DNA sequencing. The first set of primers chosen, were based on those used by Miele *et al.*, 1995. The results obtained from PCR, southern blot/hybridisation and DNA sequencing using these primers was inconclusive. The results did not relate to each other and were also discrepant with the MIC values obtained.

The second set of primers developed by Dukta-Malen *et al.*, 1995, proved to be successful in screening for resistance determinants in the strains used in this study. Results obtained from the southern blots and sequencing reaffirmed accurately those attained by PCR. These also relate to the MIC values. This implies that the genotypes of the strains were reliably correlated with their phenotypes. This proves that PCR is efficient and consistent enough to be used in the elucidation of vancomycin resistant genes. It is also convenient since it is a rapid and simple technique that is sensitive enough to also detect low-level resistance such as that of the VanC phenotype. The addition of already well-characterized vancomycin resistant Enterococci served as a guide to differentiate the false positives from the false negatives.

Additional analysis was performed using HPLC. This method took a closer look at the cytoplasmic peptidoglycan precursors arrested with the antibiotics, vancomycin and bacitracin. The accumulation of UDP-MurNAc-tetrapeptides and UDP-MurNAc-pentapeptides further confirmed the resistance status of these strains. The molecular masses obtained by mass spectrometry correctly identified the precursors. Hence, in this study the confirmatory tests were accurately concurrent with the conventional phenotypic screening tests. From this study, the conventional screening method, such as susceptibility testing via micro-titre plate dilution or disk-diffusion assays should serve as a first screen.

However, PCR seemed to be the most specific, reliable and rapid amongst the assays tested, in particular for the detection of VanC resistant genes, which have low levels of resistance to vancomycin.

Innovative techniques must be developed for the rapid microbiological diagnosis of these strains. Pulsed-Field Gel Electrophoresis (PFGE) seems to be a useful discriminatory DNA-based technique that provides reproducible results. Another promising method for the detection of resistant genes and mutational resistance is DNA microarray technology (98). The continued research into the development of new drugs and sound drug design offer the prospect of possible eradication of the emerging resistant phenotypes. The key to solving this problem is to find an antibiotic that is active against these strains, one that binds to peptidoglycan precursor terminating with the depsipeptide, D-Ala-D-Lac. Another would be to find an inhibitor of the D-Ala: D-Ala ligase (5). A set of carbohydrates derivatives of vancomycin that are active against bacterial strains that are resistant were discovered (48). Long-term solutions, such as vaccine-based immunotherapies and gene therapies need to be explored to abolish the problem of drug resistance, like vancomycin resistance (50).

CHAPTER FIVE

REFERENCES

1. **Allen, N. E., J. N. Hobs, Jr., and T. I. Nicas.** 1996. Inhibition of peptidoglycan biosynthesis in vancomycin-susceptible and -resistant bacteria by a semisynthetic glycopeptide antibiotic. Antimicrobial Agents and Chemotherapy **40:**2356-2362.

2. **Al-Obeid, S., E. Collatz, and L. Gutmann.** 1990. Mechanism of resistance to vancomycin in *Enterococcus faecium* D366 and *Enterococcus faecalis* A256. Antimicrobial Agents and Chemotherapy **34:**252-256.

3. **Arias, C. A., P. Courvalin, and P. E. Reynolds.** 2000. VanC cluster of vancomycin-resistant *Enterococcus gallinarum* BM4174. Antimicrobial Agents and Chemotherapy **44:**1660-6.

4. **Arias, C. A., J. Weisner, J. M. Blackburn, and P. E. Reynolds.** 2000. Serine and alanine racemase activities of VanT: a protein necessary for vancomycin resistance in *Enterococcus gallinarum* BM4174. Microbiology **146:**1727-34.

5. **Arthur, M., and P. Courvalin.** 1993. Genetics and mechanisms of glycopeptide resistance in Enterococci. Antimicrobial Agents and Chemotherapy **37:**1563-1571.

6. **Arthur, M., F. Depardieu, L. Cabanie, P. E. Reynolds, and P. Courvalin.** 1998. Requirement of the VanY and VanX D, D-peptidases for glycopeptide resistance in Enterococci. Molecular Microbiology **31**.

7. **Arthur, M., F. Depardieu, C. Molinas, P. Reynolds, and P. Courvalin.** 1995. The vanZ gene of Tn1546 from *Enterococcus faecium* BM4147 confers resistance to teicoplanin. Gene **154:**87-92.

8. **Arthur, M., F. Depardieu, H. A. Snaith, P. E. Reynolds, and P. Courvalin.** 1994. Contribution of VanY D, D-carboxypeptidase to glycopeptide resistance in *Enterococcus faecalis* by hydrolysis of peptidoglycan precursors. Antimicrobial Agents and Chemotherapy **38:**1899-903.

9. **Arthur, M., C. Molinas, T. D. H. Bugg, G. D. Wright, C. T. Walsh, and P. Courvalin.** 1992. Evidence for In Vivo incorporation of D-lactate into

peptidoglycan precursors of vancomycin-resistant Enterococci. Antimicrobial Agents and Chemotherapy **36:**867-869.

10. **Arthur, M., C. Molinas, and P. Courvalin.** 1992. The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. Journal of Bacteriology **174:**2582-2591.

11. **Arthur, M., C. Molinas, F. Depardieu, and P. Courvalin.** 1993. Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. Journal of Bacteriology **175:**117-27.

12. **Arthur, M., and R. Quintiliani, Jr.** 2001. Regulation of VanA- and VanB-type glycopeptide resistance in Enterococci. Antimicrobial Agents and Chemotherapy **45:**375-81.

13. **Arthur, M., P. Reynolds, and P. Courvalin.** 1996. Glycopeptide resistance in Enterococci. Trends in Microbiology **4:**401-7.

14. **Baptista, M., F. Depardieu, P. Courvalin, and M. Arthur.** 1996. Specificity of induction of glycopeptide resistance genes in *Enterococcus faecalis*. Antimicrobial Agents and Chemotherapy **40:**2291-2295.

15. **Baptista, M., F. Depardieu, P. E. Reynolds, P. Courvalin, and M. Arthur.** 1997. Mutations leading to increased levels of resistance to glycopeptide antibiotics in VanB-type Enterococci. Molecular Microbiology **25:**93-105.

16. **Baptista, M., P. Rodrigues, F. Depardieu, P. Courvalin, and M. Arthur.** 1999. Single-cell analysis of glycopeptide resistance gene expression in teicoplanin-resistant mutants of a VanB-type *Enterococcus faecalis*. Molecular Microbiology **32:**17-28.

17. **Beauregard, D. A., A. J. Maguire, D. H. Williams, and P. E. Reynolds.** 1997. Semiquantitation of cooperativity in binding of vancomycin-group antibiotics to vancomycin-susceptible and -resistant organisms. Antimicrobial Agents and Chemotherapy **41:**2418-2423.

18. **Billot-Klein, D., L. Gutmann, D. Bryant, D. Bell, J. van Heijenoort, J. Grewal, and D. M. Shlaes.** 1996. Peptidoglycan synthesis and structure in *Staphylococcus haemolyticus* expressing increasing levels of resistance to glycopeptide antibiotics. Journal of Bacteriology **178:**4696-4703.

19. **Billot-Klein, D., D. M. Shlaes, D. Bryant, D. Bell, J. van Heijenoot, and L. Gutmann.** 1996. Peptidoglycan structure of *Enterococcus faecium* expressing vancomycin resistance of the VanB type. Biochemistry Journal **313:**711-715.

20. **Bouhss, A., N. Josseaume, D. Allanic, M. Crouvoisier, L. Gutmann, J. L. Mainardi, D. Mengin-Lecreulx, J. van Heijenoort, and M. Arthur.** 2001. Identification of the UDP-MurNAc-pentapeptide:L-alanine ligase for synthesis of branched peptidoglycan precursors in *Enterococcus faecalis*. Journal of Bacteriology **183:**5122-7.

21. **Boyd, D. A., J. Conly, H. Dedier, G. Peters, L. Robertson, E. Slater, and M. R. Mulvey.** 2000. Molecular characterization of the vanD gene cluster and a novel insertion element in a vancomycin-resistant Enterococcus isolated in Canada. Journal of Clinical Microbiology **38:**2392-4.

22. **Brisson-Noel, A., S. Dukta-Malen, C. Molinas, R. Leclercq, and P. Courvalin.** 1990. Cloning and heterospecific expression of the resistant determinant *vanA* encoding high-level resistance to glycopeptides in *Enterococcus faecium* BM4147. Antimicrobial Agents and Chemotherapy **34:**924-927.

23. **Brotz, H., G. Bierbaum, K. Leopold, P. E. Reynolds, and H. G. Sahl.** 1998. The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. Antimicrobial Agents and Chemotherapy **42:**154-60.

24. **Bugg, T. D. H., S. Dukta-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Identification of vancomycin resistant protein VanA as a D-alanine: D-alanine ligase of altered substrate specificity. Biochemistry **30:**2017-2021.

25. **Bugg, T. D. H., G. D. Wright, S. Dukta-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: Biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry **30:**10408-10415.

26. **Casadewall, B., and P. Courvalin.** 1999. Characterization of the *vanD* glycopeptide resistance gene cluster from *Enterococcus faecium* BM4339. Journal of Bacteriology **181:**3644-3648.

27. **Cetinkaya, Y., P. Falk, and C. G. Mayhall.** 2000. Vancomycin-resistant Enterococci. Clinical Microbiology Reviews **13:**686-707.

28. **Cookson, B.** 2005. Clinical significance of emergence of bacterial antimicrobial resistance in the hospital environment. Journal of Applied Microbiology **10:**1365-1377.

29. **Cooper, M. A., and D. H. Williams.** 1999. Binding of glycopeptide antibiotics to a model of a vancomycin-resistant bacterium. Chemistry & Biology **6:**891-899.

30. **Courvalin, P.** 2002. Glycopeptide resistance in enterococci. Identifying Resistance International Newsletter **2:**1-4.

31. **Courvalin, P., and P. Trieu-Cuot.** 2001. Minimizing potential resistance: The molecular view. Clinical Infectious Disease **33:**S138-46.

32. **David, V., B. Bozdogan, J.-L. Mainardi, R. Legrand, L. Gutmann, and R. Leclercq.** 2004. Mechanism of intrinsic resistance to vancomycin in *Clostridium innocuum* NCIB 10674. Journal of Bacteriology **186:**3415-3422.

33. **de Jonge, B. L., S. Handwerger, and D. Gage.** 1996. Altered peptidoglycan composition in vancomycin-resistant *Enterococcus faecalis*. Antimicrobial Agents and Chemotherapy **40:**863-9.

34. **Depardieu, F., M. G. Bonara, P. E. Reynolds, and P. Courvalin.** 2003. The *vanG* glycopeptide resistance operon from *Enterococcus faecalis* revisited. Molecular Microbiology **50:**931-948.

35. **Depardieu, F., P. Courvalin, and T. Msadek.** 2003. A six amino acid deletion, partially overlapping the $VanS_B$ G2 ATP-binding motif, leads to constitutive glycopeptide ressiatance in VanB-type *Enterococcus faecium*. Molecular Microbiology **50:**1069-1083.

36. **Depardieu, F., P. Reynolds, and P. Courvalin.** 2003. VanD-Type vancomycin-resistant *Enterococcus faecium* 10/96A. Antimicrobial Agents and Chemotherapy **47:**7-18.

37. **Dukta-Malen, S., S. Evers, and P. Courvalin.** 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant Enterococci by PCR. Journal of Clinical Microbiology **33:**1434.

38. **Dutka-Malen, S., S. Evers, and P. Courvalin.** 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant Enterococci by PCR. Journal of Clinical Microbiology **33:**1434.

39. **Dutta, I., and P. E. Reynolds.** 2003. The vanC-3 vancomycin resistance gene cluster of *Enterococcus flavescens* CCM 439. Journal of Antimicrobial Chemotherapy **51:**703-6.

40. **(ESCMID), E. C. f. A. S. T. E. o. t. E. S. o. C. M. a. I. D.** 2003. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. European Society of Clinical Microbiology and Infectious Diseases **5.1**.

41. **Evers, S., and P. Courvalin.** 1996. Regulation of VanB-type vancomycin resistance gene expression by the $VanS_B$-$VanR_B$ two-component regulatory system in *Enterococcus faecalis* V583. Journal of Bacteriology **178:**1302-1309.

42. **Evers, S., P. E. Reynolds, and P. Courvalin.** 1994. Sequence of the vanB and ddl genes encoding D-alanine:D-lactate and D-alanine:D-alanine ligases in vancomycin-resistant *Enterococcus faecalis* V583. Gene **140:**97-102.

43. **Evers, S., D. F. Sahm, and P. Courvalin.** 1993. The vanB gene of vancomycin-resistant *Enterococcus faecalis* V583 is structurally related to genes encoding D-Ala:D-Ala ligases and glycopeptide-resistance proteins VanA and VanC. Gene **124:**143-4.

44. **Fines, M., B. Perichon, P. Reynolds, D. F. Sahm, and P. Courvalin.** 1999. VanE, a new type of acquired glycopeptide resistance in *Enterococcus faecalis* BM4405. Antimicrobial Agents and Chemotherapy **43:**2161-4.

45. **Fluit, A. C., M. R. Visser, and F. J. Schmitz.** 2001. Molecular detection of antimicrobial resistance. Clinical Microbiology Reviews **14:**836-71.

46. **Gao, Y.** 2002. Glycopeptide antibiotics and development of inhibitors to overcome vancomycin resistance. Natural Products Report **19:**100-7.

47. **Garnier, F., S. Taourit, P. Glaser, P. Courvalin, and M. Galimand.** 2000. Characterization of transposon Tn1549, conferring VanB-type resistance in Enterococcus spp. Microbiology **146 :**1481-9.

48. **Ge, M., Z. Chen, H. R. Onishi, J. Kohler, L. L. Silver, R. Kerns, S. Fukuzawa, C. Thompson, and D. Kahne.** 1999. Vancomycin derivatives that inhibit peptidoglycan biosynthesis without binding D-Ala-D-Ala. Science **284:**507-11.

49. **Gilmore, M. S., and J. A. Hoch.** 1999. Antibiotic resistance. A vancomycin surprise. Nature **399:**524-5, 527.

50. **Gold, H. S.** 2001. Vancomycin-resistant Enterococci: Mechanisms and clinical observations. Clinical Infectious Disease **33:**210-219.

51. **Goldman, R. C., and D. Gange.** 2000. Inhibition of transglycosylation involved in bacterial peptidoglycan synthesis. Current Medicinal Chemistry **7:**801-820.

52. **Gutmann, L., D. Billot-Klein, S. Al-Obeid, I. Klare, E. Francoual, E. Collatz, and J. van Heijenoot.** 1992. Inducible carboxypeptidase activity in vancomycin-resistant Enterococci. Antimicrobial Agents and Chemotherapy **36:**77-80.

53. **Handwerger, S., M. J. Pucci, K. J. Volk, J. Liu, and M. S. Lee.** 1992. The cytoplasmic peptidoglycan precursor of vancomycin-resistant *Enterococcus faecalis* terminates in lactate. Journal of Bacteriology **174:**5982-4.

54. **Healy, V. L., I. A. Lessard, D. I. Roper, J. R. Knox, and C. T. Walsh.** 2000. Vancomycin resistance in Enterococci: reprogramming of the D-Ala-D-Ala ligases in bacterial peptidoglycan biosynthesis. Chemistry & Biology **7:**109-119.

55. **Healy, V. L., L. S. Mullins, X. Li, S. E. Hall, F. M. Raushel, and C. T. Walsh.** 2000. D-Ala-D-X ligases: evaluation of D-alanyl phosphate intermediate by MIX, PIX and rapid quench studies. Chemistry & Biology **7:**505-14.

56. **Henriques Normark, B., R. Novak, A. Ortqvist, G. Kallenius, E. Tuomanen, and S. Normark.** 2001. Clinical isolates of *Streptococcus pneumoniae* that exhibit tolerance of vancomycin. Clinical Infectious Disease **32:**552-8.

57. **Hogan, D., and R. Kolter.** 2002. Why are bacteria refractory to antimicrobials? Current Opinions in Microbiology **5:**472-7.

58. **Kawalec, M., M. Gniadkowski, J. Kedzierska, A. Skotnicki, J. Fiett, and W. Hryniewicz.** 2001. Selection of a teicoplanin-resistant *Enterococcus faecium* mutant during an outbreak caused by vancomycin-resistant Enterococci with the VanB phenotype. Journal of Clinical Microbiology **39:**4274-4282.

59. **Kirst, H. A., D. G. Thompson, and T. I. Nicas.** 1998. Historical yearly usage of vancomycin. Antimicrobial Agents and Chemotherapy **42:**1303-4.

60. **Koch, A. L.** 2001. Autolysis control hypotheses for tolerance to wall antibiotics. Antimicrobial Agents and Chemotherapy **45:**2671-5.

61. **Kolar, M., I. Vagnerova, T. Latal, and I. Kohnova.** 1999. Occurrence of vancomycin-resistant Enterococci in relation to the administration of glycopeptide antibiotics. Acta Universitatis Palackianae Olomucensis Facultatis Medicae **142:**69-71.

62. **Kuhn, I., A. Iversen, M. Finn, C. Greko, L. G. Burman, A. R. Blanch, X. Vilanova, A. Manero, H. Taylor, J. Caplin, L. Dominguez, I. A. Herrero, M. A. Moreno, and R. Mollby.** 2005. Occurrence and relatedness of vancomycin-resistant Enterococci in animals, humans, and the environment in different European regions. Applied and Environmental Microbiology **71:**5383-90.

63. **Lessard, I. A. D., and C. T. Walsh.** 1999. VanX, a bacterial D-alanyl-D-alanine dipeptidase: Resistance, immunity, or survival function? Proceedings of National Academic Scientific. USA **96:**11028-11032.

64. **Marshall, C. G., G. Broadhead, B. K. Leskiw, and G. D. Wright.** 1997. D-Ala-D-ala ligases from glycopeptide antibiotic-producing organisms are highly homologous to the enterococcal vancomycin-resistance ligases VanA and VanB. Proceedings of National Academic Scientific. USA **94:**6480-6483.

65. **Marshall, C. G., I. A. D. Lessard, I.-S. Park, and G. D. Wright.** 1998. Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. Antimicrobial Agents and Chemotherapy **42:**2215-2220.

66. **Martinez, J. L., and F. Baquero.** 2000. Mutation frequencies and antibiotic resistance. Antimicrobial Agents and Chemotherapy **44:**1771-7.

67. **McCullers, J. A., B. K. English, and R. Novak.** 2000. Isolation and characterization of vancomycin-tolerant *Streptococcus pneumoniae* from the cerebrospinal fluid of a patient who developed recrudescent meningitis. Journal of Infectious Diseases **181:**369-73.

68. **McKessar, S. J., A. M. Berry, J. M. Bell, J. D. Turnidge, and J. C. Paton.** 2000. Genetic characterization of vanG, a novel vancomycin resistance locus of *Enterococcus faecalis*. Antimicrobial Agents and Chemotherapy **44:**3224-3228.

69. **Mendez-Alvarez, S., X. Perez-Hernandez, and F. Claverie-Martin.** 2000. Glycopeptide resistance in Enterococci. International Microbiology **3:**71-80.

70. **Miele, A., M. Bandera, and B. P. Goldstein.** 1995. Use of primers for vancomycin resistance genes to determine *van* genotype in Enterococci and to study gene organization in VanA isolates. Antimicrobial Agents and Chemotherapy **39:**1772-1778.

71. **Mongodin, E., J. Finan, M. W. Climo, A. Rosato, S. Gill, and G. L. Archer.** 2003. Microarray transcription analysis of clinical *Staphylococcus aureus* isolates resistant to vancomycin. Journal of Bacteriology **185:**4638-43.

72. **Nagarajan, R.** 1991. Antibacterial activities and modes of action of vancomycin and related glycopeptides. Antimicrobial Agents and Chemotherapy **35:**605-609.

73. **Navarro, F., and P. Courvalin.** 1994. Analysis of genes encoding D-alanine-D-alanine ligase-related enzymes in *Enterococcus casseliflavus* and *Enterococcus flavescens*. Antimicrobial Agents and Chemotherapy **38:**1788-1793.

74. **Nieto, M., and H. R. Perkins.** 1971. Modifications of the acyl-D-alanyl-D-alanine terminus affecting complex-formation with vancomycin. Biochemistry Journal **123:**789-803.

75. **Normark, B. H., R. Novak, A. Ortqvist, G. Kallenius, E. Tuomanen, and S. Normark.** 2001. Clinical isolates of *Streptococcus pneumoniae* that exhibit tolerance to vancomycin. Clinical Infectious Diseases **32:**552-558.

76. **Novais, C., T. M. Coque, J. C. Sousa, F. Baquero, and L. Peixe.** 2004. Local genetic patterns within a vancomycin-resistant *Enterococcus faecalis* clone isolated in three hospitals in Portugal. Antimicrobial Agents and Chemotherapy **48:**3613-3617.

77. **Novais, C., T. M. Coque, J. C. Sousa, F. Baquero, L. Peixe, and P. R. S. Group.** 2004. Local genetic patterns within a vancomycin-resistant *Enterococcus faecalis* clone isolated in three hospitals in Portugal. Antimicrobial Agents and Chemotherapy **48:**3613-3617.

78. **Novak, R., E. Charpentier, J. S. Braun, and E. Tuomanen.** 2000. Signal transduction by a death signal peptide: uncovering the mechanism of bacterial killing by penicillin. Molecular Cell **5:**49-57.

79. **Novak, R., B. Henriques, E. Charpentier, S. Normark, and E. Tuomanen.** 1999. Emergence of vancomycin tolerance in *Streptococcus pneumoniae*. Nature **399:**590-3.

80. **Palladino, S., I. D. Kay, J. P. Flexman, I. Boehm, A. M. Costa, E. J. Lambert, and K. J. Christiansen.** 2003. Rapid detection of vanA and vanB genes directly from clinical specimens and enrichment broths by real-time multiplex PCR assay. Journal of Clinical Microbiology **41:**2483-6.

81. **Panesso, D., L. Abadia-Patino, N. Vanegas, P. Reynolds, P. Courvalin, and C. A. Arias.** 2005. Transcriptional analysis of the *vanC* cluster from *Enterococcus gallinarum* strains with constitutive and inducible vancomycin resistance. Antimicrobial Agents and Chemotherapy **49:**1060-1066.

82. **Philips, I.** 1999. The use of bacitracin as a growth promoter in animals produces no risk to human health. Journal of Antimicrobial Chemotherapy **44:**725-728.

83. **Podmore, A. H., and P. E. Reynolds.** 2002. Purification and characterization of VanXY(C), a D,D-dipeptidase/D,D-carboxypeptidase in vancomycin-resistant *Enterococcus gallinarum* BM4174. European Journal of Biochemistry **269:**2740-2746.

84. **Podmore, A. H. B., and P. E. Reynolds.** 2002. Purification and characterization of $VanXY_C$, a D,D-dipeptidase/D,D-carboxypeptidase in vancomycin-resistant *Enterococcus gallinarum* BM4174. European Journal of Biochemistry **269:**2740-2746.

85. **Pootoolal, J., J. Neu, and G. D. Wright.** 2002. Glycopeptide antibiotic resistance. Annual Review of Pharmacology and Toxicology. **42:**381-408.

86. **Reynolds, P., and P. Courvalin.** 2005. Vancomycin-resistance in Enterococci due to the synthesis of precursors terminating in D-Alanyl-D-Serine. Antimicrobial Agents and Chemotherapy **49:**21-25.

87. **Reynolds, P. E., C. A. Arias, and P. Courvalin.** 1999. Gene *van*XY_C encodes D,D-dipeptidase (VanX) and D,D-carboxypeptidase (VanY) activities in vancomycin-resistant *Enterococcus gallinarum* BM4147. Molecular Microbiology **34:**341-349.

88. **Rice, L. B.** 2001. Emergence of vancomycin-resistant Enterococci. Emerging Infectious Diseases **7:**183-187.

89. **Robertson, G. T., J. Zhao, B. V. Desai, W. H. Coleman, T. I. Nicas, R. Gilmour, L. Grinius, D. A. Morrison, and M. E. Winkler.** 2002. Vancomycin tolerance induced by erythromycin but not by loss of vncRS, vex3, or pep27 function in *Streptococcus pneumoniae*. Journal of Bacteriology **184:**6987-7000.

90. **Rohrer, S., and B. Berger-Bachi.** 2003. FemABX peptidyl transferases: a link between branched-chain cell wall peptide formation and beta-lactam resistance in Gram-positive cocci. Antimicrobial Agents and Chemotherapy **47:**837-846.

91. **Rothfield, L., and D. Romeo.** 1971. Role of lipids in the biosynthesis of the bacterial cell envelope. Bacteriological Reviews **35:**14-38.

92. **Sahm, D. F., L. Free, and S. Handwerger.** 1995. Inducible and constitutive expression of vanC-1-encoded resistance to vancomycin in *Enterococcus gallinarum*. Antimicrobial Agents and Chemotherapy **39:**1480-1484.

93. **Salyers, A. A., and C. F. Amabile-Cuevas.** 1997. Why are antibiotic resistance genes so resistant to elimination? Antimicrobial Agents and Chemotherapy **41:**2321-2325.

94. **Severin, A., S. W. Wu, K. Tabei, and A. Tomasz.** 2004. Penicillin-binding protein 2 is essential for expression of high-level vancomycin resistance and cell wall synthesis in vancomycin-resistant *Staphylococcus aureus* carrying the Enterococcal vanA gene complex. Antimicrobial Agents and Chemotherapy **48:**4566-4573.

95. **Sinha Roy, R., P. Yang, S. Kodali, Y. Xiong, R. M. Kim, P. R. Griffin, H. R. Onishi, J. Kohler, L. L. Silver, and K. Chapman.** 2001. Direct interaction of a vancomycin derivative with bacterial enzymes involved in cell wall biosynthesis. Chemistry & Biology **8:**1095-1096.

96. **Srinivasan, A., J. D. Dick, and T. M. Perl.** 2002. Vancomycin resistance in Staphylococci. Clinical Microbiology Reviews **15:**430-438.

97. **Stone, K. J., and J. L. Strominger.** 1971. Mechanism of action of bacitracin: complexation with metal ion and C55-Isoprenyl pyrophosphate. Proceedings of the National Academy of Science USA **68:**3223-3227.

98. **Sundsfjord, A., G. S. Simonsen, B. C. Haldorsen, H. Haaheim, S.-O. Hjelmevol, P. Littauer, and K. H. Dahl.** 2004. Genetic methods for detection of antimicrobial resistance. Acta Pathologica, Microbiologica et Immunologica Scandinavica **112:**815-837.

99. **Sussmuth, R. D.** 2002. Vancomycin resistance: small molecule approaches targeting the bacterial cell wall biosynthesis. Chemistry & Biochemistry **3:**295-298.

100. **Tenover, F. C.** 2001. Development and spread of bacterial resistance to antimicrobial agents: an overview. Clinical Infectious Diseases **33 Supplement 3:**S108-S115.

101. **Ton-That, H., L. A. Marraffini, and O. Schneewind.** 2004. Protein sorting to the cell wall envelope of Gram-positive bacteria. Biochimica et Biophysica Acta **1694:**269-278.

102. **Toye, B., J. Shymanski, M. Bobrowska, W. Woods, and K. Ramotar.** 1997. Clinical and epidemiological significance of Enterococci intrinsically resistant to vancomycin (possessing the vanC genotype). Journal of Clinical Microbiology **35:**3166-3170.

103. **Van Bambeke, F., M. Chauvel, P. E. Reynolds, H. S. Fraimow, and P. Courvalin.** 1999. Vancomycin-dependent *Enterococcus faecalis* clinical isolates and revertant mutants. Antimicrobial Agents and Chemotherapy **43:**41-47.

104. **van Heijenoort, J.** 2001. Formation of the glycan chains in the synthesis of bacterial peptidoglycan. Glycobiology **11:**25R-36R.

105. **Walsh, T. R., and R. A. Howe.** 2002. The prevalence and mechanisms of vancomycin resistance in *Staphylococcus aureus*. Annual Reviews of Microbiology **56:**657-675.

106. **Woodford, N., A. P. Johnson, D. Morrison, and D. C. Speller.** 1995. Current perspectives on glycopeptide resistance. Clinical Microbiology Reviews **8:**585-615.