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The molecular basis of glycopeptide Resistence in two Clinical Isolates: Bacillus Lentus RSA1208 and Paenibacillus Thiaminolyticus RSA 1221

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CHAPTER 1

LITERATURE REVIEW

1.1 Antimicrobial chemotherapy

The control of microorganisms is critical for the prevention and treatment of diseases. Antibiotics are microbial products, which kill or inhibit the growth of susceptible bacteria. They can be classified according to their biochemical structure and by their mechanism of action. They act by inhibiting bacterial cell wall synthesis, protein synthesis, or deoxyribonucleic acid (DNA) replication and transcription. Antibiotics can damage bacterial membrane structures or disrupt membrane function, or block metabolic pathways through the inhibition of crucial enzymes. This brief review of the mode action of antibiotics (section 1.1.1) and mechanisms of antibiotic resistance (section 1.1.2) was summarized from Prescott *et al*. (1999).

1.1.1 Mode of action of antibiotics

1.1.1. a Inhibition of nucleic acid synthesis

DNA gyrase is an enzyme required during DNA replication, transcription and DNA repair. It regulates the super coiling of DNA. The ciprofloxacin and other quinolone antibiotics bind to the DNA gyrase-DNA helix complex and inhibit the function of the gyrase

enzyme, resulting in inefficiency of DNA replication, repair or transcription, leading to cell death. Rifampicin blocks RNA synthesis by binding to and inhibiting DNA-dependent ribonucleic acid (RNA) polymerase.

1.1.1. b Inhibition of protein synthesis

Prokaryotic ribosomes contain two multimeric subunits: 30S and 50S. Antibiotics can bind to one or both ribosomal subunits leading to misreading of the messenger RNA (mRNA) and the formation of non-functional proteins. For example, aminoglycosides are antibiotics that bind to the 30S ribosomal subunit and induce codon misreading and prevents protein translocation. Various other antibiotic classes affect other steps in protein synthesis, such as peptide bond formation between the amino acids in the growing peptide chain (e.g. chloramphenicol binds to the 50S ribosomal subunit and blocks peptide bond formation by sterically inhibiting the peptidyl transferase enzyme) or the antibiotic tetracycline binding to the 30S ribosomal subunit and inhibiting the binding of aminoacyl transfer RNA (tRNA), as a result preventing the incorporation of new amino acids to the growing peptide chain.

1.1.1. c Disruption of metabolic pathways

Antimetabolite antibiotics block vital cellular pathways by competitively inhibiting the use metabolites by key enzymes. For example, sulfonamides, which are structurally similar to *p*-aminobenzoic acid (PABA), inhibit the folate biosynthesis pathway by acting as

competitive antagonists to PABA. The sulfonanmides compete with PABA for the active site of an enzyme required in folic acid synthesis. This decreases the folate concentration and subsequently the inhibition of purine and pyrimidine synthesis, which are required for nucleic acid synthesis.

1.1.1. d Disruption of the plasma membrane

The polymyxins are polypeptides with a long hydrophobic tail which act as cationic detergents. The antibiotic binds to the bacterial plasma membrane and disrupts the structure and permeability of the membrane resulting in leakage of the cytoplasmic contents. Colistin belongs to the polymyxin group of antibiotics.

1.1.1. e Inhibition of cell wall synthesis

The cell wall is one of the most important parts of a bacterium, except for mycoplasmas and some archaebacteria, which have cell membranes instead of cell walls. It helps maintain the shape of the bacterium and prevents osmotic lysis. The cell wall also functions as a physical barrier against toxic compounds and in certain bacteria, the cell wall components also contribute to pathogenicity. Many selective antibiotics interfere with cell wall synthesis by inhibiting key enzymes.

Cell wall synthesis inhibitors have a high therapeutic index because bacterial cell walls have a unique structure not found in eukaryotic cells. Gram-positive cell walls consist of a

single, thick, homogenous peptidoglycan (PG) or murein layer adjacent to the plasma membrane. The PG in the cell wall is interlinked with teichoic acid. Some teichoic acid molecules have lipids attached and are called lipoteichoic acid (Fig. 1.1). In contrast, Gram-negative cell walls are fairly complex, consisting of two layers: a thin PG layer surrounded by a thick outer lipid bilayer containing lipopolysaccharides (LPS), lipoproteins, phospholipids and proteins (Fig. 1.2). Cell wall inhibitors are in part only effective in Gram-positive bacteria due to their cell wall structure since glycopeptides are large polar molecules, unable to penetrate the outer lipopolysaccharide membrane of Gram-negative bacteria and as a result have a limited activity against these organisms.

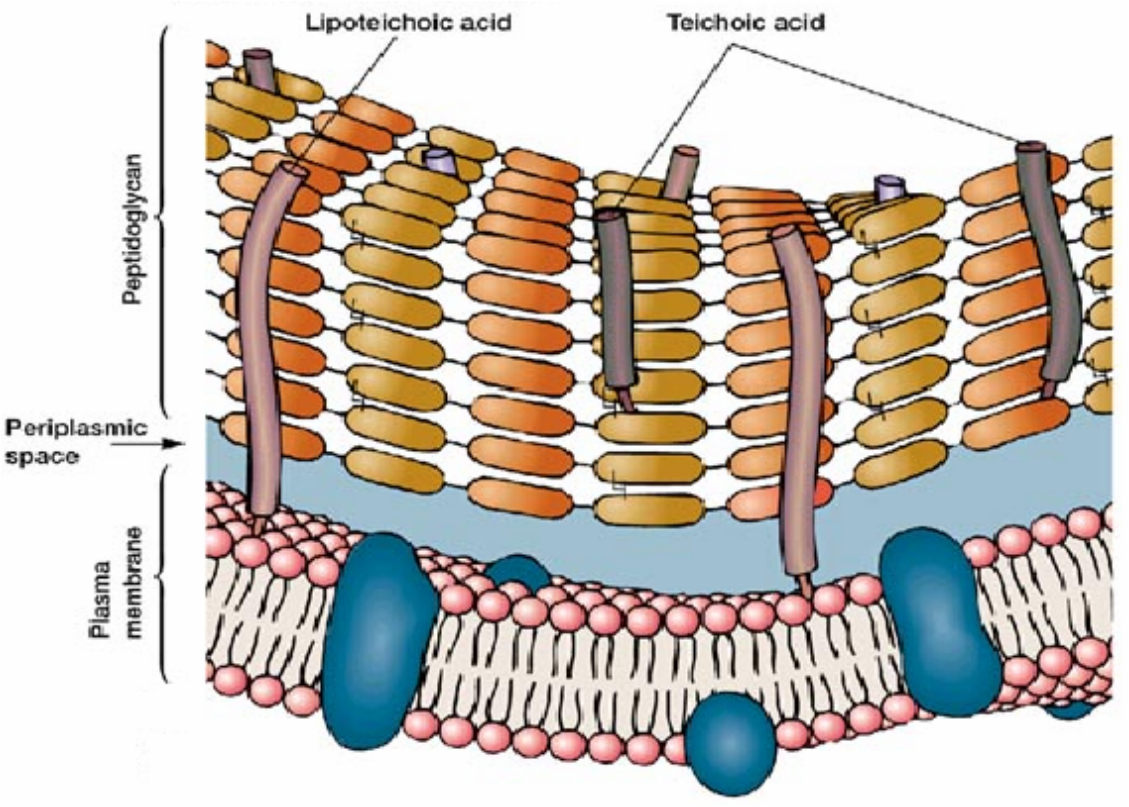


Figure 1.1: Gram-positive cell wall. Diagram indicating a thick homogenous layer composed primarily of peptidoglycan (Prescott *et al*., 1999).

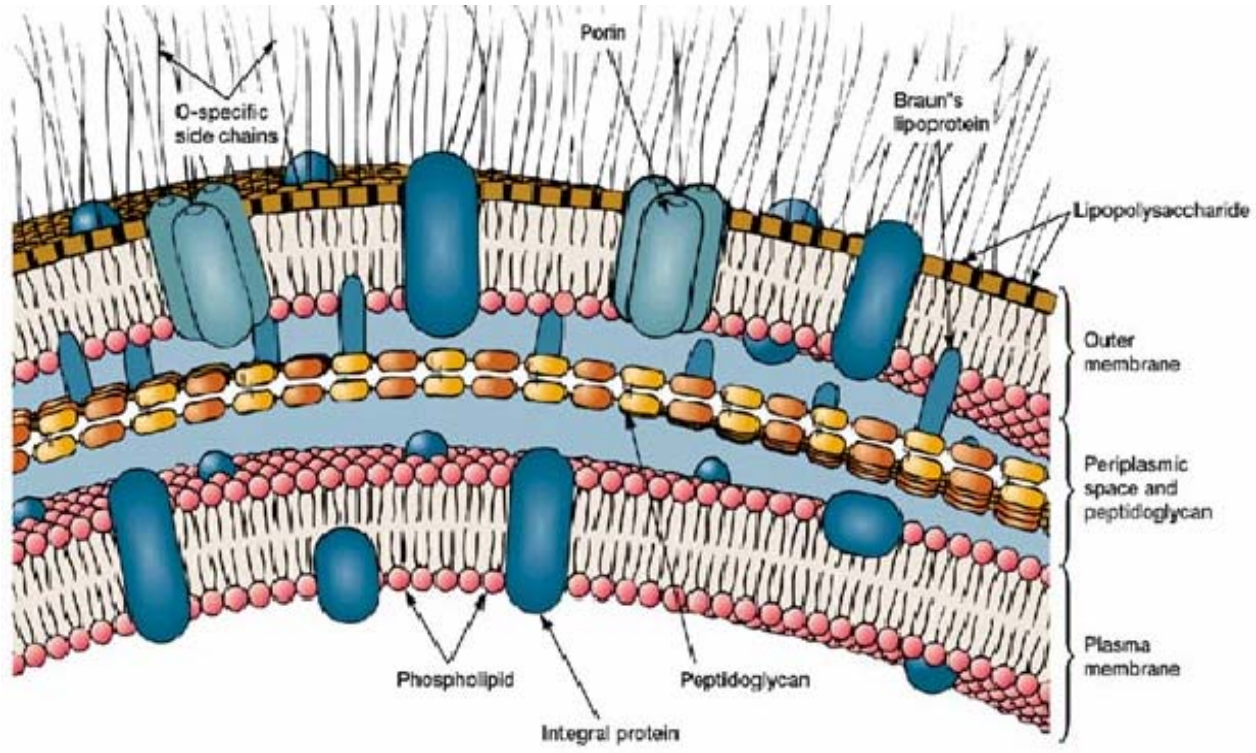


Figure 1.2: Gram-negative cell wall. Diagram indicating a thin peptidoglycan layer adjacent to the plasma membrane. The lipopolysaccharide outer membrane lies adjacent to the peptidoglycan layer and they are connected to each other via Braun's lipoproteins (Prescott *et al*., 1999).

Peptidoglycan is a polymer made up of identical, interlocking glycopeptide subunits. Each subunit consists of two sugar derivatives, N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG). A tetra-peptide side chain is composed of alternating D- and L-amino acid isomers and is attached to the NAM amino sugar moiety (Fig. 1.3). The monomeric subunits are synthesized in the cytoplasm of the bacterium and are transported to the growing cell wall by lipid carriers. The monomers are joined together by a process of transglycosylation via their sugars to form long nascent PG chains. Transpeptidases,

also known as penicillin-binding proteins (PBPs), cross-link these PG chains between the tetra-peptides (Fig. 1.4). The cross-linking process ultimately strengthens the cell wall against osmotic lysis.

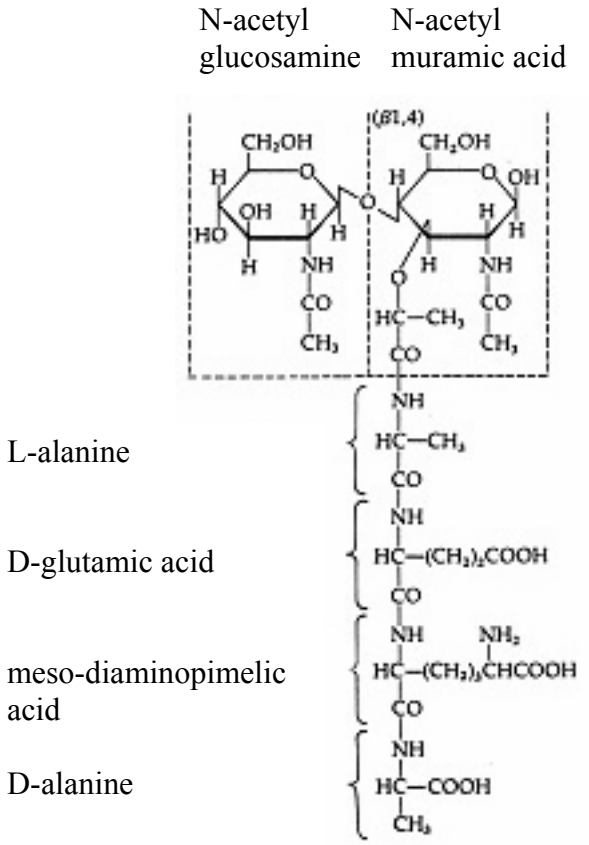


Figure 1.3: Peptidoglycan subunit of *Bacillus megaterium*. The backbone of this polymer is composed of alternating N- acetylglucosamine and N-acetylmuramic acid residues. The tetrapeptide side chain is composed of four alternating D- and L- amino acids, which is connected to the carboxyl group of N-acetylmuramic acid (Prescott *et al*., 1999).

a) **b)**

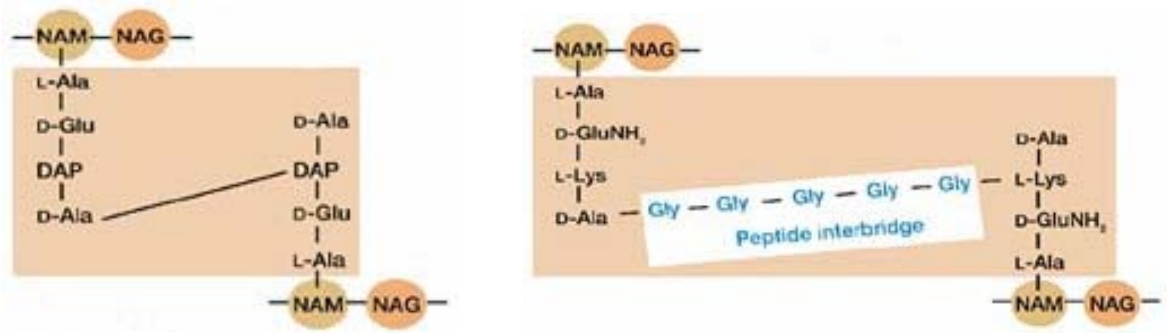


Figure 1.4: Cross-linking of peptidoglycan subunits. **a**) Direct cross-linking between the tetra peptide amino acids. This is typically seen in Gram-negative bacteria e.g. *Escherichia coli*. **b**) In Gram- positive bacteria e.g. *Staphylococcus aureus*, peptidoglycan subunits are joined together via cross-linking (pentaglycine peptide interbridge) between the peptide side chains (Prescott, *et al*., 1999).

In order for a bacterium to grow and divide, the peptide cross-links in the cell wall must be hydrolyzed, new PG monomers incorporated into the cell wall and the peptide cross-links reformed. Autolysins are endogenous bacterial enzymes that hydrolyze the peptide bonds and new PG monomers are added to the pre-existing PG layer by glycosyltransferases. Transpeptidases reform the peptide bonds between the PG chains. Interference with this process results in a weakened cell wall, which is more susceptible to osmotic lysis. Beta-lactams (β-lactams) such as penicillins, cephalosporins and the carbapenems bind to transpeptidases and inhibit cross-linking of the PG monomers resulting in a weakened cell wall and cell death. A contributing factor to the antibacterial properties of the β-lactam antibiotics is unopposed action of autolysins that occur when PBPs are inhibited by this group of antibiotics (Mandell *et al*., 2000)

1.1.2 Mechanisms of antimicrobial resistance

Resistance to antimicrobials can develop as a result of a single or multiple mutations within a bacterium or can be intrinsic to a particular bacterial species. Acquired resistance is commonly associated with the presence of extra chromosomal DNA e.g. plasmids carrying resistance determinants. Bacteria can transfer plasmids containing these resistance genes to other bacteria via horizontal gene transfer mechanisms: conjugation, transduction and transformation. A single plasmid can harbour resistance genes to multiple classes of antimicrobials. There are different types of resistance mechanisms. The bacterium can modify the antimicrobial target molecule, chemically modify the antimicrobial drug, and prevent the entry of the antimicrobial into the cell or actively efflux the antimicrobial from within the cell to the outside.

1.1.2. a Alterations of the drug target in the bacterium

The modification of the drug target molecule leads to an inability of the antimicrobial to bind to its target and therefore unable to have an effect. For example, changes in the DNA gyrase enzyme, caused by a genetic mutation, can lead to resistance to fluoroquinolones by preventing the quinolone from binding to the enzyme.

1.1.2. b Decreasing accessibility of the drug target molecule

Decreasing the accessibility of the target molecule can occur in many ways. Changes in the permeability of the bacterial cell membrane can prevent entry of the antibiotic into the cell

or the bacterium can actively remove the drug from the cell e.g. plasmid-mediated tetracycline resistance is a result of increased efflux of the antibiotic via efflux pumps.

1.1.2. c Inactivation or destruction of the drug

The production of an enzyme that hydrolyzes the drug can result in resistance to that antibiotic. The most well known example is the production of β-lactamase (penicillinase) that hydrolyzes the β-lactam ring of many pencillins.

1.1.3 Emergence of resistance

The potential of many different species of bacteria, including human pathogens, to resist the inhibitory actions of antimicrobial agents has become a worldwide problem. Antibiotic resistance continues to spread not only between nosocomial pathogens but also amongst community-acquired pathogens (Tenover and McGowan, 1996).

The incidence of antibiotic resistant infections has increased globally in the last few decades (Dzidic and Bedekovic, 2003). This increase in antimicrobial resistance can be attributed to a combination of changes in intrinsic bacterial characteristics, the selective pressure of antimicrobial use and misuse in the clinical settings and in animal husbandry practices, increase in invasive procedures and usage of invasive devices, a larger number of susceptible hosts, and breakdown in infection control practices resulting in spread of resistant organisms. Social and technological advances, such as air transport, have

increased population mobility and therefore propagate the spread of resistant organisms (Dzidic and Bedekovic, 2003).

The dissemination of resistance genes is facilitated by the integration of these sequences into naturally occurring mobile genetic elements and dispersed within the microbial populations through conjugation, transduction, or transformation. The emergence of multidrug-resistant bacteria and the spread of resistance genes highlight the need for stringent prevention policies, which include changes in antibiotic prescription practices, and implementation of strict infection control practices to control the transmission of nosocomial pathogens (Dzidic and Bedekovic, 2003).

1.2 Glycopeptide Antibiotics

The glycopeptide antibiotics vancomycin and teicoplanin were isolated from *Streptomyces orientalis* (renamed *Amycolatopsis orientalis*) and *Actinoplanes teichomyceticus*, respectively (Barna *et al*., 1984).

Biochemical studies on the mode of action of glycopeptides showed that these agents inhibit cell wall PG synthesis, by interacting with a substrate of the transpeptidase enzyme, thus preventing substrate binding to the active site of the enzyme (Jawetz *et al*., 1991).

1.2.1 Teicoplanin

Teicoplanin (Fig. 1.5) is produced by the actinomycete *Actinoplanes teichomyceticus*. It has a mode of action and spectrum of activity similar to vancomycin. It is commonly used in neutropenic patients, as it is more efficacious and tolerable than vancomycin. Teicoplanin activity is enhanced by its anchoring to the membrane via a lipid tail (Kureishi *et al*., 1990).

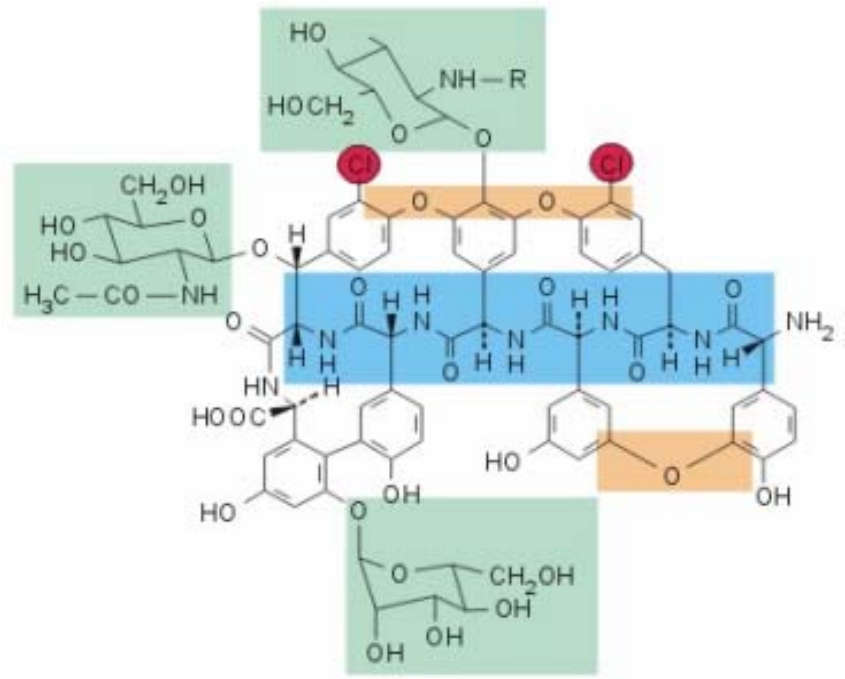


Figure 1.5: Chemical structure of teicoplanin. The molecule has 3 sugar derivatives (green), a peptide backbone (blue) and seven aromatic rings (http://www.biologie.uni-hamburg.de/lehre/mikro/allgmi00/alg06m31.jpg).

1.2.2 Vancomycin

The molecular structure of vancomycin (Fig. 1.6) is a linear heptapeptide molecule with five aromatic rings. Vancomycin acts by inhibiting growth and multiplication of susceptible bacteria by inhibiting PG biosynthesis. Vancomycin binds to the PG precursors and prevents it from binding to the transpeptidase active site. Peptide cross-linking cannot occur, and the structural integrity of the cell wall is compromised. Vancomycin is a large molecule, 1.5-kilo Daltons (kDa), which cannot penetrate the cytoplasmic membrane. The molecular target of vancomycin is the D-alanyl-D-alanine (D-ala-D-ala) moiety at the carboxy- terminus (C-terminus) of PG precursors (Barna *et al*., 1984).

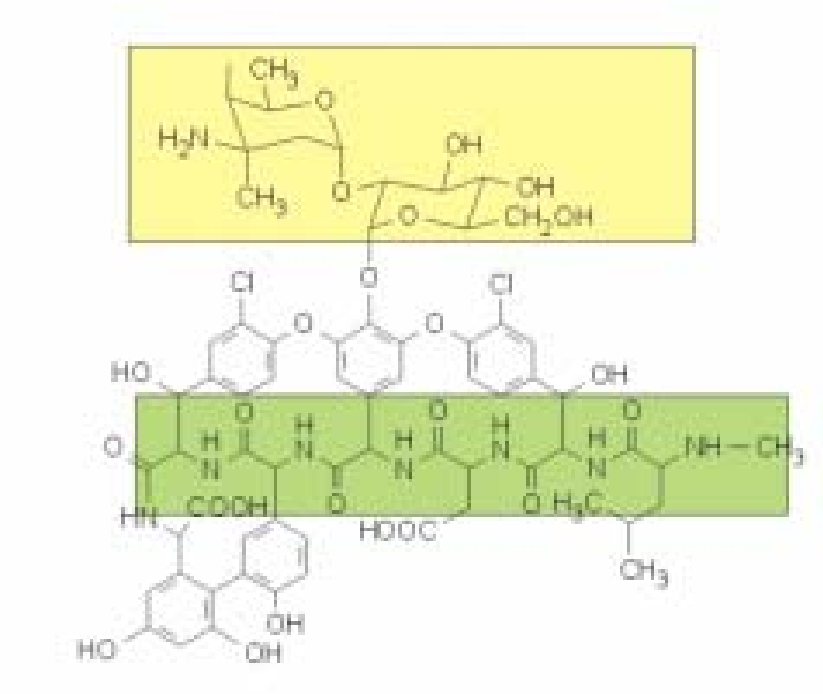


Figure 1.6: Chemical structure of vancomycin. The molecule has 5 aromatic rings and a branched-chain hexose sugar derivative (yellow) and a peptide backbone (green) (http://www.biologie.uni-hamburg.de/lehre/mikro/allgmi00/alg06m31.jpg).

The binding of the vancomycin molecule to its target is facilitated by hydrogen bonding between the D-ala-D-ala moiety and the heptapeptide backbone of the antibiotic (Fig. 1.7). These bonds are further strengthened by hydrophobic interactions between the peptide methyl groups of the PG precursor and the hydrocarbons of the antibiotic molecule (Barna *et al*., 1984).

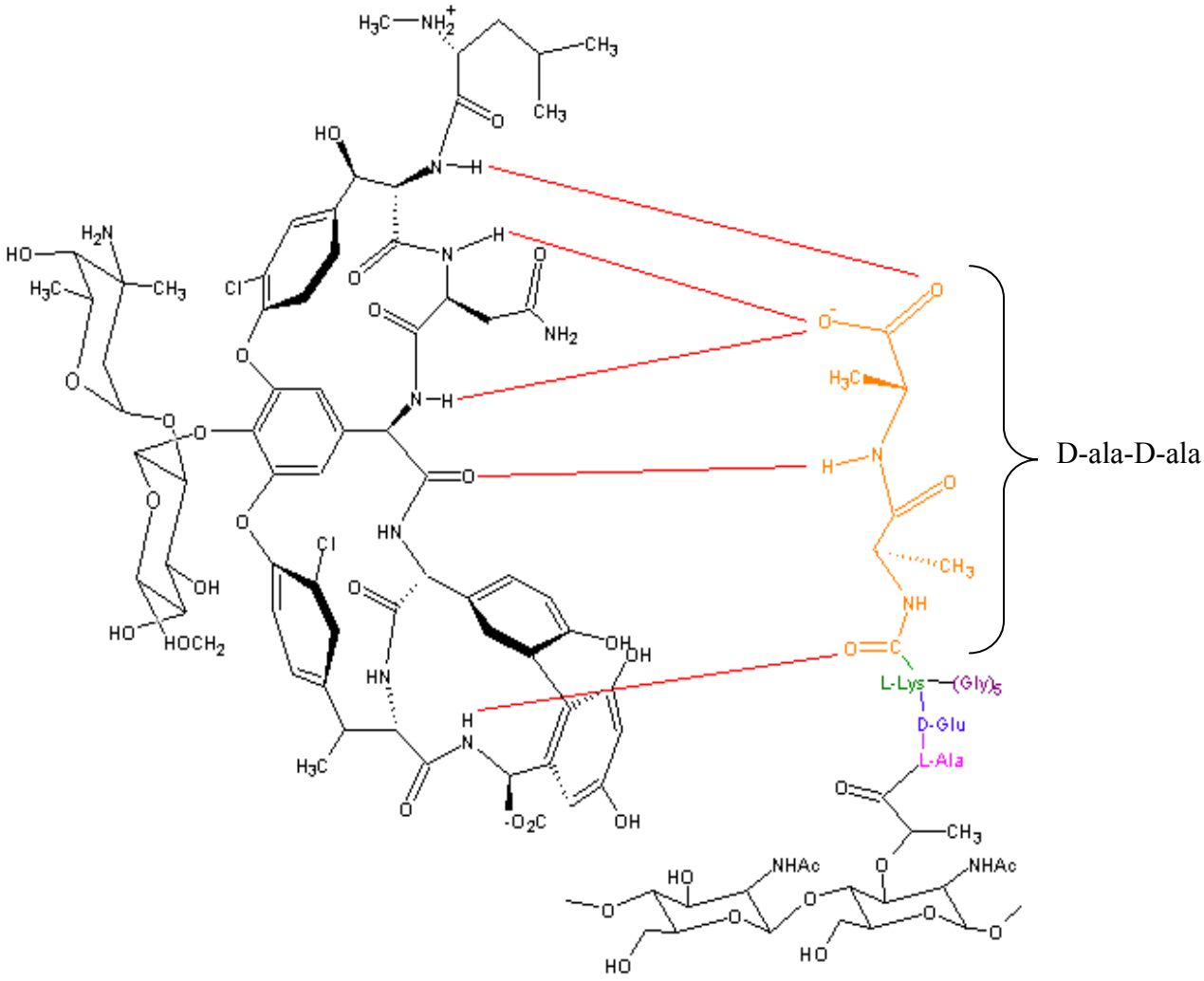


Figure 1.7: Mechanism of vancomycin action. The formation of 5 hydrogen bonds between the D-ala-D-ala moiety of the PG precursor and the heptapeptide backbone of vancomycin prevents transpeptidation and transglycosylation. NOTE 5 hydrogen bonds (www.omedon.co.uk/ vrsa/vancomycin/).

The cleavage of the terminal D- alanine (D-ala) amino acids from the PG chains allows for the formation of peptide bonds during transpeptidation. These peptide bonds are the basis of the formation of cross-links in the cell wall. The high affinity binding of vancomycin to the terminal D-ala-D-ala moiety inhibits the cleavage of the terminal D-ala and subsequently inhibits cross linking. Vancomycin susceptible microorganisms are unable to form cross-links between the PG chains. Vancomycin also blocks transglycosylation by sterically inhibiting the incorporation of new PG precursors into the nascent PG chain and by the sequestration of PG precursors. Vancomycin activity is increased by dimerization of the antibiotic (Arthur *et al.*, 1996).

Vancomycin is a narrow spectrum drug only effective against Gram-positive bacteria, including staphylococci, streptococci (e.g. enterococci, Group B streptococci, *Streptococcus pneumoniae*, and penicillin-resistant *Streptococcus pneumoniae*), *Bacillus* species (spp.), *Listeria* spp., and *Clostridium difficile*. Vancomycin is unable to cross the cell wall or the cell membrane of bacteria, but is active on the cell surface. Hence bacterial cells are unable to use efflux pump mechanisms or metabolism of the antibiotic to protect themselves, relying only on changing the antibiotic target molecule to become resistant (http://www.uic.edu/pharmacy/courses/pmpr342/itokazu/glycopeptide.html).

Vancomycin exhibits time-dependent killing i.e. the optimal bactericidal effect is achieved when the antibiotic concentration is constantly above the minimum inhibitory concentration (MIC). The rate and degree of the bactericidal effect remains relatively constant once the antibiotic concentration is approximately four times the MIC for the microorganism. The goal of antimicrobial therapy is to maintain these levels for as long as

possible during the dosing interval. The optimal duration that the antibiotic concentration should remain above the MIC is unknown (www.aic.cuhk.edu.hk/web8/antibacterials.htm#killing_characteristics).

Vancomycin was first used in 1956 to treat penicillin-resistant staphylococci. However, the toxicity of vancomycin and the introduction of penicillinase-resistant β-lactams antibiotics (such as methicillin) soon made vancomycin a less useful drug. Vancomycin regained popularity in the 1970s as an effective alternative for the treatment of bacteria that had developed resistance against the more popular drugs such as penicillin, cloxacillin and the cephalosporins. Until the late 1980s, medical practitioners were dependent upon vancomycin to effectively treat infections caused by Gram-positive bacteria such as staphylococci and streptococci. However, vancomycin resistant enterococci are emerging as an important worldwide problem as there are few reliable alternative antimicrobial therapies for these organisms (http://www.uic.edu/pharmacy/courses/pmpr342/itokazu/glycopeptide.html).

1.3 Enterococci

Enterococci are non-motile, Gram-positive cocci commonly found in the gastro-intestinal tract of humans and animals. Enterococci form part of the dominant microflora in a number of dairy products. At present, enterococci are the third most frequently isolated bacteria associated with nosocomial infections, after staphylococci and *Escherichia coli*. They are intrinsically resistant to many antibiotics including cephalosporins, polymyxins, lincomycin and clindamycin. They are able to acquire resistance to macrolides, tetracyclines, chloramphenicol, trimethoprim/ sulfamethoxazole, rifampicin, aminoglycosides and ampicillin (Peters *et al*., 2003). They are susceptible only to synergistic penicillin/ aminoglycoside therapy combined with a cell wall acting antibiotic (e.g. vancomycin or ampicillin).

Due to their importance as causative agents of nosocomial infections, enterococci are becoming increasingly clinically significant. Enterococcal infections are becoming more and more difficult to treat as resistance to antibiotics increases (Mannu *et al*., 2003).

1.4 Glycopeptide resistant enterococci

The discovery of an acquired, self-transferable resistance mechanism to vancomycin and teicoplanin in enterococci (VRE) in 1988 caused considerable alarm, not only because of the immediate problem of how to treat infections when no other viable antibiotics are available, but also since VRE may serve as a reservoir of vancomycin resistance (Van^R) genes, which may pass to more virulent organisms, such as those of the genus *Staphylococcus* (Marshall *et al*., 1997). Since 1988, VRE (now termed glycopeptide resistant enterococci [GRE]) have been reported to cause nosocomial outbreaks and serious infections worldwide but primarily in the United States of America (USA) (Poyart *et al*., 1997). The clonal spread of GRE and plasmid dissemination between strains is known to occur (Poyart *et al*., 1997).

Glycopeptide-resistant *E. faecium* (GREF) has in particular established itself as a significant causative agent of nosocomial infections and colonization in patients in specialized hospital units such as renal, bone marrow transplant and intensive care units, in the USA, Europe and elsewhere. Most vancomycin resistant strains of *E. faecium* are commonly associated with nosocomial infections. GRE are frequently resistant to high-levels of aminoglycosides, and many other antimicrobial agents (Woodford *et al*., 1997 and Woodford, 2001).

1.5 Vancomycin resistance

The explosive emergence of GRE worldwide has led to intensive research of the molecular determinants of glycopeptide resistance. These investigations have revealed one of the most complex molecular systems of resistance and a model of genetic adaptation. Vancomycin resistance is usually associated with a strategy of remodelling the termini of PG precursors in cell wall synthesis, from D-ala-D-ala to D-alanyl-D-lactate (D-ala-D-lac) or D-alanyl-D-serine (D-ala-D-ser), depending on the specificity of the Van ligase protein encoded by a resistance gene cassette (Lessard and Walsh, 1999).

The modified PG binds vancomycin 1000-fold less avidly. The modification of the terminus from D-ala-D-ala to D-ala-D-lac results in the loss of a central hydrogen bond (H-bond) (Fig. 1.8). This decrease in the strength of vancomycin binding results in clinical antibiotic resistance (Roper *et al*., 2000).

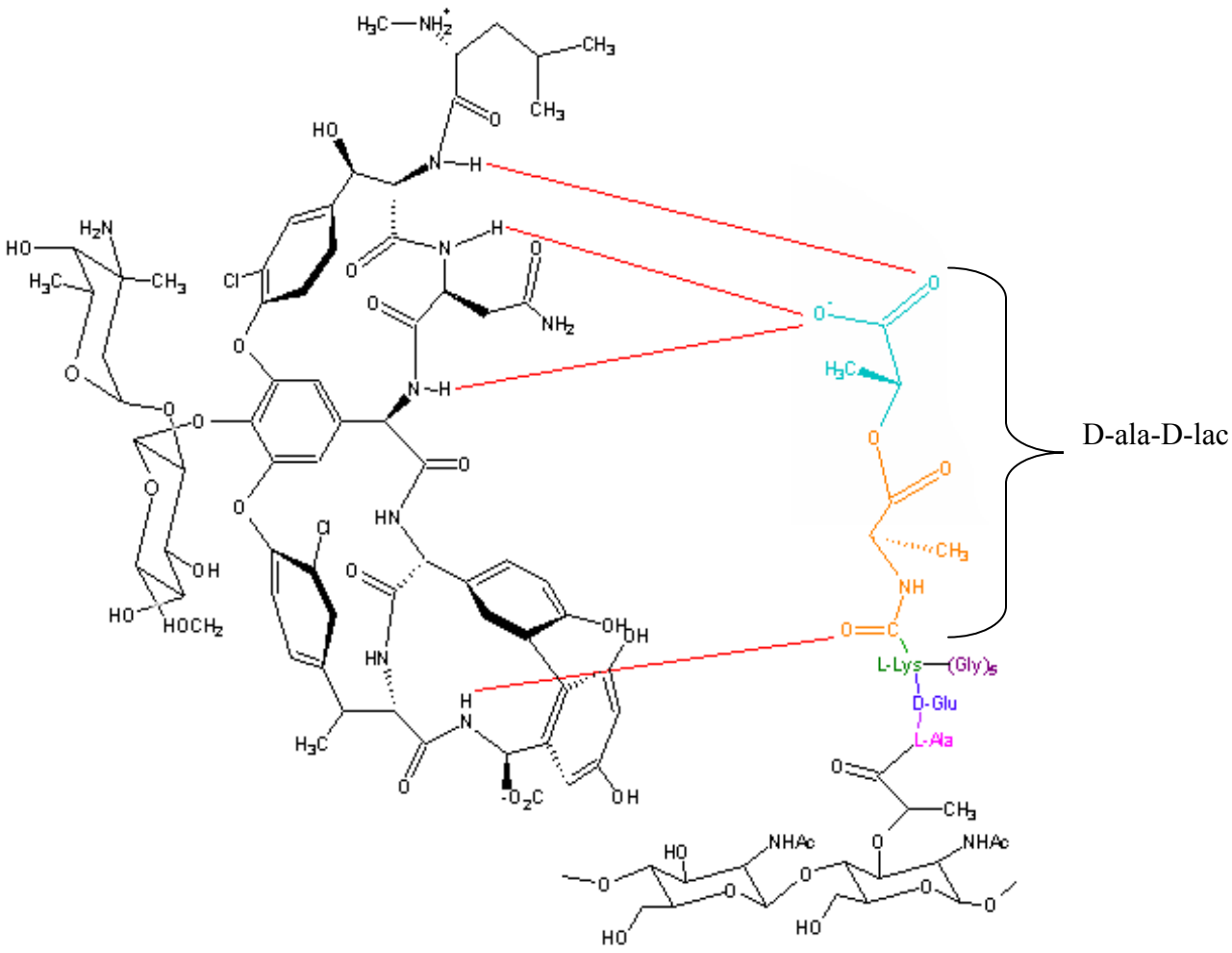


Figure 1.8: Mechanism of resistance to vancomycin. Vancomycin binds with low affinity to the terminal D-ala-D-lac. The change of the terminal D-ala-D-ala to D-ala-D-lac results in a loss of a crucial H-bond. NOTE Only 4 hydrogen bonds (www.omedon.co.uk/ vrsa/vancomycin/).

GRE have a wide geographical distribution and are phenotypically and genotypically heterogeneous. GRE can be found in hospitals as well as in animal husbandry, where the glycopeptide avoparcin until recently was used as an antimicrobial growth-promoting food additive, administered primarily to pigs and poultry.

There are currently seven vancomycin resistance phenotypes (Table 1.1); five acquired resistance phenotypes, (vanA, vanB, vanD, vanE, and vanG), and one intrinsic (vanC) phenotype. The final glycopeptide resistance phenotype identified so far is vanF, which has been assigned to the biopesticide *Paenibacillus popilliae*. It has a putative ligase gene homologous to a portion of the *vanA* and *vanB* ligase gene found in enterococci (Patel *et al*., 2000).

Of the five acquired resistance phenotypes, only two have been characterized as transferable by conjugation: the vanA-type confers high-level inducible resistance to both vancomycin (MIC ≥ 64 μg/mL) and teicoplanin (MIC ≥ 8 μg/mL). The vanB-type exhibits variable levels of inducible resistance to vancomycin only (MIC from 4 to ≥ 1024 μg/mL) (Perichon *et al*., 1997). The vanA phenotype of enterococcal glycopeptide resistance is the most commonly observed; its main reservoir is *E. faecium*. The *van* genes found primarily in enterococci are described in more detail in section 1.6.

Table 1.1: Summary of the defining characteristics of the different vancomycin resistance phenotypes.

Phenotype	**vanA**	**vanB**	**vanC1 and vanC2/C3**	**vanD**	**vanE**	**vanF**	**vanG**
Resistance	Acquired	Acquired	Intrinsic	Acquired	Acquired	Acquired	Acquired
Resistant to	Vancomycin and teicoplanin	Vancomycin	Vancomycin	Vancomycin and teicoplanin	Vancomycin	Vancomycin	Vancomycin
Level of resistance	High	Variable	Low	Intermediate	Moderate	High	Moderate
Vancomycin MIC (µg/mL)	>128	16-1024	4-16	64-128	16	400-800	16
Teicoplanin MIC (µg/mL)	≥64	<2	0.5-1	4-8	0.5	---	---
Terminal amino acid	D-lac	D-lac	D-ser	D-lac	D-ser	D-lac	D-ser
Expression	Inducible	Inducible	Constitutive	Inducible or constitutive	Inducible	Unknown	Inducible
Location of *van* genes	Plasmid or chromosome	Plasmid or chromosome	Chromosome	Chromosome	Unknown	Unknown	Chromosome
Transferable plasmids	Yes	Yes	No	No	No	Unknown	No
Transposon	Tn*1546*	Tn*1549*	--	--	--	--	--

1.6 Mechanisms of glycopeptide resistance

To date only two mechanisms of glycopeptide resistance have been described. The first resistance mechanism was observed in GRE where resistance was associated with the presence of a *van* gene cluster. An alternate mechanism of glycopeptide resistance was observed in studies on vancomycin intermediate *Staphylococcus aureus* (VISA) isolates. Resistance in VISA is associated with an accumulation of excess amounts of PG, which results in a thickened cell wall (discussed in section 1.8.1).

1.6.1 vanA resistance phenotype

The vanA phenotype is the most frequently identified form in GRE (Woodford *et al*., 1997). The genetic and biochemical basis of vancomycin resistance is also best understood for vanA. The vanA phenotype was first observed in *E. faecium* BM4147 in the late 1980s. The resistance genes of this isolate are located on a 34-kilobase (kb) non-conjugative plasmid designated pIP816 (Leclercq *et al*., 1988).

Genetic analysis of the plasmid revealed a gene cluster consisting of seven designated genes *vanR*, *vanS*, *vanH, vanA, vanX*, *vanY*, and *vanZ*. The widespread dissemination of the *vanA* genotype is a result of the gene cluster being part of a transposable element. Sequence analysis of the flanking regions of the *van* gene cluster carried on pIP816, identified a prototype *vanA*-carrying resistance transposon designated Tn*1546*, a member of the Tn*3* transposon family (Fig. 1.9). This element contains the seven *van* genes located immediately downstream of open reading frame (*orf*) 1 (transposase) and *orf2* (resolvase), which are associated with transposition functions. The transposase and resolvase genes are

transcribed in opposite directions. Flanking the transposon are imperfect inverted repeat (IR) sequences (Arthur *et al*., 1993).

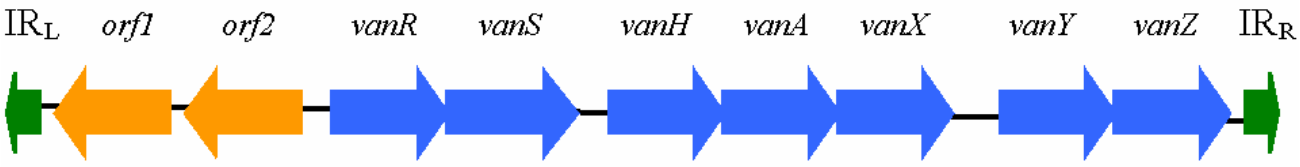


Figure 1.9: Schematic representation of the glycopeptide resistance transposon Tn*1546* from *E. faecium* BM4147. The arrows represent the coding sequences and the direction of transcription. The green arrows labelled IR_L and IR_R indicate the left and right inverted repeats of the transposon, respectively.

The most upstream (or 5') genes in the *vanA* operon are *vanR* and *vanS*, which encode a two component regulatory system that directs transcription of *vanH, vanA,* and *vanX*, in response to the presence of vancomycin or the related antibiotics (Fig. 1.10) (Woodford *et al*., 1995).

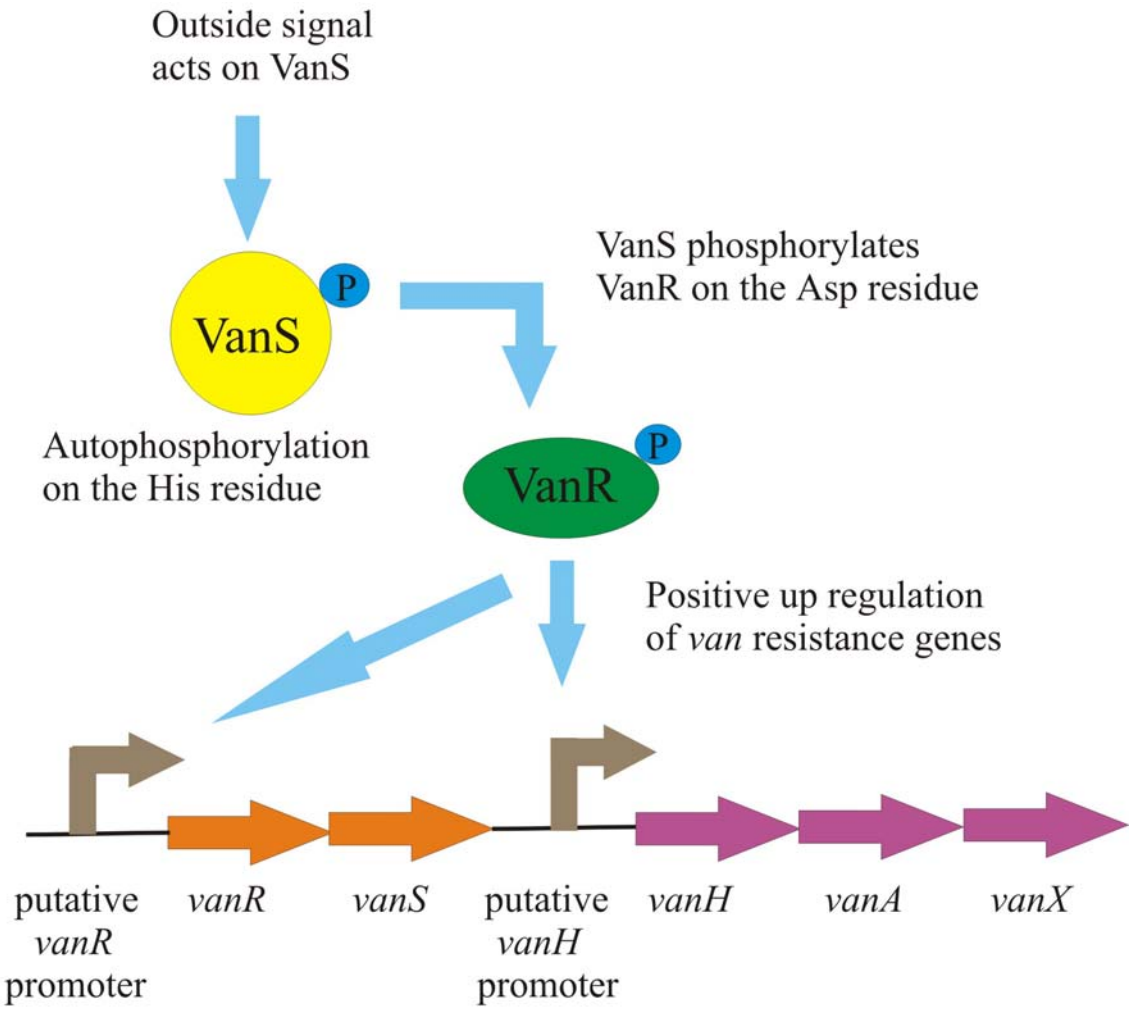


Figure 1.10: The induction of vancomycin resistance expression. An outside signal (vancomycin or related antibiotics) triggers the autophosphorylation of VanS. Phosphorylated VanS readily donates a phosphate group to VanR. Phosphorylated VanR binds to the putative *vanH* and *vanR* promoter regions and acts as a transcription activator, which upregulates the expression of *van* resistance genes: *vanR*, *vanS*, *vanH*, *vanA* and *vanX*. (His= histidine, Arg= arginine)

The brief review of the vancomycin resistance genes and the function of their respective proteins encoded by the *vanA* resistance operon were summarized from Woodford (2001) (Table 1.2).

VanS is a membrane-bound histidine kinase sensor protein and VanR is a response regulator protein. Arthur and colleagues (1992) demonstrated that VanS and VanR transcriptionally regulate the expression of *vanH*, *vanA* and *vanX*. Wright and colleagues (1992) showed that histidine $(His)_{164}$ on the cytoplasmic domain of VanS is autophosphorylated in the presence of adenosine triphosphate (ATP), in response to a stimulus. The phosphorylated VanS readily donates a phosphate group to the aspartic acid $(Asp)_{53}$ on VanR. Phosphorylated VanR (Phos-VanR), and to a lesser extent unphosphorylated VanR, acts as a transcription activator by binding to the DNA at the putative *vanH* (P_H) promoter region between *vanS* and *vanH*, and *vanR* (P_R) promoter region upstream of *vanR*, thereby activating the transcription of *vanR*, *vanS*, *vanH, vanA*, *and vanX*.

The *vanH* gene encodes for a D-specific α-ketoacid dehydrogenase, which reduces pyruvate to D-lactate (D-lac), one of the substrates for the Van ligase protein encoded by *vanA*.

vanA is a D:ala:D:ala ligase (*ddl*) with an altered specificity. The normal cellular ligase (*ddl* gene product) ligates two D-ala molecules to produce the D-ala-D-ala dipeptide that is required for the synthesis of PG precursors. VanA ligates D-ala to D-lac, to produce D-ala-D-lac. When this moiety is incorporated into the C-terminus of PG precursors, vancomycin is unable to bind as effectively to the precursors.

The last essential vancomycin resistance gene is *vanX*. *vanX* is a D, D-dipeptidase, which hydrolyzes the cytoplasmic dipeptide D-ala-D-ala produced by the chromosomal *ddl*, thus preventing the incorporation of the susceptible dipeptides into PG precursors. The five genes (*vanR*, *vanS*, *vanH, vanA, vanX*) and their respective protein products are essential for the establishment of vancomycin resistance in most bacterial cells.

The *vanA* operon encodes two accessory proteins, VanY and VanZ. The *vanY* gene encodes a D, D-carboxypeptidase, which cleaves the terminal D-ala residue from the D-Ala-D-Ala- containing cytoplasmic PG precursors thus preventing these vancomycin susceptible PG precursors from being incorporated into the cell wall. VanZ is an unknown gene product that confers teicoplanin resistance.

Table 1.2: Summary of *vanA* resistance genes, their respective gene products and the function of the proteins encoded by the resistance operon.

Gene	Gene product	Function
vanR	Transcriptional response regulator	Phosphorylated VanR binds to DNA and upregulates the transcription of the *van* genes
vanS	Membrane bound histidine sensor kinase	An outside signal triggers the autophosphorylation of VanS, which donates the phosphate group to VanR
vanA	D-ala: D-lac ligase	Synthesis of the depsipeptide D-ala-D-lac
vanH	D-specific α-ketoacid dehydrogenase	Reduces pyruvate to D-lac, one of the substrates for the VanA ligase

vanX	D, D-dipeptidase	Hydrolyzes cytoplasmic dipeptide D-ala-D-ala produced by the chromosomal *ddl* gene product, thus preventing the incorporation of the susceptible dipeptides into PG precursors
vanY	D, D-carboxypeptidase	Removing the terminal D-ala amino acid from cytoplasmic PG precursors
vanZ	Unknown	Encodes a protein that confers teicoplanin resistance

1.6.2 vanB resistance phenotype

The vanB phenotype confers variable levels of vancomycin resistance but the organism remains susceptible to teicoplanin. Originally, enterococci displaying the vanB resistance phenotype were associated with non-transferable resistance. However, the increased detection of the vanB phenotype in VRE isolated from clinical specimens prompted speculation that the *vanB* genes are associated with transposable elements on either conjugative plasmids or chromosomes (Woodford, 2001).

Molecular analysis of the *vanB* operon revealed a similar gene cluster to the one carried on Tn*1546*, which mediates the *vanA*-type resistance (Fig. 1.11). The *vanB* transposable element was designated Tn*1549*. The $vanR_B$, $vanS_B$, $vanH_B$, *vanB*, $vanX_B$, and $vanY_B$ genes are homologous to the *vanA* genes. The gene arrangement of Tn*1547* is however different

to that of Tn*1546*. The production of a novel protein, designated VanW, was discovered but its function remains unknown (Quintiliani and Courvalin, 1996). The *vanB* genotype is divided into 3 distinct subtypes: *vanB1*, *vanB2*, and *vanB3* based on DNA sequence variations. There is no correlation between the subtypes and levels of vancomycin resistance (Dahl *et al*., 1999).

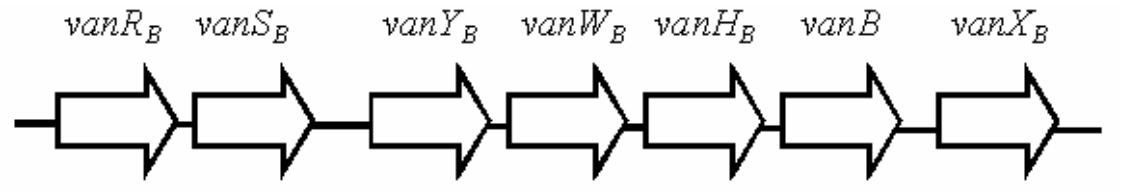


Figure 1.11: Schematic representation of the *vanB* gene cluster. Open arrows represent the coding sequences and the direction of transcription.

1.6.3 vanC resistance phenotype

Certain enterococcal species are intrinsically resistant to low levels of vancomycin, but are susceptible to teicoplanin. This expression of vancomycin resistance is specific to *E. gallinarum*, *E. casseliflavus*, and *E. flavescens*. The *vanC* resistance genes is not acquired or transferred. The *vanC1* ligase gene is specific for *E. gallinarum*, while the *vanC2/C3* ligase gene has been described in *E. casseliflavus*, and *E. flavescens* (Leclercq *et al*., 1992 and Navarro *et al*., 1994).

VanC resistance can be either inducible or constitutive, and is characterized by the incorporation of D-ala-D-ser at the C-terminus of PG precursors. Vancomycin has a reduced affinity for this moiety (Billot-Klein *et al*., 1994). In addition to the *vanC* ligase,

two additional, but essential proteins are produced: VanT is a membrane-bound serine racemase, which converts L-ser to D-ser, and $VanXY_C$, a protein that has D, D-dipeptidase and D, D-carboxypeptidase activities (Fig. 1.12). This allows for the hydrolysis of the susceptible dipeptide D-ala-D-ala and removal of the terminal D-ala from pentapeptide of PG precursors (Arias *et al*., 1999 and Reynolds *et al*., 1999).

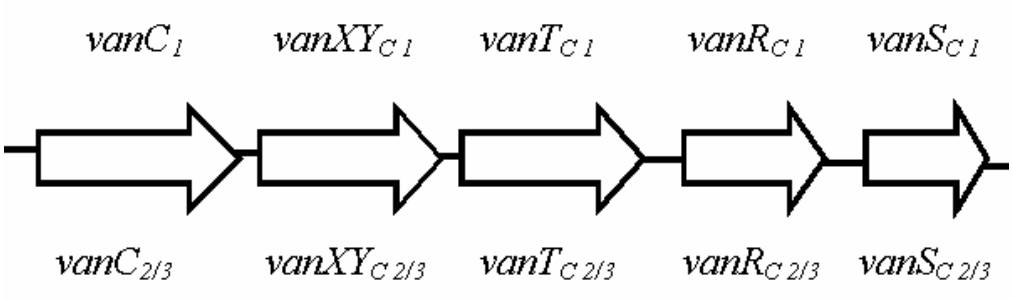


Figure 1.12: Schematic representation of the genetic arrangement of the *E. gallinarum vanC-1* gene cluster and the *E. casseliflavus* and *E. flavescens vanC2/3* gene cluster. Open arrows represent the coding sequences and the direction of transcription.

1.6.4 vanD resistance phenotype

In 1997, Perichon and co-workers first described a clinical isolate of *E. faecium* BM4339 that was constitutively resistant to vancomycin (MIC 64 μg/mL) and to low levels of teicoplanin (MIC 4 μg/mL). This new type of vancomycin resistance phenotype was designated vanD (Perichon *et al*., 1997). By 2001, only four strains of *E. faecium* had been found to express the *vanD* gene clusters (Fig. 1.13) (Woodford, 2001).

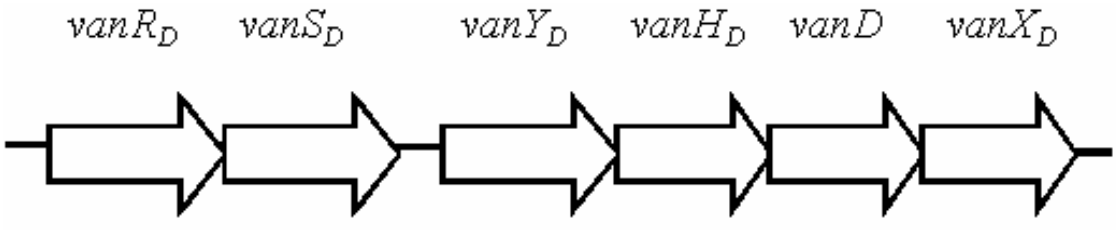


Figure 1.13: Schematic representation of the *vanD* gene cluster. Open arrows represent the coding sequences and the direction of transcription.

1.6.5 vanE resistance phenotype

In 1999, Fines and colleagues described the first VanE resistant phenotype in a clinical isolate of *E. faecalis*. Isolate BM4405 expressed moderate, inducible levels of vancomycin resistance (MIC 16μg/mL) and was susceptible to teicoplanin. It was discovered that this organism synthesized PG precursors terminating in D-ala-D-ser and had serine racemase (VanT) activity. Genetic analysis of the *vanE* gene (Fig. 1.14) showed it had a >50% identity with the *vanC* gene (Fines *et al*., 1999).

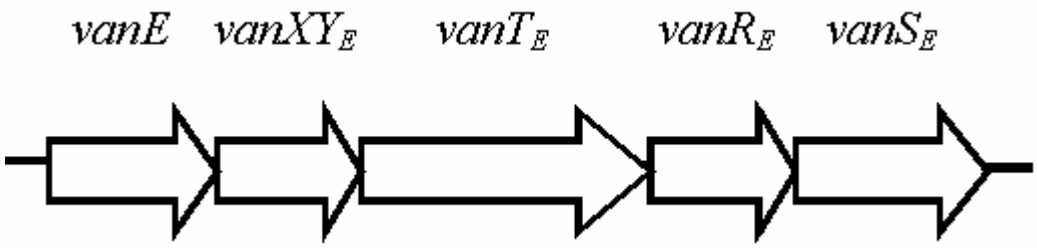


Figure 1.14: Schematic representation of the *vanE* gene cluster. Open arrows represent the coding sequences and the direction of transcription.

1.6.6 vanF resistance phenotype

Paenibacillus popilliae is the causative agent of milky disease in Japanese beetle larva, an agricultural pest. Biopesticidal powders containing the spores of *P. popilliae* have been used for more 50 years in the USA for the control of the Japanese beetle (*Popilliae japonica* Newman) population. In 1960, it was first noted that the isolate *P. popilliae* NRRL B2309 was resistant to vancomycin (MIC= 400-800μg/mL) but susceptible to teicoplanin (MIC<1μg/mL). Thereafter several more *P. popilliae* isolates resistant to vancomycin were identified (Rippere *et al*., 1998).

In 1998, Rippere and colleagues detected a putative ligase gene with a 77% homology to a portion of the enterococcal *vanA* gene in *P. popilliae* (Rippere *et al*., 1998). The putative ligase gene was designated *vanE*. In was later changed to *vanF* since *vanE* was described in *E. faecalis*. In 2000, Patel and colleagues investigated the presence of other *van*-like genes were present in *P. popilliae*. Their study revealed the presence of 4 genes homologous to the enterococcal *vanH, vanX, vanY* and *vanZ* (Fig. 1.15) (Patel *et al*., 2000).

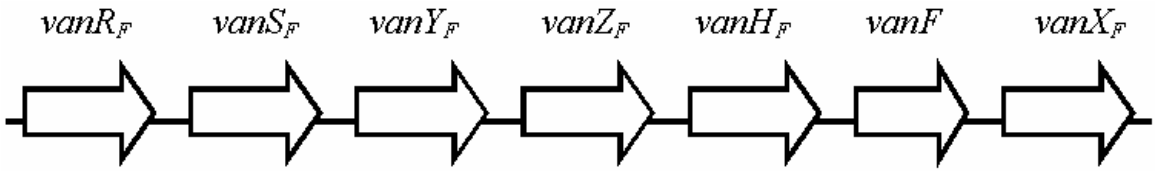


Figure 1.15: Schematic representation of the *vanF* gene cluster. Open arrows represent the coding sequences and the direction of transcription.

1.6.7 vanG resistance phenotype

McKessar and co-workers (2000) reported four *E. faecalis* strains, indistinguishable by macro-restriction analysis, that expressed moderate levels of vancomycin resistance (MIC = 16 μg/mL) but were susceptible to teicoplanin. This phenotype is similar to the VanB and VanE phenotypes but was polymerase chain reaction (PCR) negative using primers specific for all the enterococcal vancomycin resistance genotypes. This novel vancomycin resistance genotype was designated VanG. Genetic analysis of the *vanG* gene cluster showed that the gene arrangement was different to the other *van* clusters (McKessar *et al*., 2000).

Depardieu and colleagues (2003b) performed further analysis on the *vanG* cluster in *E. faecalis* WCH9 and 5 new *E. faecalis* strains BM4518-21 showing similar resistance to WCH9. The *vanG* gene cluster was determined to be chromosomal and vancomycin resistance a result of the synthesis of PG precursors terminating in D-ala-D-ser. The *vanG* operon encodes eights proteins: $VanU_G$, $VanR_G$, $VanS_G$, $VanY_G$, $VanW_G$, VanG, $VanXY_G$, and $VanT_G$. $vanU_G$, $vanR_G$ and $vanS_G$ encode a three component putative regulatory system as opposed to the two-component regulatory system observed in the other *van* genotypes (Fig. 1.16). Present in the gene cluster is $VanXY_G$, a putative bi-functional D, D-dipeptidase and D, D-carboxypeptidase, *vanG* a D:ala:D:ser ligase, $vanT_G$ a serine racemase and $vanW_G$, the function of which is unknown (Depardieu *et al*., 2003b).

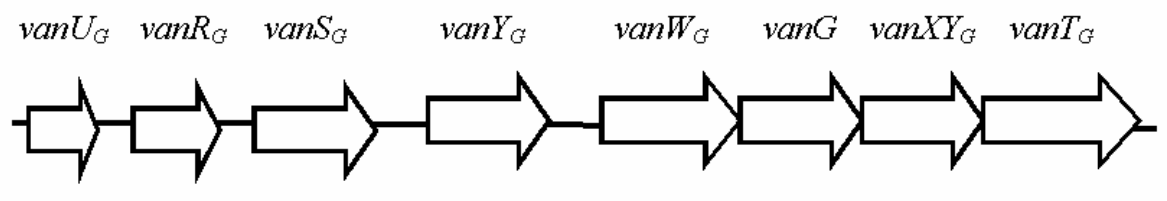


Figure 1.16: Schematic representation of the *vanG* gene cluster from *E. faecalis* WCH9 and BM4518. Open arrows represent the coding sequences and the direction of transcription.

1.6.8 vanB phenotype- *vanA* genotype

The *vanB* gene cluster confers resistance to vancomycin only. The *vanA* gene cluster confers resistance to both vancomycin and teicoplanin. In the *vanA* gene operon, *vanZ* is an accessory gene with an unknown gene product that confers teicoplanin resistance. Mutations in the *vanZ* gene and the absence of functional VanZ protein could result in teicoplanin susceptibility and the manifestation of a vanB phenotype. Hashimoto and colleagues (2000) characterized four *vanA*- type VRE isolates that expressed high-level vancomycin resistance and low-level teicoplanin resistance (VanB phenotype).

Their studies found that all four isolates had the identical amino acid substitutions at the same position in the deduced VanS amino acid sequence. They concluded that three point mutations in the *vanS* gene resulted in the three amino acid substitutions in the N-terminal region of VanS, which confers reduced teicoplanin resistance. The three substitutions are leucine [Leu/ L]$_{50}$→valine [Val/ V], glutamate [Glu/ E]$_{54}$→ glutamine [Gln/ Q] and Gln$_{68}$→histidine [His/ H] (Hashimoto *et al*., 2000).

In Taiwanese retail chickens, Lauderdale and colleagues (2002) found *E. faecium* and *E. faecalis* isolates that were highly resistant to vancomycin and susceptible to teicoplanin (vanB phenotype) but PCR positive for *vanA*. In addition, they also found four human *E. faecalis* isolates that were highly resistant to vancomycin and intermediately resistant to teicoplanin but were characterized as *vanA* genotypically (vanB phenotype- *vanA* genotype incongruence). All of the VRE chicken isolates had the same three point nucleotide mutations and the resultant amino acid substitutions described by Hashimoto *et al*. (2000). One of the three above mentioned human isolates had all three mutations and the other two had two of the three mutations. The *vanZ* gene sequences of all isolates showed no changes irrespective of whether the isolates were resistant or susceptible to teicoplanin. These results suggest that the incongruity in the isolates described by Lauderdale and colleagues is not a result of mutations in the *vanZ* gene (Lauderdale *et al*., 2002).

1.7 Glycopeptide resistance in non-enterococcal species

Since the appearance of GRE in the late 1980s, the potential spread of vancomycin resistance determinants to other clinically relevant Gram-positive bacteria has been anticipated. This was confirmed by the discovery of the *van* genes in organisms such as *Streptococcus bovis* and *S. aureus* (discussed in section 1.8).

1.7.1 Glycopeptide resistance determinants in clinically relevant Gram-positive bacteria

Streptococcus bovis is a group D streptococcus frequently found in the intestinal flora of humans and animals. Under certain pathological conditions, this organism can cause bacteraemia, endocarditis, neonatal infection and meningitis. Until recently, all strains of *S. bovis* described have been susceptible to glycopeptides. In 1997, Poyart and colleagues described the first clinical isolate of *S. bovis* to express inducible resistance to vancomycin. Genetic analysis on this isolate showed that this resistance phenotype is a result of a chromosomally-borne *vanB*-related gene, with a 95.5% sequence homology to the prototype *vanB* sequence of *E. faecalis* V583. This is the first time the role of streptococci other than enterococci has been implicated in the distribution of vancomycin resistance genes. This highlighted the potential danger of a spread of vancomycin resistance genes to other streptococci, such as penicillin-resistant pneumococci, for which vancomycin is used in combination with a third generation cephalosporin (Poyart *et al*., 1997).

1.7.2 Glycopeptide resistance determinants in other non-enterococcal clinical isolates and environmental organisms

In 1995, Power and colleagues reported clinical isolates of *Oerskovia turbata* and *Arcanobacterium haemolyticum* demonstrating high-level, constitutive resistance to both vancomycin and teicoplanin. Molecular analysis showed the presence of a *vanA* gene carried on a plasmid. This was the first report confirming the presence of a *vanA* gene cluster in genera other than enterococci. (Powers *et al*., 1995).

In 2001, Stinear and colleagues isolated two vancomycin resistant anaerobic bacteria from human faeces namely, *Eggerthella lenta* and *Clostridium innocuum*. Molecular analysis of these isolates showed that they were *vanB* PCR positive. The authors propose that the *vanB* resistance arose from horizontal gene transfer in the human bowel (Stinear *et al*., 2001).

In 2004, David and associates described the mechanism of intrinsic vancomycin resistance in *C. innocuum* NCIB 10674. Low-level resistance to vancomycin was a result of the production of PG precursors terminating in D-ala-D-ser. Sequencing of a chromosomal fragment from *C. innocuum* NCIB 10674 revealed the presence of an endogenous *ddl* gene and a racemase gene. The authors conclude that these two putative D-ala: D-ser ligase and racemase genes are associated with the resistance observed in the this isolate i.e. permitting the synthesis of PG precursors terminating in D-ser, which as previously mentioned, has a low affinity for vancomycin (David *et al*., 2004).

Bacillus circulans is a naturally occurring glycopeptide susceptible bacterium. In 1998, Ligozzi and co-workers reported the isolation of the first clinical *B. circulans* strain with

glycopeptide resistance. Genetic analysis of the *van* genes revealed that this strain contains a chromosomally-borne *vanA*-like gene cluster, with a high degree of sequence similarity to the *vanA* gene cluster found in enterococci. This resistance cluster was not associated with Tn*1546* as determined by PCR analysis using the ORF1 and ORF2 primers. The authors identified one *orf* upstream of *vanR*. The deduced protein showed a 28% amino acid sequence similarity with the Tn*1546*-encoded resolvase, suggesting that this *vanA*-like is associated with a novel mobile genetic element (Ligozzi *et al*., 1998).

A gene resembling *vanA* and *vanB* in *P. popilliae* was identified in 1998 (See section 1.6.6). The putative ligase gene was amplified from an isolate stored in dry haemolymph since 1945. This suggests that the ligase present in *P. popilliae* was not transferred from a GRE since GRE was only described in the late 1980s. The putative ligase detected in *P. popilliae* showed similarity to both *vanA* and *vanB* ligase genes, suggesting that this gene may be the precursor for the *vanA* and *vanB* genes found in current GRE strains. The similarity of the entire *vanF* resistance cluster in *P. popilliae* to that found in *vanA*-VRE would suggest a common ancestral origin (Patel *et al*., 2000).

Very recently, *Paenibacillus thiaminolyticus* PT-2B1 and *Paenibacillus apiarius* PA-B2B isolated from soil samples in Denmark were found to harbour a *vanHAX*-like gene cluster homologous to the *vanHAX* enterococcal gene cluster (Guardabassi *et al*., 2004). The putative D-ala-D-lac ligase gene amplified using degenerate primers in *P. thiaminolyticus* PT-2B1 and *P. apiarius* PA-B2B exhibited a 92% and 87% similarity to *vanA* (Guardabassi *et al*., 2004).

The partial *vanH*- and *vanX*-like sequences flanking the putative D-ala-D-lac ligase gene in *P. thiaminolyticus* PT-2B1 and *P. apiarius* PA-B2B exhibited the highest percentage identity that have been reported for non-enterococcal environmental isolates to the genes in Tn*1546* (Table 1.3). The presence of these *vanA*-like genes in *Paenibacillus* species would suggest that members of this genus could have played a role in the evolution of enterococcal *vanA* glycopeptide resistance (Guardabassi *et al*., 2004).

Table 1.3. Nucleotide identity (%) between *vanHAX*-like genes in *P. thiaminolyticus* PT-2B1, *P. apiarius* PA-B2B and vanA *E. faecium* BM4147

	Partial *vanH*-like sequence	Complete *vanA*-like sequence	Partial *vanX*-like sequence
P. thiaminolyticus PT-2B1	92%	92%	94%
P. apiarius PA-B2B	79%	87%	84%

Adapted from Guardabassi *et al*.(2004)

1.8 Vancomycin resistance in *Staphylococcus aureus*

Staphylococcus aureus is the most common causative agent of nosocomial- and community-acquired infections (Appelbaum *et al*., 2004). Vancomycin is the drug of choice for the treatment of infections caused by methicillin-resistant *S. aureus* (MRSA). MRSA harbours a chromosomal *mecA* gene that encodes a MRSA-specific PBP 2' or PBP 2a. β-lactams have a reduced affinity for this variant PBP. Since the identification of VRE in the late 1980s, the emergence of vancomycin-resistant *S. aureus* (VRSA) was anticipated (Hiramatsu, 2001).

Staphylococci and enterococci naturally exchange antibiotic resistance determinants, so it was surprising that clinical isolates of staphylococci carrying *van* genes had never been isolated until recently. The transfer of the *vanA* gene by conjugation from *E. faecalis* NCTC 12201 to *S. aureus* B111 had been demonstrated under laboratory conditions. The researchers of this study showed that high-level glycopeptide resistance could be transferred from enterococci to *S. aureus* under *in vitro* conditions similar to those in nature (Noble *et al*., 1992).

1.8.1 Vancomycin intermediate resistant *Staphylococcus aureus*

In May 1996, the first MRSA clinical isolate with intermediate resistance to vancomycin was isolated in Japan. This vancomycin-intermediate *S. aureus* (VISA), designated Mu50 had an MIC = 8μg/mL (Hiramatsu *et al*., 1997). Investigations into the molecular mechanism of resistance revealed that this isolate did not possess the enterococcal *van*

genes (*vanA*, *vanB*, *vanC1* or *vanC2/3*) when tested by PCR using *van* specific primers. The absence of *van* genes suggested that vancomycin resistance in Mu50 is not a result of the production of PG precursors terminating in D-ala-D-lac, which was confirmed by high performance liquid chromatography (HPLC) analysis of the PG monomers. Studies of the mechanism of Mu50 resistance showed that the isolate produced 3-5 times more PBP 2 and PBP 2' and a 3-8-fold increase in intracellular PG monomers when compared to vancomycin-susceptible control strains. Transmission electron microscopy (TEM) showed a significant increase in the cell wall thickness in Mu50 compared with the control strains (Fig. 1.17) (Hanaki *et al*., 1998a).

Further analysis of the PG composition of Mu50 showed an increase in NAG incorporation into the cell wall and increased amounts of non-amidated PG monomers, which results in reduced cross-linking (Hanaki *et al*., 1998b). Non-amidated PG monomers have a greater binding affinity to vancomycin, which decreases the concentration of the antibiotic available to bind the PG precursors (Cui *et al*., 2000). In the growing Mu50, vancomycin molecules are trapped in the mesh-like outer PG layers and in combination with the effect of reduced cross linking of the cell wall, the antibiotic is unable to bind to exert its antimicrobial effect. This phenomenon of trapping the antibiotic molecules is termed "affinity trapping". The fundamental feature of all VISA isolates is a thickened cell wall, in all probability due to the increased amounts of PG (Cui *et al*., 2003).

a) b)

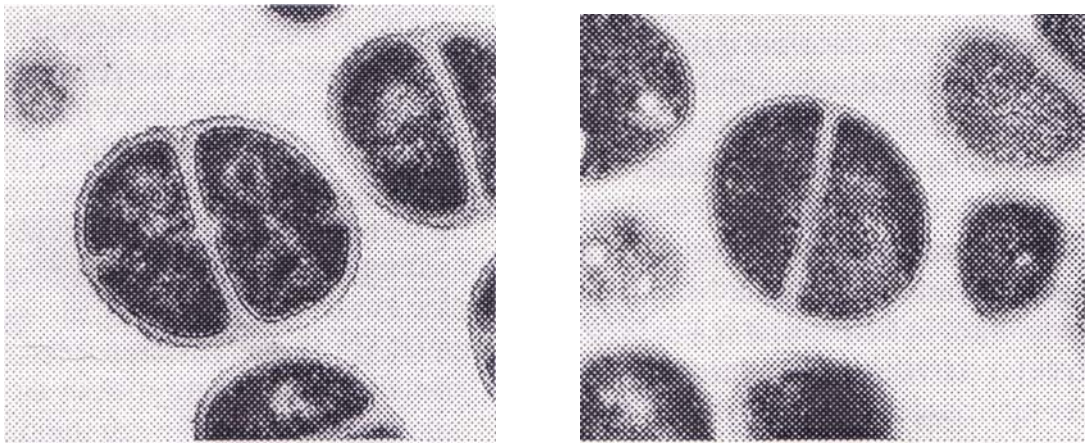


Figure 1.17: Transmission electron microscopy of Mu50 (a) and H1 (b). Electron micrographs shows that Mu50 cell wall is approximately twice as thick as compared to the control H1 strain (Hanaki *et al*., 1998a).

1.8.2 Vancomycin resistant *Staphylococcus aureus*

In July 2002, the first clinical isolate of VRSA, with a MIC >128 μg/mL, was documented in Michigan, USA (*MMWR*, 5 July 2002). In September 2002, a second VRSA isolate with a MIC= 64 μg/mL was detected in Pennsylvania, USA (*MMWR*, 11 October 2002). In April 2004, a third VRSA clinical isolate, with an MIC= 64 μg/mL was reported in New York, USA (*MMWR*, 23 April 2004). All three VRSA isolates contained both the *mecA* and *vanA* genes, which confers oxacillin and vancomycin resistance, respectively. All three isolates were shown to be unrelated by macro-restriction analysis. The presence of the *vanA* gene in these VRSA suggests that these organisms acquired the vancomycin resistance determinant from a GRE, via conjugative events. Additional cases of *van* genes associated VRSA are likely to occur, either by the spread of a few clones, or by multiple transfer events (*MMWR*, 11 October 2002 and *MMWR*, 23 April 2004).

1.9 Origins of the vancomycin resistance genes

The origin of antibiotic resistance determinants is of importance for several reasons, such as permitting a prediction of the emergence and spread of resistance, the design of new antimicrobial drugs, and the identification of potential reservoirs of resistance genes.

Acquired antibiotic resistance can occur either through spontaneous mutation of the gene encoding the antibiotic target, thereby modifying it and rendering it unrecognizable by the antibiotic, or by the acquisition of external genetic elements, such as plasmids or transposons that carry resistance determinants. The origins of acquired resistance genes are diverse, but it has long been acknowledged that the potential reservoirs of these genes are the antibiotic-producing organisms that naturally harbour resistance genes to protect themselves from the actions of the synthesized antibiotic (Marshall *et al*., 1998b).

The origins of the *van* resistance genes in enterococci have not been unequivocally established. One hypothesis is that the appearance and widespread dissemination of the *van* resistance genes is a direct result of selective pressures due to the increased oral and parenteral use of vancomycin in clinical practice and the use of avoparcin in animal husbandry. The increasing number of GRE isolated in the clinical setting is due to the burgeoning use of vancomycin as a prophylactic agent and their use in patients that are sensitive to penicillin (Klein *et al*., 1998). The European Union, banned the use of avoparcin in 1997 due to the potential link of the spread of VRE from food animals to humans. Avoparcin is used as an antibacterial growth-promoter in animal feed in the pig and poultry industries (Patel *et al*., 2000).

Markopulous and co-workers (1998) demonstrated that vancomycin resistance in enterococci could not develop as a result of continuous exposure to the antibiotic, but that *S. epidermidis*, was able to develop increased resistance to teicoplanin. All starting strains of *E. faecium, E. faecalis* and S. *epidermidis* used in the study were susceptible to both vancomycin and teicoplanin. The strains were grown in the presence of vancomycin or teicoplanin using two selection methods, agar- and broth- dilution. Each strain was subjected to 20 passages and the MICs re-evaluated after 5 passages. After every 5 passages, cultures were checked for contamination. The tests were performed in duplicate and changes in the MICs were documented for each strain and antibiotic.

Results showed that in the *E. faecalis* strains, resistance to both vancomycin and teicoplanin increased slightly (2- to 4- fold higher than the starting MICs). In *E. faecium* strains, the teicoplanin MICs increased by 2- to 8- fold but no increase in vancomycin MICs were observed. In *S. epidermidis* the MICs for vancomycin did not change but the MICs for teicoplanin increased 64-fold.

Their results do not support the hypothesis that the development of resistance in a particular patient is a result of prolonged glycopeptide therapy but rather supports the hypothesis that the emergence of glycopeptide resistance *in vivo* is a result of the spread of glycopeptide resistant organisms (Markopulos *et al*., 1998).

Marshall and colleagues (1998b) presented an alternate hypothesis to the origins of the *van* genes. The intrinsically vancomycin-resistant lactic acid bacteria i.e. from the genera *Leuconostoc* and *Lactobacillus*, and the glycopeptide-producing bacteria e.g. *Amycolatopsis orientalis* and *Streptomyces toyocaensis*, are potential reservoirs of the *van*-

like genes that may have been transferred to enterococci. Intrinsic genes encoding a VanA/B homologue designated *DdlM*, from *Streptomyces toyocaensis* and the ligase gene (*ddlN*) from *Amycolatopsis orientalis* were cloned and sequenced. Sequence analysis of the *DdlM*, *ddlN*, *vanA* and *vanB*, showed a high degree of homology between these genes. These results suggest that the enterococcal VanA/B enzymes might have originated in glycopeptide-producing organisms (Marshall *et al*., 1998a).

Cloning and sequencing of homologous *vanH* and *vanX* genes in the glycopeptide-producing organisms revealed the presence of a *vanHAX*- like gene cluster. This strong homology of the gene products and the arrangement of the cloned genes indicate a common origin for the vancomycin resistance genes in GRE and antibiotic-producing organisms (Marshall *et al*., 1998b).

Guardabassi *et al*. (2004) state that due to the harsh and competitive nature of an environment such as soil, this would most likely encourage environmental bacteria to develop or acquire resistance mechanisms (Guardabassi *et al*., 2004). Therefore an alternate hypothesis to the origins of van^R genes is that vancomycin resistance determinants found in environmental microorganisms that do not produce glycopeptides, could have been transferred to enterococci, resulting in the emergence and establishment of GRE.

Environmental organisms containing genes resembling *van*A and *vanB* were not identified until 2000, when resistance genes in *P. popilliae* were detected. The use of *P. popilliae* spores in biopesticidal preparations in agriculture may have had an impact on vancomycin resistance observed in present GRE (Rippere *et al*., 1998).

To support the above hypothesis the nucleotide sequence similarity of the *vanHAX*-like genes in *P. thiaminolyticus* PT-2B1 and *P. apiarius* PA-B2B when compared to the *vanA, vanH* and *vanX* genes from *vanA*- carrying *E. faecium*, is the highest percentage identity that have been reported for non-enterococcal glycopeptide resistant environmental isolates (section 1.7.2). Phylogenetic analysis of the *vanA*-like genes in *P. thiaminolyticus* PT-2B1, *P. apiarius* PA-B2B, *vanA* gene in *B. circulans* VR0709 and enterococcal *vanA* would suggest that the four genes have a close evolutionary relationship (Guardabassi *et al*., 2004).

CHAPTER 2

AIMS OF THE STUDY

Two glycopeptide resistant Gram-positive clinical isolates, *Bacillus lentus* RSA1208 and *Paenibacillus thiaminolyticus* RSA1221, were studied to investigate the molecular mechanisms of glycopeptide resistance and to establish a relationship to the known glycopeptide resistance mechanisms.

The investigations of each isolate have been split into Study A: *B. lentus* RSA1208 and Study B: *P. thiaminolyticus* RSA1221, except the methodology is presented as a single chapter since many techniques used were common to both studies. In view of the diversity of the isolates and the differences found in their resistance mechanisms, it was deemed that each organism should be discussed separately under the headings; results, discussion and conclusion. The *B. lentus* RSA1208 and *P. thiaminolyticus* RSA1221 studies are presented in Chapter 4 and 5, respectively. A final general conclusion is presented in Chapter 6.

Study A: Glycopeptide resistance characterization of human clinical isolate *Bacillus lentus* RSA1208

In February 1999, a Gram-positive *Bacillus* spp. resistant to vancomycin was isolated from the blood culture of a newborn infant admitted to a South African hospital with neonatal sepsis. The isolate, referred to as RSA1208, was later identified as *B. lentus*. *B. lentus* is a

large aerobic, gram-positive bacillus. The genus *Bacillus* is rarely implicated or associated with infections, but the presence of high vancomycin resistance in this non-enterococcal species (MIC >256 μg/mL), may reflect the mobility of the *van* resistance gene clusters.

Molecular analysis of the *van* gene clusters would emphasize the evolutionary processes of vancomycin resistance. Characterization of the vancomycin resistance mechanisms will lead to an enhanced understanding of the transfer and selection of resistant organisms. This could impact on both the knowledge of the global epidemiology of glycopeptide-resistant enterococci, and aid in the prudent design and usage of antibiotics.

Study A specific objectives:

1. Determine whether *van* genes are responsible for the vancomycin resistant phenotype of *Bacillus lentus* RSA1208.
2. If *van* genes are present, they will be sequenced and characterized.
3. If no *van* genes are found, other mechanisms of resistance will be investigated

Study B: Characterization of glycopeptide resistance of human clinical isolate *Paenibacillus thiaminolyticus* RSA1221

In October 1998, a Gram-positive organism (strain RSA1221) was isolated from a blood culture collected from an acquired immunodeficiency syndrome (AIDS) patient diagnosed with pulmonary tuberculosis. Susceptibility testing showed the organism to be highly resistant to vancomycin (MIC >256μg/mL).

The organism was provisionally identified as *Paenibacillus alvei*, but further characterization was performed in Dr N. Logan's department at the Glasgow Caledonian University. This reference laboratory used the 74 phenotypic characteristics from the API identification systems, 20E and 50CHB, together with the Biotype 100 assimilation kit and morphological examination, and the results were subjected to numerical analysis using an extensive database comprising species of *Bacillus* and related genera. The final identification was *Paenibacillus thiaminolyticus*.

Preliminary molecular analysis of the strain was performed prior to this project to determine the vancomycin resistance genotype and to find similarities to either the *vanA* genotype commonly found in GRE or the genotype described in *B. circulans* strain VR0709. PCR analysis revealed the presence of a *vanA* gene and the absence of both the open reading frames associated with the *vanA*-carrying transposon Tn*1546* and the *orf* found in *B. circulans* VR0709.

Documentation of the presence of the *vanA* gene in *P. thiaminolyticus* may be of importance in elucidating the origin and evolution of vancomycin resistance genes in Gram-positive bacteria and needs to be investigated further.

Study B specific objectives:

1. Determine the extent of homology of the *vanA* gene detected in *P. thiaminolyticus* RSA1221 with other documented *vanA* genes.
2. To detect and sequence other *van* associated resistance genes (*vanR*, *vanS*, *vanH*, *vanA*, *vanX*, *vanY*, and *vanZ*) and compare to known *vanA* cassettes in terms of gene orientation and sequence similarity.
3. Determine whether the resistance determinant resides on the bacterial chromosome or a plasmid.
4. Determine whether the *vanA* gene cluster is associated with a known transposon or mobile genetic elements other than those previously documented.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemical and reagents, media and kits

All chemicals and reagents used in this study were of molecular or analytical grade. A list of all reagents and kits used and the composition of media, see appendices I-III. For a complete list of the composition of buffers and the restriction endonucleases used in this study, see section 3.15.

3.2 Strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 3.1 and Table 3.2, respectively. All strains were cultured overnight on 5% blood agar plates and in 10ml brain heart infusion (BHI) broth at 37^{o}C (IncoTherm, Labotec, Midrand, South Africa).

Short-term stock cultures were prepared by stab-inoculating semi solid agar vials with each strain, which were incubated at 37^{o}C overnight and stored at room temperature. Long-term stock cultures were made by adding 1mL of either 100% sterile glycerol (vol/vol) or 100% dimethylsulfoxide (DMSO) (vol/vol) to 1mL of an overnight culture grown in BHI broth. The cultures were mixed and stored at –70^{o}C (Kelvinator Series 1000, Manitowoc, Wisconsin, USA).

Table 3.1: Bacterial strains used in this study

Strain	Relevant genotype	Relevant phenotype	Source or reference
Bacillus lentus RSA1208	Unknown	High-level glycopeptide resistant	Clinical isolate[a]
Paenibacillus thiaminolyticus RSA1221	*vanA*	Glycopeptide resistant	Clinical isolate[a]
Enterococcus faecium BM4147	*vanA* (plasmid-borne)	Glycopeptide resistant	Prof. P Courvalin Institut Pasteur, Paris France
Bacillus circulans VR0709	*vanA* (chromosomally-borne)	Glycopeptide resistant	Prof. R Fontana Universita Degli Studi Di Verona, Verona Italy
Bacillus lentus B0498	---	Glycopeptide susceptible	Dr. N Logan Glasgow Caledonian University, Glasgow Scotland
Bacillus lentus B1090	---	Glycopeptide susceptible	Dr. N Logan Glasgow Caledonian University, Glasgow Scotland

Bacillus lentus B1048	---	Glycopeptide susceptible	Dr. N Logan Glasgow Caledonian University, Glasgow Scotland
DH5α™ Chemically Competent *Escherichia coli* cells	*gyrA96* (mutation in the DNA gyrase gene) ϕ*80dlacZΔM15* (partial deletion in β-galactosidase gene)	Nalidixic acid resistant Blue/white screening	Invitrogen Life Technologies Carlsbad, CA, USA
Library efficiency DH5α™ Competent *E. coli* cells	*gyrA96* (mutation in the DNA gyrase gene) ϕ*80dlacZΔM15* (partial deletion in β-galactosidase gene)	Nalidixic acid resistant Blue/white screening	Invitrogen Life Technologies Carlsbad, CA, USA

a. University of the Witwatersrand Human Research Ethics Committee clearance number M0L-05-07

Table 3.2: Bacterial plasmids used in this study

Plasmid	Relevant features	Source or reference
pUC19	- Ampicillin resistance - Multiple cloning site within the β-galactosidase gene (blue/white screening)	Invitrogen Life Technologies Carlsbad, CA, USA
pGEM®-3Zf(+)	- Multiple cloning site within the β-galactosidase gene (blue/white screening) - Ampicillin resistance gene	Promega Madison, WI, USA

3.3 Phenotypic characterization of *B. lentus* RSA1208 and *P. thiaminolyticus* RSA1221

Both bacterial isolates were characterized using conventional microbiological methods. The primary tests used to characterize each bacterium were Gram staining and bacterial cell morphology on agar plates. In addition to the primary tests, the following biochemical tests were performed: catalase activity, oxidase activity, indole production, Voges-Proskauer, nitrate reduction, carbohydrate utilization (sorbitol, arabinose, pyruvate, sucrose, mannitol, glucose, lactose, xylose, and salicin), gelatin hydrolysis, starch hydrolysis, lecithinase production, citrate utilization, urease production, aesculin hydrolysis.

Table 3.3: Biochemical characteristics of *B. lentus* (Koneman *et al*., 1997) and *P. thiaminolyticus* (Parry *et al*., 1983)

Characteristics	***B. lentus***	***P. thiaminolyticus***
Catalase activity	Positive	Positive
Oxidase activity	Positive	Positive
Indole production	Negative	Positive
Voges-Proskauer	Negative	Negative
Nitrate reduction	d	Positive
Carbohydrate utilization		
- Sorbitol	d	--
- Arabinose	Positive	Variable
- Pyruvate	Weak positive	--
- Sucrose	Variable	--
- Mannitol	Weak positive	Negative
- Glucose	Weak positive	Positive
- Lactose	Positive	--
- Xylose	Negative	--
- Salicin	Positive	Negative
Gelatin hydrolysis	d	Positive
Starch hydrolysis	d	Positive
Lecithinase production	Negative	Negative
Citrate utilization	Negative	Negative
Urease production	Positive	Positive
Aesculin hydrolysis	Positive	--

d. 11-89% of strains are positive. Adapted from (Parry *et al.,* 1983, Bergey's Manual of Systematic Bacteriology, 1984 and Koneman *et al*., 1997)

3.4 Antibiotic susceptibility testing

The minimum inhibitory concentrations (MICs) for vancomycin and teicoplanin were determined for each isolate by the E-test method (AB Biodisk, Solna, Sweden) on Mueller-Hinton (MH) agar plates as well as the broth microdilution method. The interpretative criteria of the National Committee for Clinical Laboratory Standards (NCCLS, 2003b) for *Staphylococcus* spp. were used to determine the antibiotic susceptibilities of the isolates for both the broth microdilution and disk diffusion tests (see appendix V).

3.4.1 Broth microdilution for determining MIC using the microtitre plate method

The MICs were determined by using the recommendations of the NCCLS and the broth microdilution method (NCCLS, 2003a).

One hundred microlitres of cation-adjusted Mueller-Hinton broth (CAMHB) was pipetted into 12 wells of a 12-well microtitre plate. The test antibiotic was diluted in CAMHB to a concentration of 128µg/mL. A 100µL volume of the diluted antibiotic was pipetted into well 1 and serially diluted ($^1/_{10}$) into wells 2-12. A 100µL 0.5 McFarland turbidity standard of the bacterial suspension was added to the wells. The colony count of the bacterial suspension was determined by diluting 10µL of the inoculum in 10mL of saline. Two hundred microlitres of this suspension was spread on a MH agar plate and incubated overnight at 37^{o}C. After incubation, the presence of approximately 50 colonies indicates an inoculum density of 5 x 10^5cfu/mL. The microtitre plate was covered with a lid and placed in a plastic bag to prevent evaporation, and incubated overnight at 37^{o}C. After incubation,

the colony count was determined and the microtitre plate checked for visible growth using a Microtitre tray reader (MIC 2000, Cooke Laboratory Product, Division of Dynatech Laboratories, Inc.). The lowest concentration of the antibiotic that visibly inhibits growth is the MIC (μg/mL). The MIC results were then compared with the NCCLS guidelines to determine susceptibility.

3.4.2 E-test for MIC (μg/mL) determination of vancomycin and teicoplanin

An appropriate number of colonies from an overnight agar plate were suspended in 1mL saline to a 0.5 McFarland turbidity standard. A sterile cotton swab was dipped into the suspension and spread evenly onto a 5% blood agar plate and allowed to dry for 10-15 min. The E-test antibiotic strip was placed on the inoculated agar surface. The inoculated plates were incubated overnight at 37^{o}C. After incubation, the MIC was determined as the point of intersection between the inhibition ellipse edge and the MIC value on the E-test strip. If no inhibition ellipse was seen, the MIC was reported as greater than (>) the highest value on the MIC reading scale.

3.4.3 Kirby-Bauer disc diffusion antibiotic susceptibility method

Susceptibility testing was performed using antibiotics other than vancomycin and teicoplanin using the disc diffusion method on MH agar plates, supplemented with sheep's blood. Briefly, a single colony was suspended in 1mL saline and spread evenly with a sterile cotton swab onto the agar plate. After the agar surface had dried for about 5 minutes

(min), the antibiotic discs were placed equidistant on the inoculated plates and incubated at 37^{o}C overnight. Results were interpreted by measuring the zone diameters with the use of sliding callipers. The zones of complete growth inhibition around each disc were carefully measured to within the nearest millimetre. The diameter of the disc was included. An interpretative correlation (susceptible, moderately susceptible, intermediate, or resistant) provided by the published NCCLS guidelines was used, to determine the antibiotic susceptibilities of the isolates.

3.5 Expression of vancomycin resistance

Cultures of the test organism were grown overnight in BHI without vancomycin and BHI containing a sub-inhibitory concentration of vancomycin (32µg/mL). Overnight bacterial cultures were sub-cultured into either fresh BHI with the same concentration of vancomycin or BHI containing no vancomycin. The cultures were grown at 37°C with shaking. Absorbance values (OD_{600}) were measured hourly on a Jenway Model 6300 spectrophotometer (Jenway, Division of Barloworld Scientific, Stone, Stadfordshire, England). A growth curve was plotted to determine the expression of resistance as a function of bacterial growth.

3.6 DNA preparation

3.6.1 Plasmid DNA preparations

Plasmid DNA was isolated using the alkaline lysis method (Ausubel *et al*., 1998). Bacteria were grown in 10mL BHI broth (37^{o}C overnight, shaking at 120rpm). Cells were pelleted (4500g, 15min) (Eppendorf Centrifuge 5804, Eppendorf AG, Hamburg, Germany). The bacterial pellet was resuspended in 250µL MP1 (resuspension) buffer and transferred to 1.5mL microfuge tubes. Two hundred and fifty microlitres of MP2 (lysis) buffer was added and briefly mixed, and left at room temperature for a maximum of 5min. After the brief incubation, 250µL chilled MP3 (neutralization) buffer was added and left on ice for 15min. The solution was centrifuged at 17900g for 15min (Eppendorf Centrifuge 5417C) and the clear supernatant transferred to a new 1.5mL microfuge tube. To precipitate the plasmid DNA, 525µL of 100% isopropanol was added. The DNA was harvested by centrifugation at 17900g for 15min. The plasmid DNA pellet was washed in 70% ethanol and centrifuged at 12900g for 5min. The pellet was air-dried and resuspended in d. H_2O or 10mM Tris- EDTA (TE) buffer.

3.6.2 Total genomic DNA preparations

For the preparation of total genomic DNA, bacteria were cultured in 10mL of BHI broth (37°C, overnight, shaking at 120rpm) (Incubator Shaker Series 25, New Brunswick Scientific Co. Inc, Edison, New Jersey, USA) and were harvested by centrifugation (4500g, 20min). The bacteria were suspended in 600µL of 6.7% sucrose solution; 8µL of lysozyme (100mg/mL), 8µL of RNaseA (1mg/mL), and the mixture was incubated for

30min at 37°C. Seventy five microlitres of 20% sodium dodecyl sulfate (SDS) was then added, and the mixture incubated for 30 min, followed by digestion with 15μL of proteinase K solution (20mg/mL) at 37°C for 30min. Total DNA was extracted twice with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) and Phase Lock Gel (Eppendorf AG). To the supernatant, 0.1vol of 3 M sodium acetate was added and the DNA was precipitated in equal volume of 100% isopropanol. After a 70% ethanol wash, the DNA was air-dried, and dissolved in 100-200μL d. H_2O or TE buffer.

3.7 DNA amplification and electrophoresis

Bacterial isolates were examined for the presence of *van* ligase genes (*vanA, vanB, vanC1, vanC2/C3, vanD*, and *vanE*), using gene specific primers, which are listed in Table 3.4. Other genes found in the *van* operon were also examined, and the oligonucleotide primers are listed in Table 3.5.

In the *B. lentus* RSA1208 study, degenerate primers (Table 3.6) were designed using the bioinformatics programme CODEHOP (COnsensus DEgenerate Hybrid Oligonucleotide Primer) to detect the presence of a D-specific α-ketoacid dehydrogenase. This gene is considered to be one of the essential genes required for expression of glycopeptide resistance.

The degenerate primers (VH1F, VH2F, and VH1R) were designed from multiple protein sequence alignments to amplify unknown targets that are related to the multiply aligned protein sequences. The CODEHOP strategy is a World Wide Web-based computer

programme (http://blocks.fhcrc.org/codehop.html). This programme is linked to the Blocker Maker multiple sequence alignment site for hybrid primer prediction starting with a set of related protein sequences. Each primer consists of a short 3' degenerate core region, and a longer 5' non-degenerate region called the consensus clamp region. The primer binds to the template DNA via the 5' consensus clamp during annealing. The hybrid structure of CODEHOP primers (5' consensus clamp and 3' degenerate core) allows for PCR amplification to be specific during the early cycles from the original source DNA and selective during the late cycles from the synthesized PCR products.

Two primer sets used to determine the *P. thiaminolyticus* RSA1221 DNA sequence of the region upstream of *vanR* (5') and downstream of *vanZ* (3') are listed in Table 3.7.

Table 3.4: Oligonucleotide primer sequences used for the PCR detection of *van* ligase genes

Primer pair	Nucleotide sequences (5'-3')	Product size base pair(bp)	Reference
vanA F	GGGAAAACGACAATTGC	731	Dutka-Malen *et al*., 1995
vanA R	GTACAATGCGGCCGTTA		
vanB F	ATGGGAAGCCCGATAGTC	635	Dutka-Malen *et al*., 1995
vanB R	GATTTCGTTCCTCGACC		
vanC1 F	GGTATCAAGGAAACCTC	842	Dutka-Malen *et al*., 1995
vanC1 R	CTTCCGCCATCATAGCT		
vanC2/*C4* F	CTCCTACGATTCTCTTG	431	Dutka-Malen *et al*., 1995
vanC2/*C3* R	CGAGCAAGACCTTTAAG		
vanD F	TAAGGCGCTTGCATATACCG	461	Perichon *et al*., 1997
vanD R	TGCAGCCAAGTATCCGGTAA		
vanE F	TGTGGTATCGGAGCTGCAG	513	Fines *et al*., 1999
vanE R	GTCGATTCTCGCTAATCC		

Table 3.5: Oligonucleotide primer sequences used for the PCR detection of other *van* genes

Primer pair	Target gene	Nucleotide sequences (5'-3')	Product size (bp)	Reference
A3 F A3R	*vanX*	GACCGAACTGAGATGAT AAACGGCACAATCTCGA	662	This project
A4F A4R	*vanY*	TTCACACCGCCCATTGT TCACTATAACCTGCTGG	714	This project
vanYF vanYR	*vanY*	ACTTAGGTTATGACTACGTTAAT CCTCCTTGAATTAGTATGTGTT	866	Miele *et al*., 1995
vanZF vanZR	*vanZ*	CTAGAGGATTGCTAGCTTTATATT GGTACGGTAAACGAGCAATAATA	445	Biavasco *et al*., 2001
A6F A6R	*vanZ*	GACGGTGTAATTGTGAG TGGGTACGGTAAACGAG	672	This project
A7 F A7 R	*vanH-vanA* overlap	CCGTTTGATGAGTTGCT AGTCCATCTTCACCTGA	722	This project
A8 F A8R	*vanH*	TATGGATGTGAGCAGGA GTATCTACAAGTGGACC	675	This project
A10 F A10 R	*vanS*	CTTGGGGATTGGATCTT CCCGCGGTAATGTCAAT	757	This project
A11 F A11 R	*vanR- vanS* overlap	ACAGGGTTAACAATCGG AGTACATCAATGCCGGT	743	This project
A12F A12R	*vanR*	GCCATTCTGTATTCCGC TTAATGACAAGGCCGGA	700	This project

Table 3.6: Degenerate primers used in the *B. lentus* RSA1208 study. The primers were designed using the CODEHOP bioinformatics programme to detect the presence of a D-specific α- ketoacid dehydrogenase

Primer pair	Target gene	Nucleotide sequences (5'-3')	Product size (bp)	Reference
VH1 F	*vanH*	TGCAGATTATACAATGATGCT GATGYTNATGGCNRT	VH1F + VH1R = 570	This project
VH2 F		CCACACGATCCATTGGCTGTA AYCACAYATHGA	VH2F + VH1R = 664	This project
VH1 R		TGTATAATATGCTGTATGCGGT GTAATAADNACRTTNGG		This project

D= A/G/T H= A/C/T R= A/G

N= A/C/G/T Y= C/T

Table 3.7: Oligonucleotide primer sequences used for the PCR detection of the 5'end and the *orfs* of the Tn*1546*

Primer pair	Nucleotide sequences (5'-3')	Product size (bp)	Reference
ORF1A ORF1B	TAGATCCGTCTCATGATG GATACATGGAATCAATCG	749	This project
ORF 2A ORF 2B	CGTGTCAGTTCGACTAACCA CCGCATAATTCATTCCTGCG	450	This project
VRB F VRB R	ACGCGTGTATACACCAGAGC AGGGCAAAGACACAGCTACA	662	This project
934 F	TGTGGATTTGCATCTGC	--	Willems *et al*., 1999
1292 R	TTACTCATGGATGTGGCC	--	Willems *et al*., 1999
1723 F	ACAGGTGAGTCATCAGGC	--	Willems *et al*., 1999
1913 R	CGTCCTGCCGACTATG	--	Willems *et al*., 1999
2768 F	AGGATGGACTAACACCAATC	--	Willems *et al*., 1999
3560 R	TATCCGAATAAGATCTCGCT	--	Willems *et al*., 1999
A6F	GACGGTGTAATTGTGAG	--	This project

In brief, total genomic DNA was extracted from cultures in BHI that were incubated overnight, using the High Pure Template Prep Kit (Roche Applied Sciences, Indianapolis, USA). DNA isolated from *E. faecium* BM4147 (*vanA*), *E. faecalis* (*vanB*), *E. gallinarum* (*vanC1*), *E. casseliflavus* (*vanC2/C3*) were used as PCR control strains.

All PCR reactions were set up in a laminar flow hood. PCR reactions were performed on a PCR thermal cycler, either a Hybaid cycler (Hybaid Omnigene, Middlesex, England) or an iCycler (Bio-Rad Laboratories Hercules, CA, USA), in a final volume of 50µL containing 1-10 ng of bacterial DNA template, 0.5µM of each primer, 1x Roche PCR Master Mix (Roche Applied Science). Fifty microlitres of mineral oil was pipetted over the PCR reaction if the thermal cycler used did not have a heated lid.

The cycles used were 95^{o}C for 2min for the first cycle; thereafter 35 cycles consisting of; 94^{o}C for 1min (denaturation), 1min at the calculated primer annealing temperature [(G/C X 4) + (A/T x 2)- 4] and 72^{o}C for 1min (extension), and 72^{o}C for 10min for the last cycle. The PCR products (20µL) were analyzed by electrophoresis in 1% Tris-acetate-EDTA (TAE) agarose gels (wt/vol) containing 1.5µL ethidium bromide (10mg/mL). A 100bp or 1kb ladder (Promega) was used as a molecular weight marker. Gels were visualized using a Bio-Rad Gel Doc 1000 system.

3.8 Nucleotide sequencing

Following gel electrophoresis, the bands containing DNA of the expected size were excised and eluted using the QIAquick Gel Extraction Kit (Qiagen Inc. Valencia, CA, USA). The concentration of the eluted product was estimated by gel electrophoresis (1% agarose- TAE gel (wt/vol), 6µL of Promega 100bp ladder as a comparison). The sequence reaction contained 10-100ng of PCR product, 4-8µL of Big Dye Terminator reaction mix (Applied Biosystems, Foster City, CA, USA), 2µL of 5µM primer, made up to 20µL with d. H_2O and overlaid with mineral oil. The cycling conditions were 25 cycles of 95^{o}C for 30sec, 50^{o}C for 30sec and 60^{o}C for 4min. The sequenced products were purified using the DyeEx Spin Kit (Qiagen) and the samples were analyzed on the automated Applied Biosystems 310 Genetic Analyzer. Both the sense and antisense strands were sequenced in duplicate.

3.9 Recombinant DNA techniques and nucleotide sequencing

Cloning of PCR products into a plasmid vector was done when problems were experienced with direct sequencing of PCR products. The presence of multiple templates in the sequencing reaction makes it impossible to analyze and therefore decreases the sequence quality. Cloning of PCR products into competent cells produces clones, each with a single copy insert.

Three hundred to five hundred nanograms of PCR product was cloned into 100ng of *Sma*I-restricted pGEM vector (Promega). The reaction also contained 0.43mM ATP (Promega),

10% polyethylene glycol (PEG) 6000 (Fermentas Life Sciences), 2.5U of *Sma*I restriction endonuclease to ensure the vector does not re-ligate, and T4 DNA ligase (400U/mL) (Roche Applied Sciences), and was incubated for 12-16 hours in the iCycler, alternating between 10^{o}C for 30sec and 30^{o}C for 30sec.

The ligation mix was transformed into DH5α™ Chemically Competent cells, using the manufacturer's protocol (Invitrogen Life Technologies). Transformed bacteria were plated on 50µg/mL ampicillin-containing Luria Bertani (LB) agar plates and incubated at 37^{o}C overnight. Individual colonies were selected and grown in LB broth overnight at 37^{o}C, shaking at 120rpm. Plasmid DNA was isolated using the commercial Qiagen QIAprep miniprep kit or the alkaline lysis method (section 3.6.1). Plasmid DNA was restricted with *EcoR*I (Promega) to check for the presence of an insert. Plasmid sequencing was performed using the plasmid M13 universal forward and reverse primers (Table 3. 8) and the Big Dye dideoxynucleotide chain termination method (Section 3.8). The sequenced products were purified using the DyeEx Spin Kit (Qiagen) and the samples analyzed on the 310 Genetic Analyzer (Applied Biosystems).

Table 3.8: Sequence of plasmid M13 universal primers

Primer	Nucleotide sequences (5'-3')
M13F	TCCCAGTCACGACGTCGT
M13R	GGAAACAGCTATGACCATG

Oligonucleotide primers and primer sequences were supplied by Promega

3.10 DNA-DNA hybridization

Southern Blotting is used to determine the molecular weight of a restriction fragment or to detect and locate a specific DNA sequence. Genomic DNA is digested with a restriction endonuclease and the resulting fragments are separated according to size by agarose gel electrophoresis. The DNA is denatured *in situ* and transferred from the agarose gel to a membrane. The relative positions of the DNA fragments are preserved. The filter membrane is hybridized with a labelled DNA probe. The probe can be detected by several methods including chemiluminescence (Ausubel *et al*., 1998). In both the *B. lentus* RSA1208 and *P. thiaminolyticus* RSA1221 study, Southern blotting was performed using *van* specific digoxigenin (DIG) - labelled probes to assess the presence or absence of *van* genes and to determine the location of the *van* resistance cassette (chromosomal vs. plasmid).

3.10.1 Restriction enzyme digest and Southern blots

Total DNA and plasmid DNA extracted, as in sections 3.6.1 and 3.6.2, were digested with *Eco*RI, *Xba*I, or *Sma*I restriction enzymes as recommended by the manufacturer. Undigested total and plasmid preparations, as well as the total and plasmid restricted reactions were resolved by electrophoresis on a 0.8% agarose- TAE gel (wt/vol). The gel was shaken gently at room temperature in depurination solution for 10min or until the bromophenol blue turned yellow. The gel was washed in distilled water and place in denaturation solution for 30 min. The DNA fragments (restricted and unrestricted) were transferred overnight to a Hybond-N^+ positively charged nylon membrane (Amersham

Biosciences, Uppsala, Sweden) by an alkaline downward capillary transfer action in denaturation solution (Ausubel *et al.*, 1998).

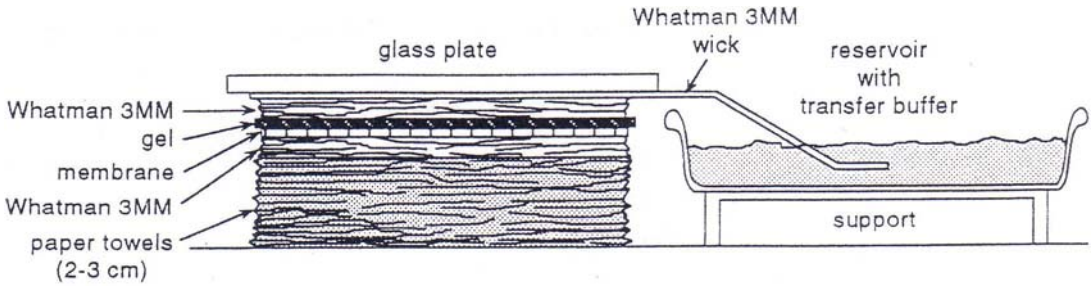


Figure 3.1: Schematic diagram of the downward capillary southern hybridization (Ausubel *et al.*, 1998).

After transfer, the membrane was washed in 2X saline sodium citrate (SSC) and air-dried on filter paper. No ultraviolet (UV) cross-linking was required.

3.10.2 DIG-labelled probe synthesis and hybridization

Using the PCR DIG probe synthesis kit (Roche Applied Sciences), hybridization probes were directly amplified and DIG-labelled using genomic DNA with the desired primer sets. A standard PCR was performed as previously mentioned in section 3.7 with the replacement of the deoxynucleoside triphosphate (dNTP) with a PCR DIG mix containing DIG-dUTP, which is incorporated during amplification. The probe mix was purified by agarose gel electrophoresis and the PCR fragment was excised and eluted. The eluted probe was boiled for 10min and 5µL of the probe reaction was added to 5mL DIG Easy Hyb buffer (Roche Applied Sciences) and stored at -20°C.

The Roche Applied Sciences protocol for prehybridization, hybridization and colorimetric detection of hybridized probe was followed with a few modifications, as described below.

Hybridizations were performed as follows: prehybridization for 1 hr at 45°C in 5mL DIG Easy Hyb buffer in Thermo Hybaid HB-CV-BS hybridization tubes, in a Mini Oven MKII (MWG-Biotech). The DIG-labelled probe was heated to 68°C for 10min prior to use. The prehybridization solution was discarded and replaced with the pre-heated probe. The membrane was left to incubate in the probe solution, overnight at 45°C.

3.10.3 Chemiluminescent detection of hybridized DIG-labelled probe

After hybridization, the membrane was washed twice; 5min per wash, in a 2X SSC, 0.1% SDS solution at room temperature, followed by two 15min washes, in a 0.5X SSC, 0.1% SDS solution at 68^{o}C. The membrane was equilibrated in 50mL 1X Wash Buffer (Roche Applied Sciences) for 1min, shaking at room temperature.

The membrane was blocked using 50mL 1X Blocking Buffer containing 1X maleic acid buffer (Roche Applied Sciences) for 45min, shaking at room temperature. Anti-DIG-alkaline phosphatase (Anti-DIG-AP) (Roche Applied Sciences) was diluted (1:10 000) in 50mL 1X Blocking buffer and added to the blot, and incubated for 45min, shaking at room temperature. The membrane was washed twice, 15min per wash, in 1X Washing buffer, shaking at room temperature. The membrane was then equilibrated in 1X Detection buffer for 2min. The disodium 3-(4-meth-oxyspiro {1, 2-dioxetane-3, 2'-(5'-chloro) tricyclo

[3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate (CSPD) (Roche Applied Sciences) substrate was diluted (1:100) in 1mL 1X Detection buffer.

The membrane was placed between two sheets of plastic, and the CSPD solution pipetted onto the DNA-bound side of the membrane. The plastic layer was slowly lowered so as to not form bubbles. The plastic bag was double sealed using a Levy and Smith Starpack sealing machine and checked for leaks and air bubbles. The bag was incubated at 37^{o}C for 15min and then positioned in a film cassette (Hypercassette, Amersham Biosciences) and taped down. In a dark room, X-Ray film (AGFA, Mortsel, Belgium) was placed in cassette. Colour development was allowed to proceed in the dark room at room temperature. Film processing was done using an automated Kodak RPX-OMAT processor at the Emergency radiography department at the Johannesburg Hospital.

3.11 Contour clamp homogenous electric field gel electrophoresis and Southern hybridization to determine the location of the resistance determinant

Contour clamp homogenous electric field (CHEF) gel electrophoresis is a molecular technique used to separate large, restricted DNA fragments (>30kb). The bacterial DNA is embedded in agarose, and cell lysis and DNA restriction occurs *in situ*. This prevents DNA shearing. The restricted fragments are separated on an agarose gel matrix by alternating the electric fields for optimal separation of large DNA fragments. CHEF gel electrophoresis is traditionally used for molecular typing in medical epidemiology. In this study, the technique was used to determine the location of the resistance determinant (chromosomal vs. plasmid). Plasmid DNA (low molecular weight) is separated from chromosomal DNA (high molecular weight) and using DNA: DNA hybridization techniques (section 3.10) and successive hybridizations with a DIG-labelled *vanA* and 16S rRNA (*rrs*) probe, the location of the resistance determinant can be determined. This technique was previously described by Depardieu *et al*. (2003b). The rationale for use of this method is that the *rrs* gene is found only on the chromosome and no *rrs* genes are expected to be found on the plasmid. Co-hybridization of the *rrs* and *vanA* probes on the same fragment would indicate that the resistance determinant is chromosomal.

3.11.1 Agarose plugs

Bacteria were grown in 10mL BHI broth (37^{o}C overnight, shaking at 120rpm). The OD_{600} readings were used to normalize the volumes of culture used per preparation, to ensure approximately equal amounts of cells per plug. The required volume of cells was aliquoted

into 2mL microfuge tubes and centrifuged (153000g for 2min). The bacterial pellet was resuspended in 500µL PIV buffer and incubated at 37°C until addition of agarose (±10min). A 1% agarose solution (wt/vol) was made with PIV buffer and cooled to 50°C and 500µL added to the bacterial suspension and pipetted into block moulds. Moulds were allowed to set at room temperature for 15min. Blocks were pressed out into 10mL VRE lysis buffer and incubated for 5 hours at 37°C with gentle shaking. The buffer was replaced with pulse field gel electrophoresis (PFGE) lysis buffer incubated overnight at 50°C. The blocks were transferred to 2ml PFGE storage buffer and stored at 2-8°C.

3.11.2 Digestion of DNA with restriction endonucleases

One third of an agarose block was cut using a clean scalpel and washed twice with 2mL PFGE storage buffer containing 1mM phenylmethylsulfonyl fluoride (PMSF) for 40min at room temperature, followed by three washes in 2mL 10mM Tris buffer, pH 8.0, for 20min at room temperature. The excess liquid was removed and 70µL restriction enzyme solution containing 10µL 10X reaction buffer, 10µL bovine serum albumin (BSA), and 26U of *Sma*I restriction endonuclease (Amersham Biosciences), was added to the block. The restriction mix was incubated overnight at 27°C. The gel electrophoresis was performed immediately after digestion.

3.11.3 Pulsed field gel electrophoresis

A CHEF DRII system (Bio-Rad Laboratories) was used for the electrophoretic separation of the DNA fragments. A 0.8% pulsed field certified agarose gel (Bio-Rad Laboratories) (wt/vol) using 0.5X Tris-borate- EDTA (TBE) buffer was prepared and left to set for 1hour. Two and a half litres of 0.5X TBE buffer was poured into the electrophoresis chamber and allowed to cool to 14^{o}C. The restricted agarose blocks were inserted into the gel wells and fixed in place with 1% molten agarose. The gel was secured in the chamber and the pre-determined current-switching parameters (6 Vcm^{-1}, 27hrs total migration time, 5sec initial switch time, 35sec final switch time and temperature, 14^{o}C) were used (Depardieu *et al*., 2003b). At the end of the run, the gel was removed and stained in d. H_2O containing 0.5µg/mL ethidium bromide for 30min and de-stained in d. H_2O for 20min. The gel was visualized and the image captured using the Bio-Rad Gel Doc 1000 system.

3.11.4 Southern hybridization using DIG-labelled *vanA* and 16S rRNA probe

This technique is described in detail in section 3.10. Briefly, the DNA fragments were transferred to a Hybond-N^{+} positively charged nylon membrane (Amersham Biosciences) by an alkaline downward capillary transfer action in denaturation solution. The DNA on the membrane was hybridized with the 16S rRNA and detected as previously described. The membrane was then stripped (see section 3.11.5) and reprobed with a *vanA* DIG-labelled probes and the hybridization detected as previously described.

The 16S rRNA probe was obtained by amplification of an internal portion of the 16S rRNA gene using primers RWO1 and DG74 (Greisen *et al*., 1994).

Table 3.9: Sequence of 16S rRNA primers

Primer	Nucleotide sequences (5'-3')	Reference
DG74	AGGAGGTGATCCAACCGCA	Greisen *et al*., 1994
RW01	AACTGGAGGAAGGTGGGGAT	Greisen *et al*., 1994

3.11.5 Stripping membrane of DIG-labelled probe after chemiluminescent detection

The membrane blot should not be allowed to dry at any stage of the prehybridization, hybridization or detection procedures.

The membrane was rinsed in d. H_2O for 1min, followed by two 15min washes, in a 0.2M NaOH, 0.1% SDS solution at 37^{o}C. The membrane was rinsed in 2X SSC for 5min and stored in 2X SSC until use.

3.12 Sub-genomic library to determine the 5' and 3'end upstream of the vancomycin resistance cassette

To determine the 5' and 3' ends of the *P. thiaminolyticus* RSA1221 *van* gene cassette sub-genomic libraries were constructed.

3.12.1 Pre-enrichment of the sequence of interest

A single primer PCR was performed using genomic DNA of *P. thiaminolyticus* RSA1221. Fragments between 1 kb and 1, 5 kb were cut out of the agarose gel and eluted using the QIAquick Gel Extraction Kit (Qiagen). Three hundred to five hundred nanograms of eluted DNA was cloned into 100ng of *Sma*I-restricted pGEM vector (Promega) using T4 DNA ligase (400U/mL) for 12-16 hours in the iCycler alternating between 10^{o}C for 30sec and 30^{o}C for 30sec. The ligation mix was transformed into DH5α™ Library efficiency cells, using the manufacturer's protocol (Invitrogen Life Technologies). Transformed bacteria were plated onto Hybond N+ membrane discs placed on 50µg/mL ampicillin-containing LB agar plates and incubated at 37^{o}C overnight. This plate was termed the master plate.

3.12.2 Colony hybridization

After overnight incubation, replicas were made of the master plate. Membrane discs were lowered gently onto the LB master plates so as to not form bubbles. The membranes were marked to ensure correct orientation of the colonies in subsequent experiments. After 60s

the replica membrane was removed using blunt-ended forceps and placed on 50 μg/mL ampicillin-containing LB agar plates and incubated at 37°C for up to 3-4 hours to ensure recovery of the colonies. The master membrane discs were kept on LB plates containing 50μg/mL chloramphenicol to prevent the further growth of colonies for long-term storage. The DNA on the replica plate was released from the bacteria by placing the membrane on filter paper saturated with denaturation solution for 10min, followed by two 10min incubation periods on a filter paper soaked in neutralization solution (1M Tris-HCl, pH 7.5, 1.5M NaCl). The membrane was washed vigorously in 2X SSC to remove protein debris and air-dried. The DNA was fixed onto the membrane by microwaving on high for 1min.

3.12.3 Hybridization and chemiluminescent detection of hybridized DIG-labelled probe

The DNA on the membrane was hybridized with the *vanS* or *vanY* DIG-labelled probes and hybridization detected as previously described (section 3.10.2 and 3.10.3).

Positive signals were traced back to the original colonies by orientating the membrane disc relative to the original agar plate. The colony was selected and grown in LB broth overnight at 37°C, shaking at 120rpm. Plasmid DNA was isolated and sequenced as previously described (section 3.8).

3.13 Transmission electron microscopy

Transmission electron microscopy (TEM) was used to assess the possible structural changes of the vancomycin resistant *B. lentus* and compare it to a control vancomycin susceptible *B. lentus* B1090. The technique used was previously described by Hanaki *et al*. (1998). Embedding, cutting and staining of the sample and TEM were performed by Mrs L van der Walt at the Electron Microscope Unit, Anatomical Pathology, National Health Laboratory Service, Johannesburg, South Africa.

Cells were cultured in BHI at 37°C overnight, shaking at 120rpm. Cells in the logarithmic phase were harvested and washed three times in 1ml PBS. Washed bacterial pellets were fixed in a solution of glutaraldehyde and formaldehyde for 1hr at 4^{o}C. The fixed pellet was rinsed in Millonig's buffer for 10min. The fixed pellet was then treated with 2% osmium tetroxide in Millonig's for 1hr at 4^{o}C. After the osmium tetroxide treatment, the pellet was rinsed in Millonig's buffer for 10min.

Cells were dehydrated with graded concentrations of ethanol: 50% ethanol for 10min, 75% ethanol for 10min, 95% ethanol for 10min, 3X 100% ethanol for 10min each and finally 2X propylene for 7min each. The bacterial pellet was embedded in Spurr resin as follows: 50% resin/propylene mixture for 15min, 75% resin/propylene for 15min, 100% resin for 30min and finally embedded in fresh Spurr resin and allowed to polymerize at 60^{o}C for 10hours.

Ultra thin sections (0.5-1micron) were cut using a Reichert ultramicrotome with glass knives. The sections were stained first with uranyl acetate and then with lead citrate.

Sections were examined using a transmission electron microscope (Hitachi H-600, Tokyo, Japan).

Evaluation of the cell wall thickness was performed using photographic images at a final magnification of 50 000X. Ten cells of each strain were selected. Each bacterium was measured at intervals of 5cm along the cell wall perimeter i.e. 8 different measurements were taken per bacterium. The average measurements of the cell wall were taken in mm across the bacterium. The results are expressed as mean ∀ standard deviation (SD).

To determine the actual measurement of the cell wall (μm), the following formula was used:

$$\frac{1000 \text{ X distance on the micrograph}}{\text{Magnification X } 2.1}$$

3.14 List of Genbank accession numbers used in this study

AF155139	*P. popilliae vanF* resistance gene cluster
AF162694	*E. gallinarum vanC1* resistance gene cluster
AF192329	*E. faecalis* transposon Tn*1549 vanB* resistance gene cluster
AF430807	*E. faecalis* strain N00-410 *vanE* resistance gene cluster
AY271782	*E. faecalis* strain BM4518 *vanG* resistance gene cluster
AY489045	*E. faecium* strain N03-0072 *vanD* resistance gene cluster
AY648035	*P. thiaminolyticus* PT-2B1 D: ala: D: ala ligase gene
AY648698	*P. apiarius* PA-B2B D: ala: D: ala ligase gene
NC 005054	*S. aureus* plasmid pLW043 *vanA* resistance gene cluster
M97297	*E. faecium* transposon Tn*1546 vanA* resistance gene cluster
X79049	*O. turbata* plasmid DNA for *vanA* resistance protein
Y15704	*B. circulans* VR0709 *vanA* gene
Y15705	*B. circulans* VR0709 *vanH* gene
Y15706	*B. circulans* VR0709 *vanR* gene
Y15707	*B. circulans* VR0709 *vanS* gene
Y15708	*B. circulans* VR0709 *vanX* gene
Y17303	*B. circulans* VR0709 *vanY* gene
Y17304	*B. circulans* VR0709 *vanZ* gene

3.15 Reagent buffers, solutions and restriction endonucleases used in this study

Table 3.10 Stock solutions used in this study

Stock solution	**Constituents**	**Directions**
Tris-Cl, 1M (pH 8.0)	121g Tris d.H_2O	Approximately 70mL concentrated HCl is needed to achieve a pH 7.4 solution and approximately 42mL for a solution with a pH of 8.0
EDTA, 0.5M (pH 8.0)	186.1g $Na_2EDTA.2H_2O$ d.H_2O	Dissolve the $Na_2EDTA.2H_2O$ powder in d.H_2O. pH to 8.0 with 10M NaOH (~ 50mL). Then add d. H_2O to 1L
HCl, 1M	86.2mL concentrated HCl d.H_2O	To 913.8mL d.H_2O add 86.2mL concentrated HCl
NaCl, 5M	292g NaCl d.H_2O	Dissolve NaCl powder in d.H_2O and make up to make 1L

NaOH, 10M	400g NaOH d.H_2O	Dissolve NaOH pellets in 450mL d.H_2O. Make up to 1L with d.H_2O
NaAc, 3M (pH 5.2)	408g NaAc. $3H_2O$ d.H_2O	Dissolve above sodium acetate in d.H_2O and make up 1L with d.H_2O. Adjust pH with 3M glacial acetic acid.
TE buffer	10mM Tris-Cl 1mM EDTA, pH 8.0 d.H_2O	----------
SDS, 20%	20g SDS d.H_2O	Dissolve SDS powder in d.H_2O and make up to 100mL.
TAE electrophoresis buffer, 50X	242g Tris base 57.1mL glacial acetic acid 37.2g $Na_2EDTA.2H_2O$ d.H_2O	Dissolve Tris and $Na_2EDTA.2H_2O$ in d.H_2O. Add the acetic acid and make up to 1L.

Table 3.11 Buffers used in the alkaline lysis plasmid preparation method

Buffer	**Constituents**
MP1- Resuspension buffer	50mM Tris-HCl, pH 8.0 10mM EDTA 100µg/ml RNase A
MP2- Lysis Buffer	200mM Sodium hydroxide 1% SDS
MP3- Neutralization buffer	3M Potassium acetate, pH 5.5

Table 3.12 Buffers used for total genomic DNA preparation

Buffer	**Constituents**
6.7% sucrose solution	0.67g sucrose Dissolve in 6mL d. H_2O. Adjust with d. H_2O to make up to 10mL

Table 3.13 Buffers used in DNA-DNA hybridization experiments

Buffer	**Constituents**
Depurination solution (0.25M HCl)	22mL concentrated HCl 978mL d. H_2O Mix and store at room temperature
Denaturation solution (0.5M NaOH, 1.5M NaCl)	87.66g NaCl 20g NaOH Add approximately 800mL d.H_2O. Mix to dissolve and make up to final volume of 1L with d.H_2O. Store at room temperature
SSC, 20X	3M NaCl (175g/L) 0.3M Na_2citrate.$2H_2O$ (88g/L) Dissolve NaCl and Na_2citrate.$2H_2O$ in d.H_2O. Make up to 1L with d.H_2O. Adjust to pH 7.0 with 1M HCl.
DIG Blocking Buffer, 10X	10g Blocking reagent 100mL Maleic acid buffer Heat at 68°C for approximately 5-6 hrs and occasional shaking.
DIG Detection buffer, 10X	100mM Tris-HCl 100mM NaCl pH9.5

Table 3.14: Buffers used in CHEF gel electrophoresis experiments

Buffer	**Constituents**
PIV buffer	1M NaCl 10mM Tris-HCl, pH 7.5
VRE lysis buffer	6mM Tris-HCl, pH 7.5 1M NaCl 100mM EDTA 0.5% Brij 58 0.2% deoxycholate 0.5% sodium lauryl sarcosine 20µg/mL RNaseA 1µg/mL lysozyme
PFGE lysis buffer	0.1M EDTA 10mM Tris-HCl, pH 8.0 0.1mg/mL proteinase K
PFGE storage buffer	10mM EDTA 10mM Tris-HCl, pH8.0

Table 3.15 List of enzymes used in this study

Enzyme	**Manufacturer**
*Eco*R1 restriction endonuclease	Promega
Fast *Taq* polymerase	Roche Applied Science
Lysozyme	Roche Applied Science
Proteinase K	Roche Applied Science
RNaseA	Roche Applied Science
*Sma*I restriction endonuclease	Amersham Biosciences
T4 DNA Ligase	Biolabs
Terminal deoxynucleotidyl transferase (*tdt* enzyme)	Promega
*Xba*I restriction endonuclease	Promega

CHAPTER 4-STUDY A

BACILLUS LENTUS RSA1208

B. lentus is an aerobic bacterium belonging to the family *Bacillaceae* that produces endospores. It is found in soil, food and spices and is considered to be non-pathogenic to humans. *B. lentus* is closely related to *B. firmus*, but is nutritionally more versatile (Parry *et al*., 1983). The basis for glycopeptide resistance in clinical isolate *B. lentus* RSA1208 was investigated.

4.1 Results

B. lentus RSA1208 is a motile, aerobic, rod-shaped, Gram-positive organism. The colony morphology on 5% blood agar is smooth, circular edged, entire, with convex colonies of about 1mm in diameter (Fig. 4.1).

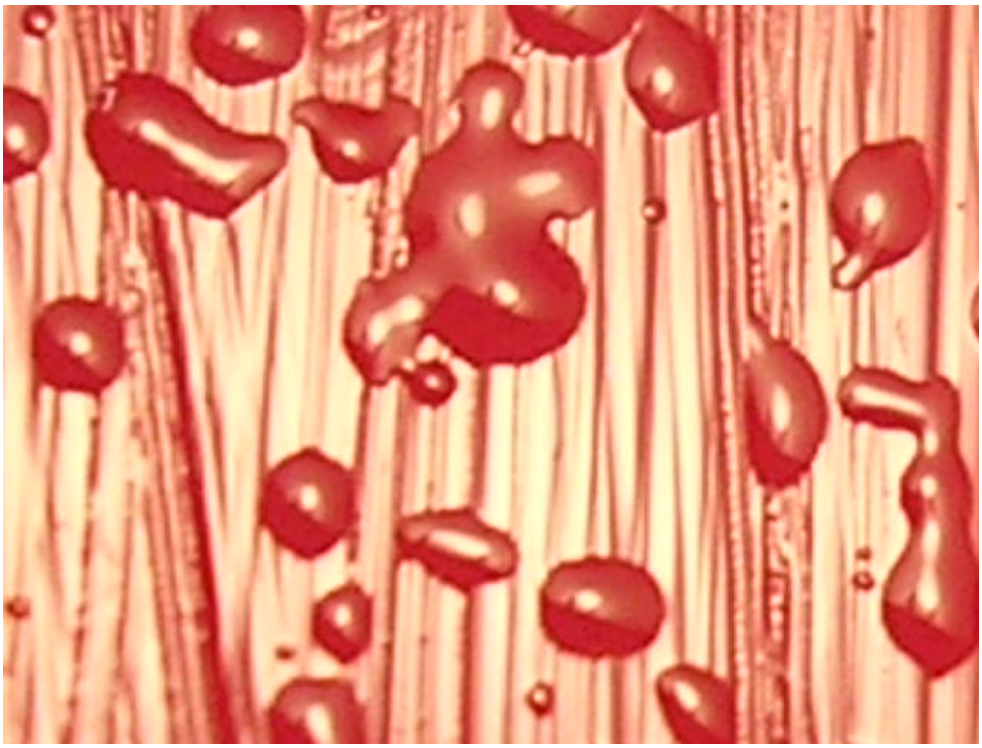


Figure 4.1: Streak plate of *B. lentus* RSA1208. *B. lentus* RSA1208 produced circular, entire, convex colonies of 1-2mm in diameter on a 5% blood agar plate. These colonies are shiny and creamy-white (Overnight incubation at 37°C).

4.1.1 Phenotypic characterization of *B. lentus* RSA1208

The biochemical characteristics of *B. lentus* RSA1208 (Table 4.1) are consistent with the description given by Koneman *et al*. (1997) and Bergey's Manual of Systematic Bacteriology (1984).

Table 4.1: Phenotypic characterization of *B. lentus* RSA1208 using conventional microbiological methods

Characteristic	***B. lentus* RSA1208**
Morphology of colonies on growth media	Circular, entire, shiny, convex colonies, 1-2mm in diameter
Atmospheric conditions	Aerobic
Motility	Positive
Gram stain	Gram-positive bacilli
Action on blood agar	No β-haemolysis
Catalase activity	Positive
Oxidase activity	Positive
Indole production	Negative
Voges-Proskauer	Negative
Nitrate reduction	Positive
Carbohydrate utilization - Sorbitol - Arabinose - Pyruvate - Sucrose - Mannitol - Glucose - Lactose - Xylose - Salicin	 Negative Weak positive Weak positive Negative Positive Positive (no gas formation) Positive Negative Positive
Gelatin hydrolysis	Negative
Starch hydrolysis	Negative
Lecithinase production	Negative
Citrate utilization	Negative
Urease production	Positive
Aesculin hydrolysis	Positive

4.1.2 Antibiotic susceptibility testing

Initial antimicrobial susceptibility testing using the broth microdilution method (van Nierop *et al*., 2000), showed that *B. lentus* RSA1208 is highly resistant to both vancomycin and teicoplanin (Table 4.2). This was confirmed in this study using the E-test method (Table 4.2 and Fig. 4.2). Other classes of antibiotics were tested against *B. lentus* RSA1208 using the Kirby-Bauer disk diffusion method (Table 4.3). The NCCLS guidelines for zone diameter interpretive standards and MIC breakpoints for *Staphylococcus* spp. were used to determine the antibiotic susceptibility of *B. lentus* RSA1208 (NCCLS, 2003b). These guidelines were used as there are currently no NCCLS guidelines for zone diameter interpretive standards and MIC breakpoints for *Bacillus* spp.

Table 4.2: *B. lentus* RSA1208 MIC determination using two different methods: broth microdilution and the E-test method

Antibiotic	**Broth microdilution** (van Nierop *et al*., 2000)		**E-test**	
	MIC	**Susceptibility**	**MIC**	**Susceptibility**
Vancomycin	32µg/mL	Resistant	>256µg/mL	Resistant
Teicoplanin	32µg/mL	Resistant	>256µg/mL	Resistant

Although both broth microdilution and E-test method clearly show that *B. lentus* RSA1208 is resistant to vancomycin and teicoplanin, the discrepancies in MIC values using these two techniques is surprising. Repeat E-test MIC susceptibility test however confirmed that the MIC value was >256µg/mL.

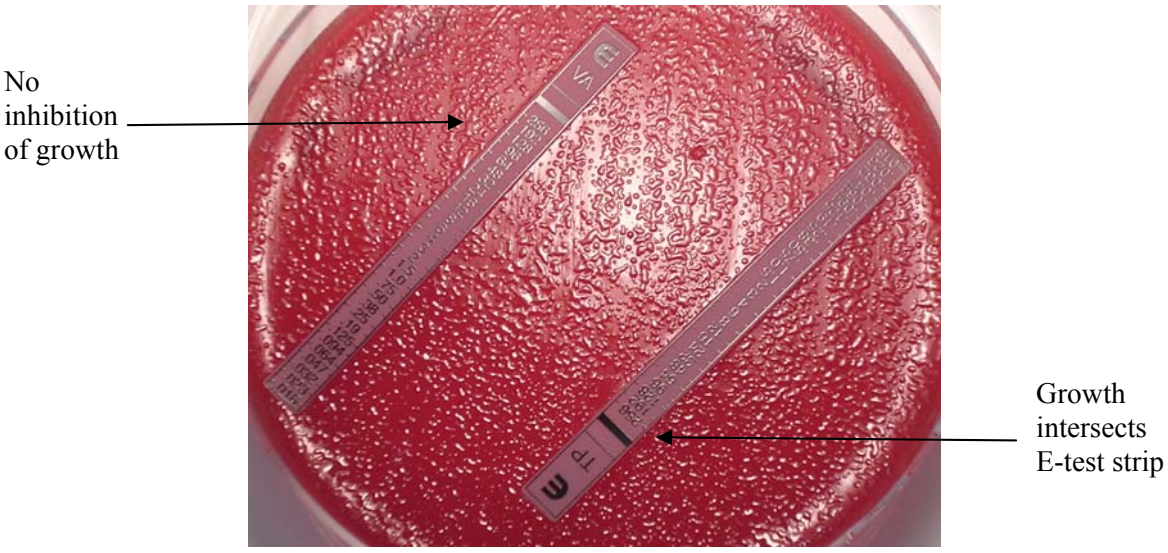


Figure 4.2: *B. lentus* RSA1208 vancomycin (VA) and teicoplanin (TP) E-test. No inhibition zones were observed around the antibiotic E-test strip i.e. growth intersects along the entire strip.

Table 4.3: *B. lentus* disk diffusion antibiotic susceptibility testing

Antibiotic class	**Zone diameter (cm) for *B. lentus* RSA1208**	**Susceptibility using zone diameter interpretative standards for *Staphylococcus aureus* (NCCLS, 2003b)**
Aminoglycoside (Gentamicin)	1.0	Sensitive
Ansamycin (Rifampicin)	1.8	Intermediate resistance
β-lactam (Penicillin)	1.9	Resistant
Carbapenem (Imipenen)	3.9	Sensitive
Cephem (Ceftazidime)	1.1	Sensitive
Fluoroquinolone (Ofloxacin)	3.1	Sensitive
Lincosamide (Clindamycin)	1.1	Resistant
Macrolide (Erythromycin)	1.0	Resistant

4.1.3 Glycopeptide resistance expression

To determine the expression of glycopeptide resistance in isolate RSA1208, inducibility experiments were performed (Fig. 4.3). The growth rates of *B. lentus* RSA1208 cultured in BHI containing no vancomycin, and *B. lentus* RSA1208 cultured in BHI containing vancomycin (32µg/mL) were the same. However when the organism was grown in BHI containing no antibiotic and then sub-cultured into BHI containing sub-inhibitory concentrations of vancomycin (32µg/mL), the growth rate was significantly altered, suggesting that expression of resistance was inducible. These experiments were performed prior to this study and the results shown below are courtesy of W. van Nierop and have been incorporated here because they are pertinent to all future discussions in this section (van Nierop *et al*., 2000).

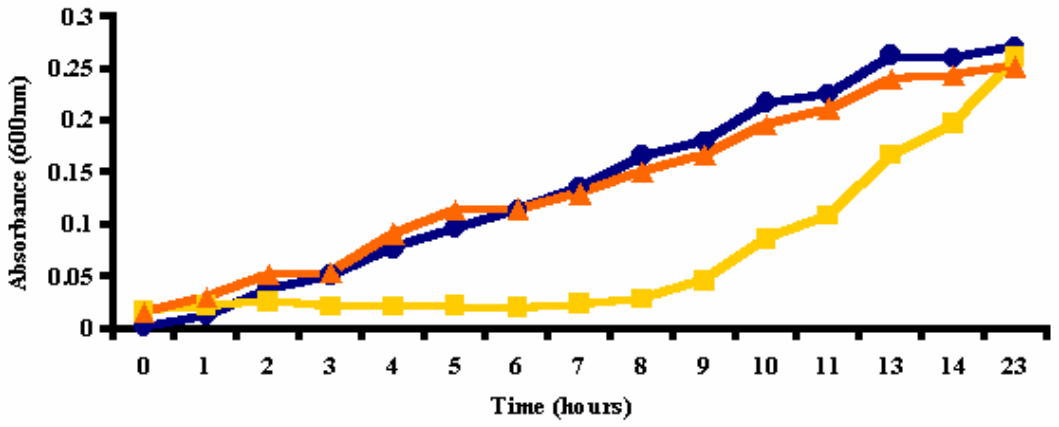


Fig. 4.3: Glycopeptide resistance expression of *B. lentus* RSA1208. *B. lentus*RSA1208 cultured in BHI containing no vancomycin (●). *B. lentus* RSA1208 pre-cultured in BHI containing no vancomycin and then sub-cultured into fresh BHI containing 32µg/mL vancomycin (■). *B. lentus* RSA1208 pre-cultured in BHI containing vancomycin 32µg/mL and then sub-cultured into fresh BHI containing 32µg/mL vancomycin (▲). Absorbance readings were taken at 600nm (van Nierop *et al*., 2000).

4.1.4 PCR detection of the *van* ligase gene

The presence or absence of the *van* ligase genes in *B. lentus* RSA1208 was investigated using enterococcal primers (listed in Table 3.4) to detect *vanA, vanB, vanC1, vanC2/C3, vanD, and vanE*. PCR products of the expected size (731bp, 635bp, 842bp, 431bp, 461bp and 513bp for *vanA*, *vanB*, *vanC1*, *vanC2/C3*, *vanD* and *vanE*, respectively) were obtained in the positive control reactions. No product was detected in any of the *B. lentus* RSA1208 PCR reactions (data not shown).

To confirm the absence of the *vanA* gene (which is most frequently associated with high-level resistance), low stringency Southern hybridization experiments were conducted. No hybridization signal was detected with the DIG-labelled enterococcal *vanA* probe in the *B. lentus* RSA1208 chromosomal and plasmid DNA extractions. Positive signals were detected in vanA-type *E. faecium* BM4147 and *B. circulans* VR0709 DNA preparations, as expected.

4.1.5 PCR detection of the D-specific α-ketoacid dehydrogenase gene

vanH is a α-ketoacid dehydrogenase and encodes an essential protein required for the expression of glycopeptide resistance. The VanH protein reduces pyruvate to D-lac, which is incorporated into the glycopeptide resistant PG precursors. An endogenous α-ketoacid dehydrogenase may provide the D-lac necessary for incorporation into vancomycin resistant PG precursors.

PCR detection of the D-specific α-ketoacid dehydrogenase gene using the CODEHOP degenerate primers (Table 3.6) and *B. lentus* RSA1208 DNA yielded no results. Positive signals were seen in the *E. faecium* BM4147 control (data not shown).

4.1.6 Cell wall morphology analysis

B. lentus RSA1208 and the control *B. lentus* B1090 strain were grown under the same conditions in BHI containing no vancomycin. Bacterial cell wall morphology was observed by transmission electron microscopy. Examination of the ultra thin sections of *B. lentus* RSA1208 showed no increase in cell wall thickness when compared to the control *B. lentus* B1090 (data not shown).

Glycopeptide resistance expression in *B. lentus* RSA1208 is inducible, therefore to observe the phenotypical effects of vancomycin exposure, *B. lentus* RSA1208 was grown in BHI containing 32μg/mL of vancomycin and as a control, *B. lentus* RSA1208 was grown in BHI containing no antibiotic. The cultures were subjected to transmission electron microscopy. In comparison to *B. lentus* RSA1208 grown in antibiotic-free media (Fig. 4.5), *B. lentus* RSA1208 grown in the presence of vancomycin, appears to have considerably thickened cell walls (Fig. 4.4), as evident in the electron micrographs. Table 4.4 shows the results of the cell wall thickness measurements.

Table 4.4: Cell wall diameter of *B. lentus* RSA1208 grown in BHI containing 32µg/mL of vancomycin compared to *B. lentus* RSA1208 grown in antibiotic-free BHI

	Average cell wall photo measurements(mm)[a]	Actual cell wall measurements (µm)[b]
B. lentus RSA1208 grown in antibiotic free media	1.13±0.23	0.011
B. lentus RSA1208 grown in 32µg/mL vancomycin	4.25±0.87	0.067

a: Mean± standard deviation

b: (distance on the print (mm) x1000) ÷ (magnification x 2.1) = distance in micrometers

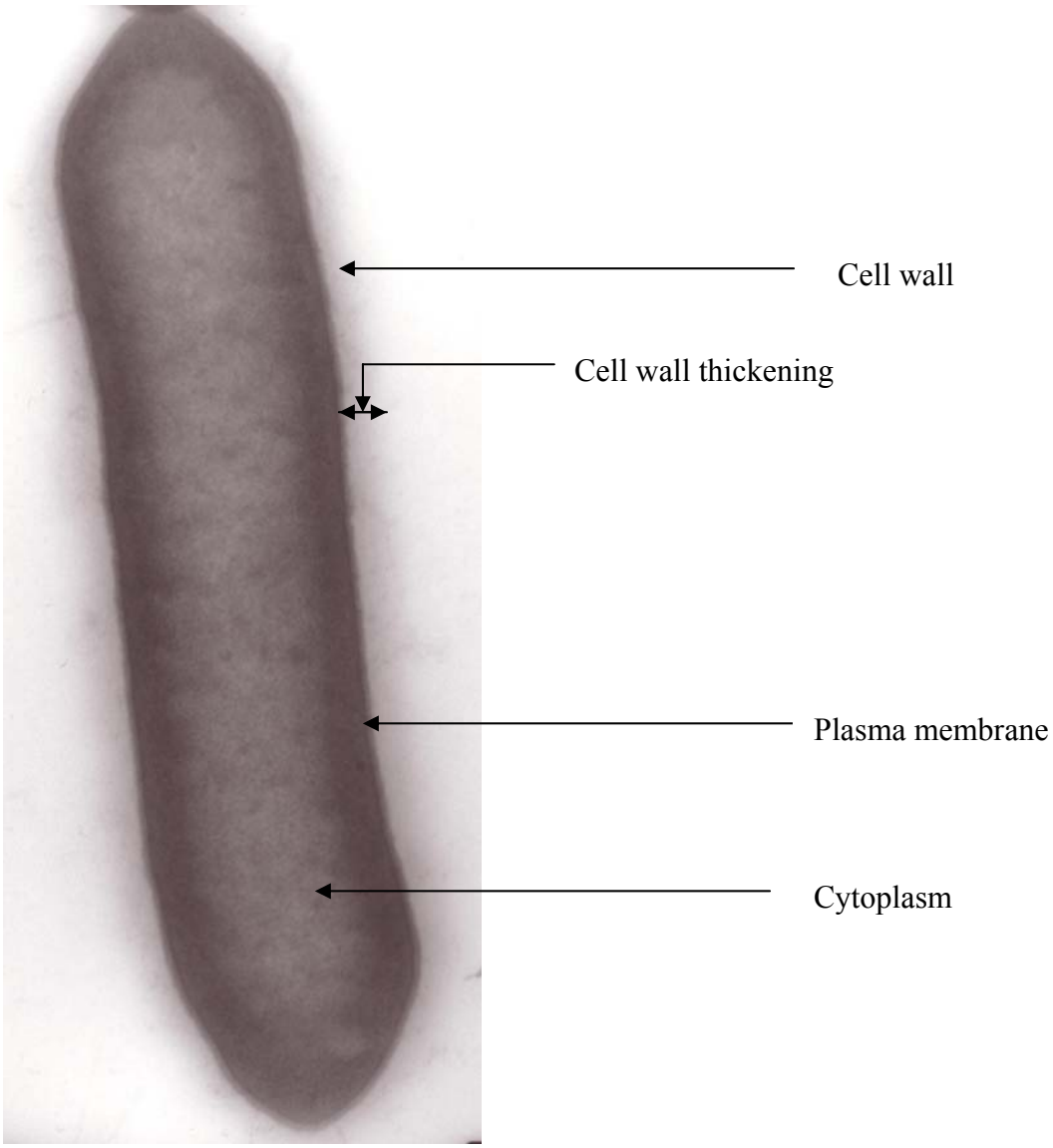


Figure 4.4: Transmission electron micrograph of *B. lentus* RSA1208 grown with antibiotic. Bacterial cells grown in BHI containing 32µg/mL vancomycin, were harvested at the mid-exponential phase of growth.
Magnification X30 000.

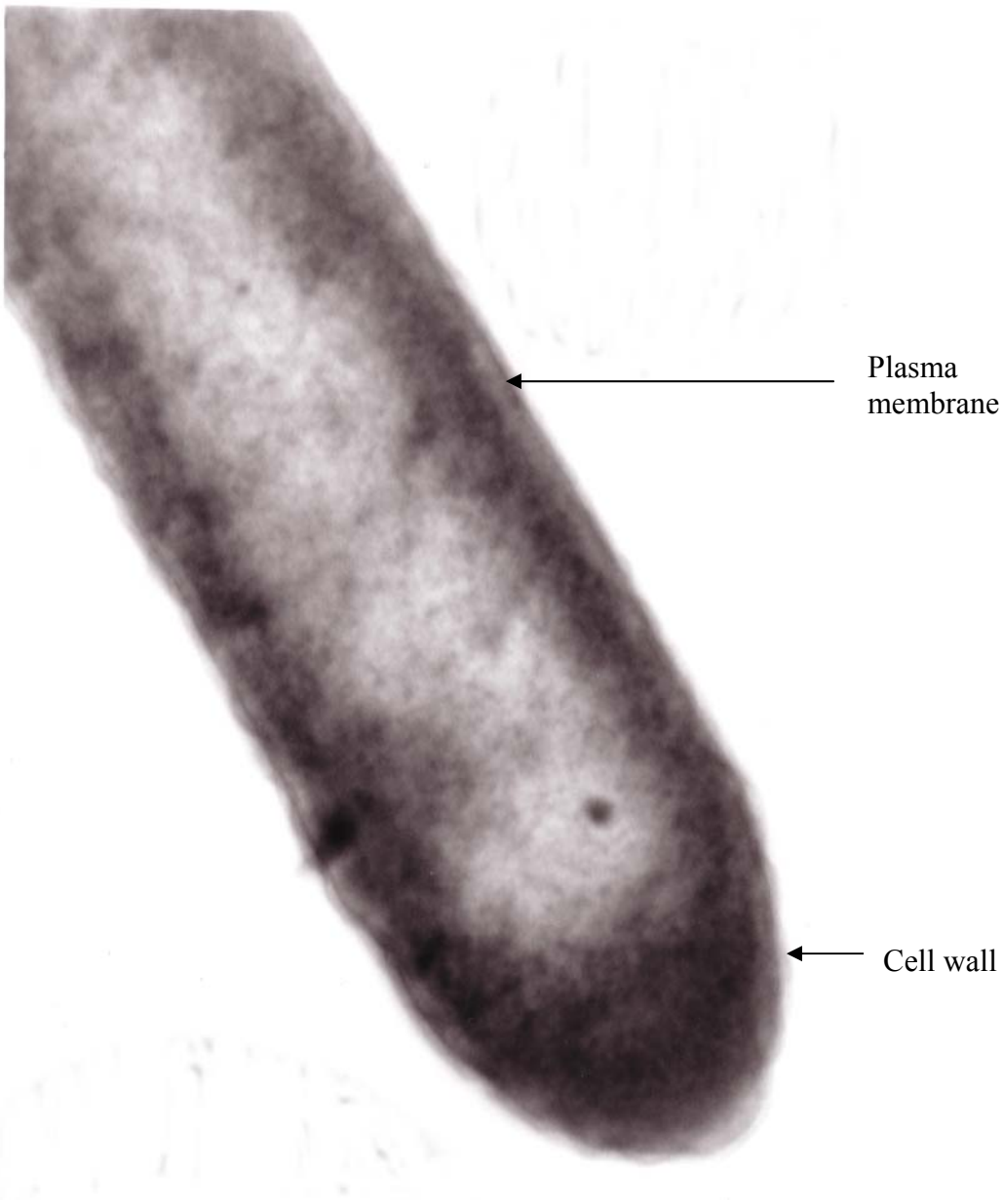


Figure 4.5: Transmission electron micrograph of *B. lentus* RSA1208 grown without antibiotic. Bacterial cells grown in BHI containing no antibiotic, harvested in mid-exponential growth phase. NOTE no evident cell wall thickening. Magnification X50000.

4.2 Discussion

B. lentus RSA1208 expressed high-level resistance to the glycopeptides as well as to the following antibiotic classes: β-lactams, lincosamides and macrolides. *B. lentus* RSA1208 can therefore be classified as a multi-drug resistant microorganism.

A bacterium can develop resistance by altering the antibiotic target as in the case of classic glycopeptide resistance, where the termini of PG chains are modified from the glycopeptide susceptible D-ala-D-ala to the glycopeptide resistant D-ala-D-lac or D-ala-D-ser. This remodelling of the termini is usually a result of the acquisition of a *van* resistance operon. This new structure does not bind vancomycin as effectively. A second resistance mechanism is decreasing the accessibility of the antibiotic target. This mechanism of resistance is seen in VISA isolate, Mu50. Accumulation of excess PG precursors results in a thickened cell wall, which traps the vancomycin molecules, in the outermost cell wall layers thereby preventing the antibiotic from exerting an effective antimicrobial action. Since these are the primary mechanisms of glycopeptide resistance, they were investigated in *B. lentus* RSA1208.

Glycopeptide inducibility experiments to determine the expression of resistance showed that resistance in isolate RSA1208 is inducible upon exposure to vancomycin. When the isolate was pre-cultured in BHI containing no vancomycin and then sub-cultured into fresh BHI containing 32μg/mL vancomycin, the slow growth, corresponding to minor changes in absorbance, between 0 hrs- 8 hrs was followed by a rapid increase in growth, indicated by an increase in absorbance, suggesting that the organism had to activate the transcription of a set of genes otherwise not expressed in the absence of glycopeptides. Pre-growth in

vancomycin reduced the lag phase considerably and resembled the negative control growth curve. This was shown in repeated experiments, confirming that resistance is inducible.

The high-level, inducible resistance to both vancomycin and teicoplanin expressed by *B. lentus* RSA1208 is commonly found in enterococci, and is associated with the presence of the *vanA* gene cluster. The absence of the *vanA* ligase gene in *B. lentus* RSA1208 was determined by PCR analysis. This result was further confirmed by Southern hybridization experiments.

None of the other known *van* ligase genes were detected in *B. lentus* RSA1208 by PCR. The absence of documented *van* ligase genes in *B. lentus* RSA1208 further strengthens the hypothesis that the high-level glycopeptide resistance phenotype observed in this organism is not a result of the presence of any of the classic enterococcal *van* resistance genes.

The absence of a D-specific α-ketoacid dehydrogenase suggests that the genome of this organism does not code for the protein that reduces pyruvate to D-lac. These results, together with the absence of the *van* ligase genes, would strongly suggest that resistance in *B. lentus* RSA1208 is not likely to be a result of modification of the termini of PG precursors from D-ala-D-ala to D-ala-D-lac or D-ala-D-ser. It is possible that glycopeptide resistance in *B. lentus* RSA1208 maybe a result of remodelling of the termini to an as yet unidentified amino acid that is unable to bind glycopeptides.

An alternate mechanism of vancomycin resistance not associated with the acquisition of a *van* operon, is the phenomenon first observed in VISA isolate, Mu50. Vancomycin resistance in Mu50 is associated with a markedly increased cell wall thickness. Preliminary

TEM results showed that the cell wall of *B. lentus* RSA1208 grown in BHI containing 32µg/mL of vancomycin was considerably thicker than when the bacterium was grown in antibiotic free media. These results suggest that *B. lentus* RSA1208 employs a similar glycopeptide resistance mechanism to that seen in VISA isolates.

It still remains to be established if this cell wall thickening resistance mechanism is solely responsible for the high-level resistance to both glycopeptides observed in *B. lentus* RSA1208. Therefore, additional HPLC analysis of the cell wall components and cell wall enzyme kinetics and metabolism studies need to be conducted to fully elucidate the molecular basis of glycopeptide resistance mechanism.

It is possible that *B. lentus* RSA1208 employs another resistance mechanism, such as the inactivation or destruction of the glycopeptide by an enzyme that hydrolyzes the antibiotic. Such an enzyme could be produced following glycopeptide induction. Since vancomycin is a large biomolecule with no known bacterial transport mechanism, it cannot penetrate the cell membrane; hence the enzyme would need to be secreted into the external environment. There is however no evidence for this hypothesis as yet.

CHAPTER 5-STUDY B

PAENIBACILLUS THIAMINOLYTICUS RSA1221

Until 1997, *P. thiaminolyticus* was known as *B. thiaminolyticus*, with the "*thiaminolyticus*" originating from the observation that it decomposes thiamine. Shida *et al*. (1997) determined the taxonomic status of six species of *Bacillus* (of which one was *B. thiaminolyticus)* possessing phenotypical characteristics similar to *Paenibacillus* spp. The results of the 16S rRNA sequence analysis combined with the fatty acid composition enabled them to validate the renaming of *Bacillus thiaminolyticus* to *Paenibacillus thiaminolyticus*. This microorganism appears to be closely related to *P. alvei* because of its ability to produce indole (Shida *et al*., 1997 and Nakamura, 1990). The molecular mechanism of glycopeptide resistance in *P. thiaminolyticus* RSA1221 was investigated.

5.1 Results

P. thiaminolyticus RSA1221 is a rod-shaped, Gram-positive bacterium. The colony morphology on 5% blood agar (Fig. 5.1) is smooth and circular, with entire colonies of about 1mm in diameter.

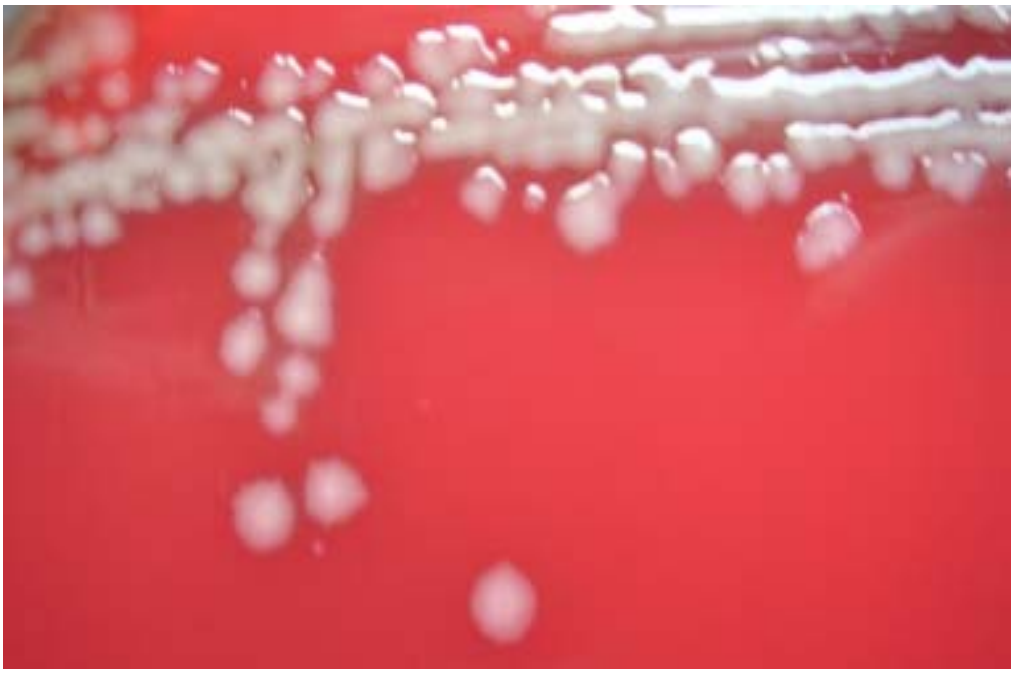


Figure 5.1: Streak plate of *P. thiaminolyticus* RSA1221. *P. thiaminolyticus* RSA1221 produced large, smooth edge, shiny, convex colonies, 1-2 mm in diameter on a 5% blood agar plate (overnight incubation at 37°C).

5.1.1 Phenotypic characterization of *P. thiaminolyticus* RSA1221

The biochemical characteristics of *P. thiaminolyticus* RSA1221 (Table 5.1) is consistent with the description given by Parry *et al*. (1984) and Bergey's Manual of Systematic Bacteriology (1984).

Table 5.1: Phenotypic characterization of *P. thiaminolyticus* RSA1221 using conventional microbiological methods

Characteristic	***P. thiaminolyticus* RSA1221**
Morphology of colonies on growth media	Large, smooth edge, shiny, convex colonies, 1mm in diameter
Atmospheric conditions	Aerobic
Motile	Positive
Gram stain	Gram-positive bacilli
Action on blood agar	B-haemolytic
Catalase activity	Positive
Oxidase activity	Positive
Indole production	Negative
Voges-Proskauer	Negative
Nitrate reduction	Positive
Carbohydrate utilization - Arabinose - Mannitol - Glucose - Salicin	 Negative Negative Positive (no gas formation) Positive
Gelatin hydrolysis	Positive
Starch hydrolysis	Positive
Lecithinase production	Negative
Citrate utilization	Negative
Urease production	Positive

5.1.2 Antibiotic susceptibility testing

Initial antimicrobial susceptibility testing using the broth microdilution method (von Gottberg *et al*., 1999), showed that *P thiaminolyticus* RSA1221 expresses high-level resistance to vancomycin and intermediate resistance to teicoplanin (Table 5.2). This was confirmed in this study using the E-test method (Table 5.2 and Fig. 5.2). As described in detail in section 4.1.2, the NCCLS guidelines for zone diameter interpretive standards and MIC breakpoints for *Staphylococcus* spp. were used to determine the antibiotic susceptibility of *P. thiaminolyticus* RSA1221 (NCCLS, 2003b).

Table 5.2: *P. thiaminolyticus* RSA1221 MIC determination using two different methods: broth microdilution and the E-test method

Antibiotic	**Broth microdilution** (von Gottberg *et al*., 1999)		**E-test**	
	MIC	**Susceptibility**	**MIC**	**Susceptibility**
Vancomycin	64μg/mL	Resistant	>256μg/mL	Resistant
Teicoplanin	16μg/mL	Intermediate resistance	8μg/mL	Intermediate resistance

Although both the broth microdilution and E-test method clearly show that *P. thiaminolyticus* RSA1221 is resistant to vancomycin and intermediately resistant to teicoplanin, the discrepancies in the vancomycin MIC values using the two techniques is surprising. Repeat vancomycin E-test MIC susceptibility test however confirmed that the MIC value was >256μg/mL.

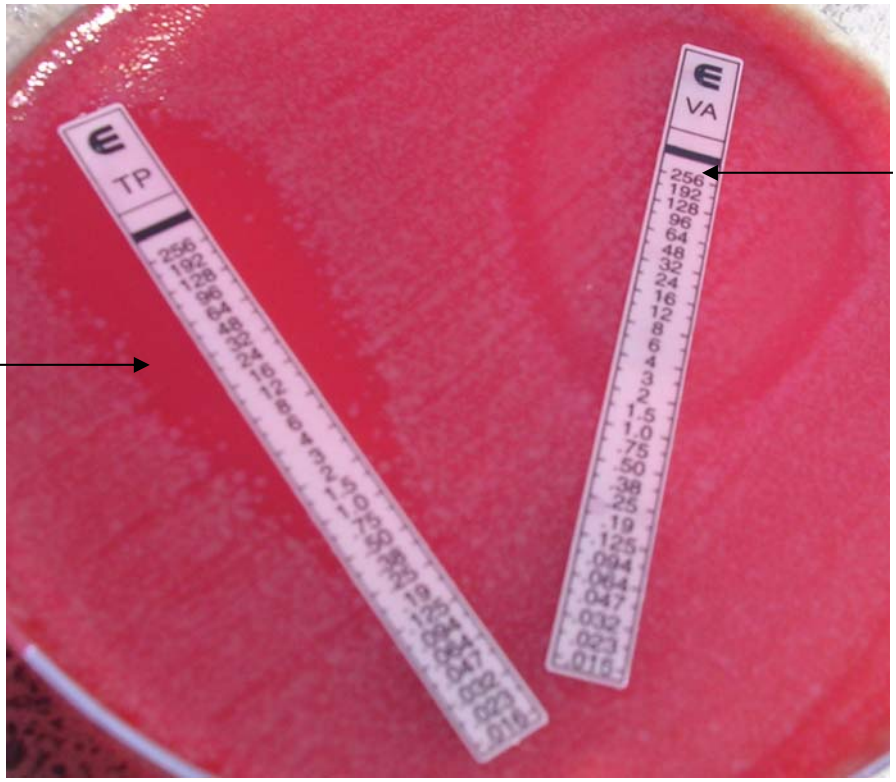


Figure 5.2: *P. thiaminolyticus* RSA1221 vancomycin (VA) and teicoplanin (TP) E-test on 5% blood agar plate. An inhibition ellipse can be seen around the teicoplanin E-test strip, MIC=8µg/mL. *P. thiaminolyticus* RSA1221 growth intersects along the vancomycin E-test strip, MIC>256µg/mL.

It is interesting to note a halo of no-growth surrounding the vancomycin E-test strip. This unexpected phenomenon was consistently observed. It would seem to be a paradoxical effect that at low vancomycin concentrations, inhibition of growth occurs with growth at higher concentrations.

5.1.3 Glycopeptide resistance expression

To determine the expression of glycopeptide resistance in *P. thiaminolyticus* RSA1221, inducibility experiments were performed (Fig. 5.3). The growth rates of isolate RSA1221 grown in the absence of and following the addition of vancomycin (32µg/mL) were very similar indicating resistance is expressed constitutively. These experiments were performed prior to this study and the results shown below are courtesy of A. von Gottberg and have been incorporated here because they are pertinent to all future discussions in this section (von Gottberg *et al*., 1999).

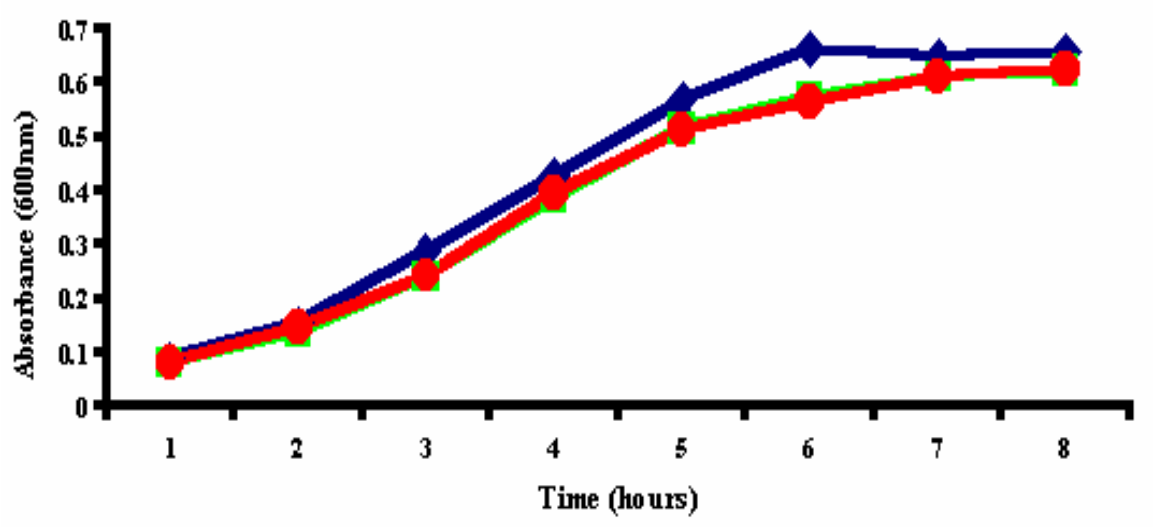


Figure 5.3: Glycopeptide resistance expression of *P. thiaminolyticus* RSA1221. RSA1221 was cultured in BHI containing no vancomycin (♦). RSA1221 pre-cultured in BHI containing no vancomycin and then sub-cultured into fresh BHI containing 32µg/mL vancomycin (■). RSA1221 pre-cultured in BHI containing 32µg/mL vancomycin and then sub-cultured into fresh BHI containing 32µg/mL vancomycin (●). Absorbance readings were taken at 600nm (von Gottberg *et al*., 1999).

5.1.4 PCR detection of the *van* ligase gene

To detect the presence of the *van* ligase gene and to determine the glycopeptide resistance genotype of RSA1221, PCR using primers specific for the enterococcal resistance genes *vanA*, *vanB*, *vanC1*, and *vanC2/C3* (Table 3.4) was performed (Fig. 5.4). The *P. thiaminolyticus* RSA1221 *vanA* PCR yielded a positive reaction and produced the expected 731bp product size (lane 1). PCR products of the expected size were obtained in the positive control reactions. The glycopeptide resistance genotype observed in *P. thiaminolyticus* RSA1221 is therefore *vanA*.

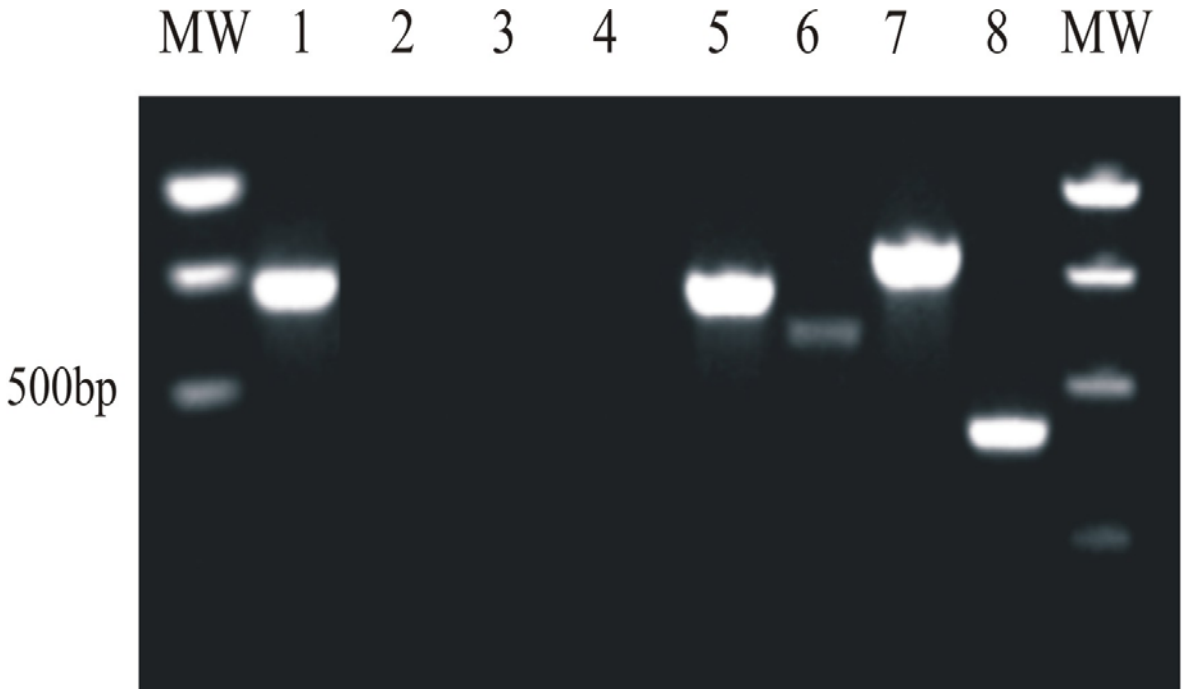


Figure 5.4: PCR analysis of *van* ligase resistance gene in *P. thiaminolyticus* RSA1221.

MW. 1kb molecular weight marker (Promega)

Lane 1. *P. thiaminolyticus* with *vanA* specific primers

2. *P. thiaminolyticus* with *vanB* specific primers

3. *P. thiaminolyticus* with *vanC1* specific primers

4. *P. thiaminolyticus* with *vanC2/C3* specific primers

5. *E. faecium vanA* positive control

6. *E. faecalis vanB* positive control

7. *E. gallinarum vanC1* positive control

8. *E. casseliflavus vanC2/C3* positive control

MW. 1kb molecular weight marker (Promega)

5.1.5 Location of the *vanA* resistance determinant

Southern hybridization experiments using a DIG-labelled enterococcal *vanA* probe was performed to determine the location of the *vanA* resistance operon. A hybridization signal was detected in both *Eco*RI restricted plasmid and total DNA preparations. The approximate fragment size was 13kb. However the degree of hybridization was stronger in the total DNA preparation suggesting the gene is chromosomally located. The weaker hybridization signal observed in the plasmid preparation, is likely to be carryover chromosomal DNA. In the positive control *E. faecium* BM4147 (where the resistance determinant is plasmid-borne), hybridization signals were detected in both the plasmid and total DNA preparations but a stronger signal was detected on a ~4-kb *Eco*RI fragment in the plasmid preparation. These results strongly suggest that the *vanA* resistance determinant of *P. thiaminolyticus* RSA1221 is chromosomally-borne.

To confirm the location (i.e. chromosomal) of the *vanA* operon, *P. thiaminolyticus* RSA1221 genomic DNA was embedded in agarose plugs and digested with *Sma*I restriction endonuclease *in situ*. The restricted fragments were separated using pulsed field gel electrophoresis. Southern hybridization experiments were performed successively with a DIG-labelled 16S rRNA (*rrs*) probe and a DIG-labelled *vanA* probe. The *rrs* probe hybridized with six *P. thiaminolyticus* RSA1221 *Sma*I-restricted fragments and the *vanA* probe co-hybridized with a ~11kb fragment. Therefore the *vanA* gene cluster in *P. thiaminolyticus* RSA1221 is chromosomal, since the *rrs* genes are not found on plasmids.

5.1.6 *vanA* nucleotide and sequence similarity analysis

In order to evaluate the similarity of the *vanA* gene of RSA1221 ($vanA_{p.t1221}$) with that of other published *vanA* genes, the 1032bp fragment amplified from *P. thiaminolyticus* RSA1221 DNA was sequenced.

The enterococcal *van* ligase genes have been well characterized in terms of sequence, structure and function. Analysis of the deduced $VanA_{p.t1221}$ protein sequence showed that the conserved motif; proline (Pro/ P)-glutamic acid (Glu/ E) - lysine (Lys/ K) - glycine (Gly/ G) found in the ω-loop of VanA and VanB D-ala: D-lac ligases (Casadewall *et al*., 1999) was also present in $VanA_{p.t1221}$ between positions 249-252 (data not shown).

The nucleotide and the deduced amino acid sequence of *P. thiaminolyticus* RSA1221 was compared with other *vanA* and *vanA*-like genes (Table 5.3). Sequence similarity analysis using the NCBI BLAST alignment tools, showed that the *vanA* gene found in *P. thiaminolyticus* RSA1221 had the highest nucleotide (97%) and amino acid (96%) identity to the *vanA*-like D-ala: D-lac gene found in *P. thiaminolyticus* PT-2B1, a recently described environmental isolate with glycopeptide resistance (Guardabassi *et al*., 2004). The amino acid sequence identity with the *vanA* gene from *E. faecium* BM4147 and *B. circulans* VR0709 was 95% and 93%, respectively.

Similarity analysis using the World Wide Web-based CLUSTAL W computer programme, of the deduced amino acid sequence of $vanA_{p.t1221}$ and *vanA* and *vanA*-like gene are shown in figure 5.5 (Chenna *et al*., 2003). It can be seen from the phenogram that the *vanA* gene in *P. thiaminolyticus* RSA1221 is closely related to the *vanA*-like genes found in *P.*

thiaminolyticus PT-2B1 and *P. apiarius* PA-B2B, as well as the *vanA* gene found in *E. faecium* BM4147.

Table 5.3: Comparison of the nucleotide and deduced amino acid sequence of $vanA_{p.t1221}$ with other documented *vanA* genes, the D-ala: D-lac ligase gene from the glycopeptide-producing organism *A. orientalis*, the putative D-ala: D-lac ligase enterococcal-like ligases from two *Paenibacillus* spp. and other *van* ligase genes.

	P. thiaminolyticus RSA1221	*E. faecium* BM4147 (*vanA*)	*B. circulans* VR0709 (*vanA*)	*O. turbata* (*vanA*)	*S. aureus* (*vanA* type VRSA, Michigan)	*A. orientalis*	*P. thiaminolyticus* PT-2B1 putative D:ala:D:lac ligase	*P. apiarius* PA-B2B putative D:ala:D:lac ligase	*E. faecalis* Tn*1549* (*vanB*)	*E. faecium* N03-0072 (*vanD*)	*P. popilliae* (*vanF*)
Length (bp)	1032	1032	1032	786	1032	1047	1032	1032	1029	1032	1032
% Nucleotide identity	----	91%	91%	91%	91%	---	**97%**[a]	95%	79%	---	80%
% Amino acid identity	---	95%	93%	93%	95%	75%	**96%**[a]	93%	85%	80%	87%
% G+C content	46%	45%	44%	46%	45%	66%	46%	48%	49%	47%	46%

a. The numbers in boldface indicate the highest percentage of identity with *P. thiaminolyticus* RSA1221

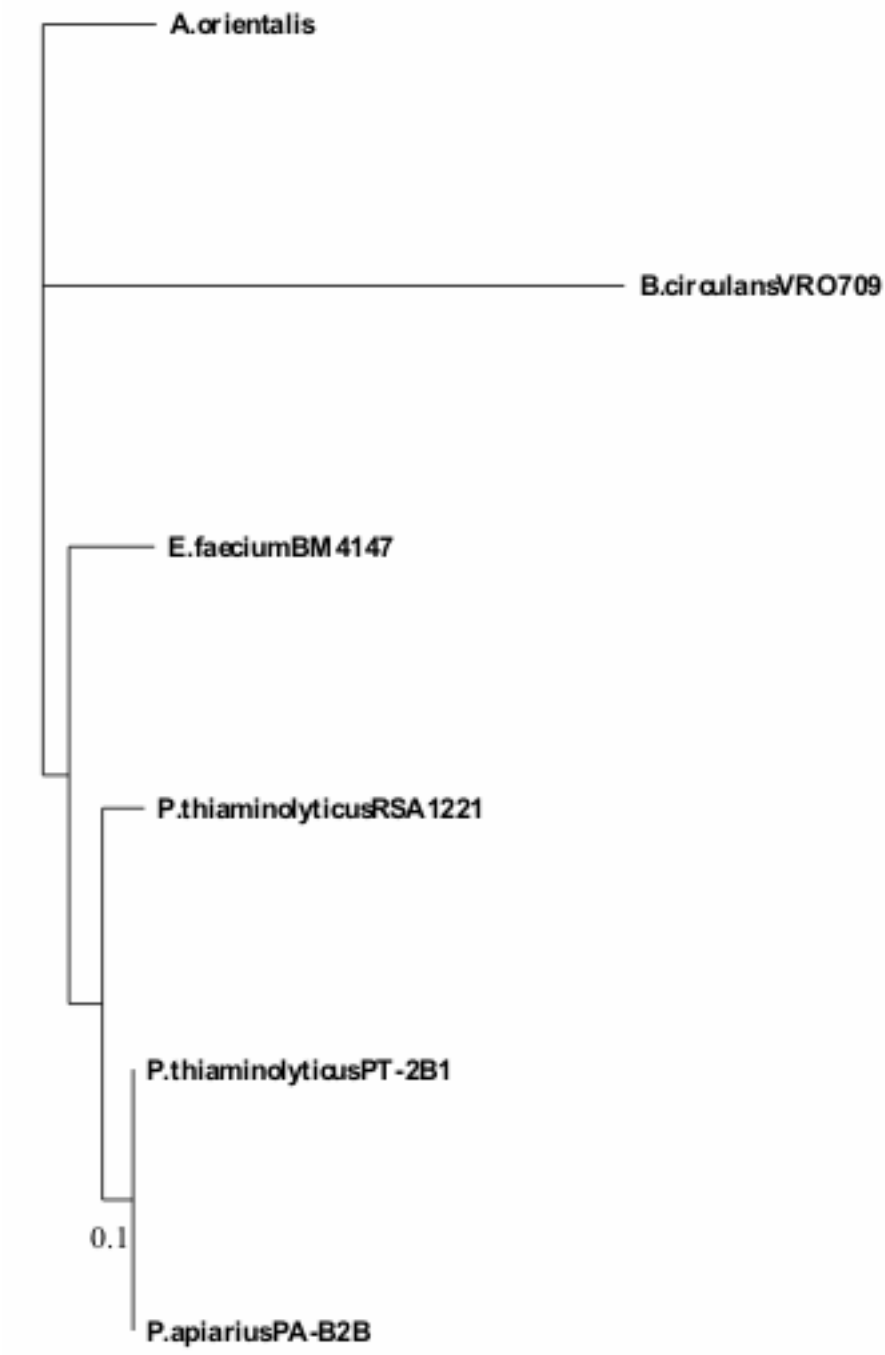


Figure 5.5: Phenogram of VanA proteins from *E. faecium* BM4147, *B. circulans* VR0709 and *P. thiaminolyticus* RSA221, the endogenous Ddl from the glycopeptide-producing organism *A. orientalis*, the putative D-ala:D-lac ligase enterococcal-like ligases from two *P. thiaminolyticus* PT-2B1 and *P. apiarius* PA-B2B. This phylogenetic tree was obtained using the programme CLUSTAL W. 0.1=10%

5.1.7 Investigation of other genes found in the RSA1221 *vanA* gene cluster

The prototypical *vanA* operon in the *E. faecium* BM4147 has been well characterized. It possesses seven *van* genes, *vanR, vanS, vanH, vanA*, *vanX, vanY* and *vanZ*. Investigations of other *van* genes in *P. thiaminolyticus* RSA1221 revealed the presence of *vanR, vanS, vanH, vanX* and *vanY*. No *vanZ* gene was detected. The nucleotide sequence of the gene cluster of *vanR, vanS, vanH, vanA* and *vanX* genes from *P. thiaminolyticus* RSA1221 was submitted to Genbank and given the accession number AY926880. Guardabassi *et al*. (2004) recently published the presence of a *vanA*-like gene in environmental isolate *P. thiaminolyticus* PT-2B1. They also noted the presence of *vanH*-like and *vanX*-like genes based on partial sequence information (Fig. 5.6).

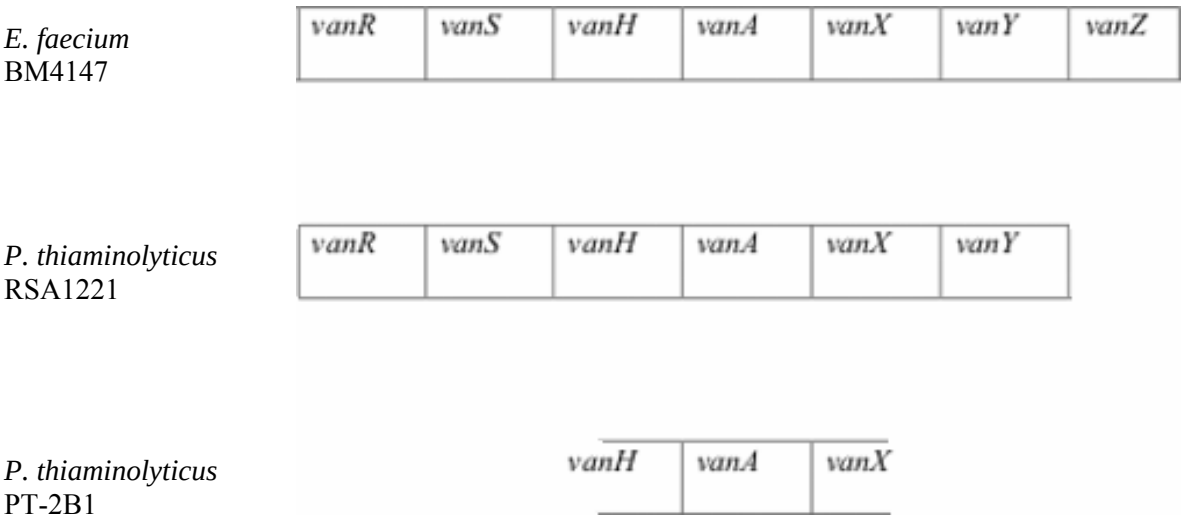


Figure 5.6: Summary of the glycopeptide resistance genes found in *E. faecium* BM4147 (Arthur *et al*., 1993), *P. thiaminolyticus* RSA1221 (this study) and *P. thiaminolyticus* PT2B1 (Guardabassi *et al*., 2004).

5.1.7.A ***vanR* and *vanS***

vanR and *vanS* encode a two-component regulatory system that activates the transcription of *vanH*, *vanA*, *vanX*, *vanY* and *vanZ* in response to an external stimulus. The presence of *vanR* and *vanS* was investigated using PCR amplification and nucleotide sequencing strategies.

The organization of *P. thiaminolyticus* RSA1221 *vanR-vanS* ($vanR_{p.t1221}$- $vanS_{p.t1221}$) genes is similar to that found in *E. faecium* BM4147, which includes the overlap of the 3' end of *vanR* with the 5' end of *vanS*. The highest BLASTn score was obtained with *vanR- vanS* two-component regulatory system from *E. faecium* BM4147, with an overall 93% nucleotide identity (data not shown).

A1. *vanR* (response regulator)

Analysis of the deduced $VanR_{p.t1221}$ amino acid sequence showed the presence of the conserved residues, aspartic acid (Asp/ D)$_{10}$, Asp_{53}, Lys_{102}, which is typical of response regulators in two-component systems from Gram-positive bacteria (Fig. 5.7) (Depardieu *et al*., 2003b). The highest degree of identity was between $VanR_{p.t1221}$ and VanR of *E. faecium* BM4147, with a 94% nucleotide and 97% amino acid identity (Table 5.4).

Table 5.4: A comparison of level of identity between the nucleotide and deduced amino acid sequence of $vanR_{p.t1221}$ with other documented *vanR* genes

	P. thiaminolyticus RSA1221	*E. faecium* BM4147 (*vanA*)	*B. circulans* VR0709 (*vanA*)	*S. aureus* (*vanA* type VRSA, Michigan)	*E. faecalis* Tn*1549* (*vanB*)	*E. gallinarum* (*vanC1*)	*E. faecium* N03-0072 (*vanD*)	*E. faecalis* N00-410 (*vanE*)	*P. popilliae* (*vanF*)	*E. faecalis* BM4518 (*vanG*)
Length (bp)	696	696	696	696	663	696	699	690	696	708
% Nucleotide identity	**---**	**94%**[a]	92%	**94%**[a]	---	---	---	---	---	---
% Amino acid identity	**---**	**97%**[a]	96%	**97%**[a]	74%	68%	72%	67%	58%	73%
% G+C content	42%	42%	42%	42%	47%	40%	44%	33%	36%	35%

a. The numbers in boldface indicate the highest percentage of identity with *P. thiaminolyticus* RSA1221

Note that *vanR* data for *P. thiaminolyticus* PT-2B1and *P. apiarius* PA-B2B were not available

vanR promoter—**TAAAAAAAGAATCATCATCTTAAGAAATTCTTAGTCATTTATTATGTAAATGCTTATAAATTCGGCCCTATAATCTGATAAATTAT--**-start of *vanR*

Asp10

atg agc aat aaa att ctt gtt gta gac **gat** gaa cat gaa att gct gat ttg gtt gaa tta tac tta aaa aac gag aat tat acg gtt

M S N K I L V V D **D** E H E I A D L V E L Y L K N E N Y T V

Asp53

ttc aaa tac tat acc gct aaa gaa gca ttg gaa tgt ata gac aag tct gag att gac ctt gcc ata ttg **gac** atc atg ctt ccc ggc

F K Y Y T A K E A L E C I D K S E I D L A I L **D** I M L P G

aca agc ggc ctt act atc tgt caa aaa ata agg gac aag cac acc tat ccg att atc atg ctg acc ggg aaa gat aca gag gta gat

T S G L T I C Q K I R D K H T Y P I I M L T G K D T E V D

Lys102

aaa att aca ggg tta aca atc ggc cgg atg gat tat ata acg **aag** ccc ttt cgc ccg ctg gag tta ttt gcg agg gtc aag gcg cag

K I T G L T I G R M D Y I T **K** P F R P L E L F A R V K A Q

ttg cgc cga tac aaa aaa tac agt gga gta gcg gag cag aac gaa aat att atc gtc cac tcc ggt ctt gtc att aat ata aac acg

L R R Y K K Y S G V A E Q N E N I I V H S G L V I N I N T

cat gag tgt ttt ctg aac gag aag cag tta tcg ctc act ccc acc gag ttt tca atc ctg cgc atc ctc tgt gaa aac aag ggg aat

H E C F L N E K Q L S L T P T E F S I L R I L C E N K G N

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gtg gtt agc tcc gag cag tta ttt cat gag gta tgg ggc gac gaa tat ttc agc aag agc aac aac acc att acc gtg cat atc cgg
 V   V   S   S   E   Q   L   F   H   E   V   W   G   D   E   Y   F   S   K   S   N   N   T   I   T   V   H   I   R

cat cta cgc gaa aaa atg aac gac acc att gat aat ccg aag tat ata aaa acg gta tgg ggg gtt ggc tat aaa att gaa aaa taa
 H   L   R   E   K   M   N   D   T   I   D   N   P   K   Y   I   K   T   V   W   G   V   G   Y   K   I   E   K STOP
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Figure 5.7: Nucleotide and deduced amino acid sequence of the *vanR*$_{p.t1221}$. The putative P_R promoter region upstream of *vanR* is indicated in black, boldface letters. The blue, boldface 12bp sequence represents the recognition site for phosphorylated-VanR to bind. Asp_{10}, Asp_{53} and Lys_{102} are the conserved residues found in the effector domains of other response regulators. Asp_{53} is phosphorylated by the histidine protein kinase VanS .

A2. *vanS* (response sensor)

The nucleotide sequence of the *vanS* gene of *P. thiaminolyticus* RSA1221 ($vanS_{p.t1221}$) was obtained by direct sequencing of the relevant PCR products. Sequence similarity analysis using the NCBI BLAST alignment tools showed the highest (95%) nucleotide and amino acid identity with the *vanS* from *E. faecium* BM4147 (Table 5.5).

Analysis of the $vanS_{p.t1221}$ nucleotide sequence revealed a 6bp (GAATAA) insertion at the 5' end of the gene (Fig. 5.8). This nucleotide insertion would effectively result in the insertion of asparagine $(Asn/N)_9$ and lysine $(Lys/K)_{10}$ towards the N-terminus of the predicted protein sequence. The sequence remains in frame with no premature stop codons introduced by the insertion. It is interesting to note that the insertion disrupts the codon for K_8, but due to the wobble position at the third base, there is no effective change of amino acid.

The C-terminal region of $VanS_{p.t1221}$ contains the five blocks of conserved amino acids, H, N, G1, F and G2 (Fig. 5.8), which is characteristic of transmitter modules in His protein kinases (Wolanin *et al*., 2002).

The CLUSTAL W multiple sequence alignment of the VanS sensor proteins from *P. thiaminolyticus* RSA1221, *E. faecium* BM4147 and *B. circulans* VR0709 is shown in figure 5.9. The catalytic centre dimerization domain of VanS is highlighted and includes His_{164} (black, boldface letter), which is the putative autophosphorylation site in *E. faecium* BM4147. The catalytic centre dimerization domain (Tyr_{161}-Ser_{178} in *E. faecium* BM4147)

is the region with which VanR interacts with Phos-VanS (Ulijasz *et al*., 2000). No changes in this domain in *P. thiaminolyticus* RSA1221 were noted.

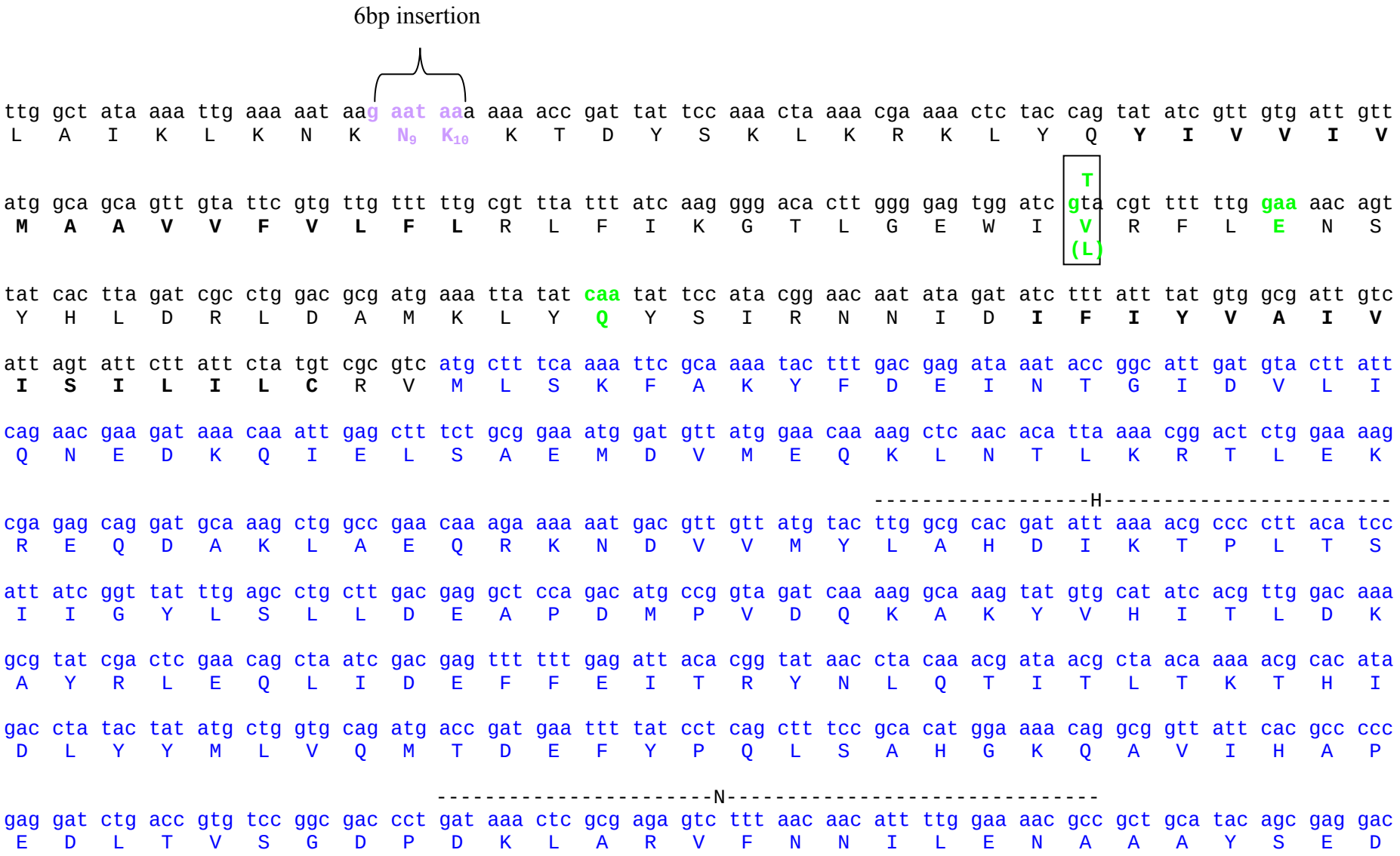
A Kyte-Doolittle hydrophobicity plot of $VanS_{p.t1221}$ using the World Wide Web-based Expasy Protscale primary sequence analysis programme is shown in figure 5.10. The clusters of hydrophobic residues (Y_{24}-I_{39} and I_{80}-C_{94}, Fig. 5.8) noted in figure 5.10, are in all likelihood the putative transmembrane regions of the $VanS_{p.t1221}$ sensor protein, similar to that found in *E. faecium* BM4147 VanS and *E. faecalis* $VanS_B$ (Batista *et al*., 1998). These hydrophobic regions at the N-terminus of the amino acid sequence suggest that the $VanS_{p.t1221}$ protein is membrane bound.

Table 5.5: A comparison of level of identity between the nucleotide and deduced amino acid sequence of *vanS*$_{p.t1221}$ with other documented *vanS* genes

	P. thiaminolyticus RSA1221	*E. faecium* BM4147 (*vanA*)	*B. circulans* VR0709 (*vanA*)	*S. aureus* (*vanA* type VRSA, Michigan)	*E. faecalis* Tn*1549* (*vanB*)	*E. gallinarum* (*vanC1*)	*E. faecium* N03-0072 (*vanD*)	*E. faecalis* N00-410 (*vanE*)	*P. popilliae* (*vanF*)	*E. faecalis* BM4518 (*vanG*)
Length (bp)	1161	1155	1140	1155	1344	1086	1146	1074	1098	1107
% Nucleotide identity	---	**95%**[a]	91%	**95%**[a]	---	---	---	---	---	---
% Amino acid identity	---	**95%**[a]	**95%**[a]	**95%**[a]	33%	59%	64%	50%	50%	60%
% G+C content	43%	39%	42%	41%	47%	42%	43%	23%	34%	37%

a. The numbers in boldface indicate the highest percentage of identity with *P. thiaminolyticus* RSA1221

Note that *vanS* data for *P. thiaminolyticus* PT-2B1and *P. apiarius* PA-B2B is not available


6bp insertion
ttg gct ata aaa ttg aaa aat aag aat aaa aaa acc gat tat tcc aaa cta aaa cga aaa ctc tac cag tat atc gtt gtg att gtt
L A I K L K N K N9 K10 K T D Y S K L K R K L Y Q Y I V V I V
T
atg gca gca gtt gta ttc gtg ttg ttt ttg cgt tta ttt atc aag ggg aca ctt ggg gag tgg atc gta cgt ttt ttg gaa aac agt
M A A V V F V L F L R L F I K G T L G E W I V R F L E N S
(L)
tat cac tta gat cgc ctg gac gcg atg aaa tta tat caa tat tcc ata cgg aac aat ata gat atc ttt att tat gtg gcg att gtc
Y H L D R L D A M K L Y Q Y S I R N N I D I F I Y V A I V
att agt att ctt att cta tgt cgc gtc atg ctt tca aaa ttc gca aaa tac ttt gac gag ata aat acc ggc att gat gta ctt att
I S I L I L C R V M L S K F A K Y F D E I N T G I D V L I
cag aac gaa gat aaa caa att gag ctt tct gcg gaa atg gat gtt atg gaa caa aag ctc aac aca tta aaa cgg act ctg gaa aag
Q N E D K Q I E L S A E M D V M E Q K L N T L K R T L E K
H
cga gag cag gat gca aag ctg gcc gaa caa aga aaa aat gac gtt gtt atg tac ttg gcg cac gat att aaa acg ccc ctt aca tcc
R E Q D A K L A E Q R K N D V V M Y L A H D I K T P L T S
att atc ggt tat ttg agc ctg ctt gac gag gct cca gac atg ccg gta gat caa aag gca aag tat gtg cat atc acg ttg gac aaa
I I G Y L S L L D E A P D M P V D Q K A K Y V H I T L D K
gcg tat cga ctc gaa cag cta atc gac gag ttt ttt gag att aca cgg tat aac cta caa acg ata acg cta aca aaa acg cac ata
A Y R L E Q L I D E F F E I T R Y N L Q T I T L T K T H I
gac cta tac tat atg ctg gtg cag atg acc gat gaa ttt tat cct cag ctt tcc gca cat gga aaa cag gcg gtt att cac gcc ccc
D L Y Y M L V Q M T D E F Y P Q L S A H G K Q A V I H A P
N
gag gat ctg acc gtg tcc ggc gac cct gat aaa ctc gcg aga gtc ttt aac aac att ttg gaa aac gcc gct gca tac agc gag gac
E D L T V S G D P D K L A R V F N N I L E N A A A Y S E D

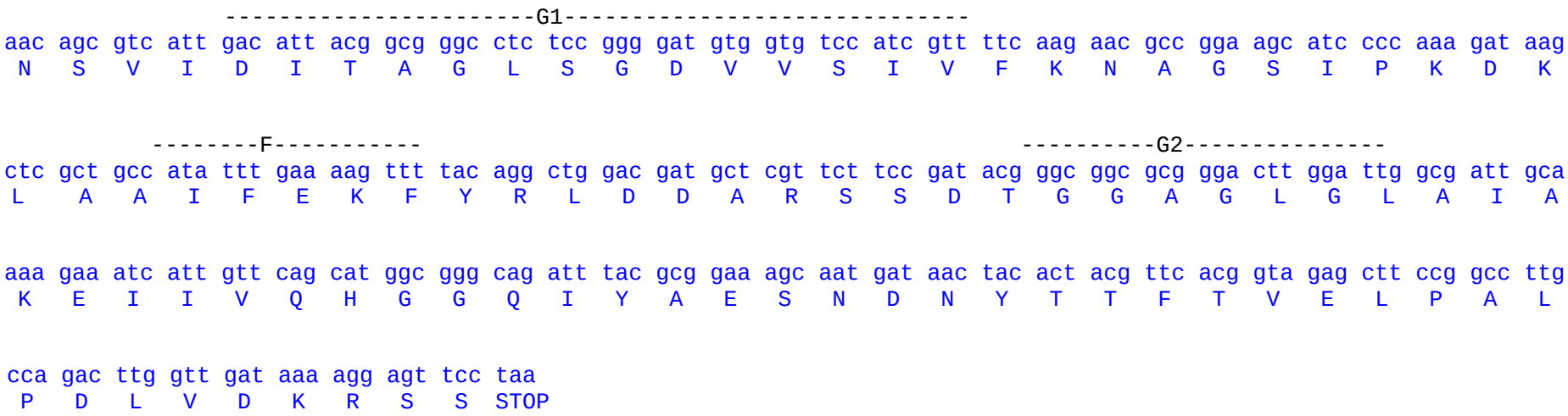


Figure 5.8: Nucleotide and deduced amino acid sequence of the *vanS*$_{p.t1221}$. In blue is the cytoplasmic C-terminus of *vanS*$_{p.t1221}$ beginning at Met$_{97}$. The purple letters indicate the location of the six-nucleotide insertion and the corresponding two amino acid insertions. The green letters mark the location of the nucleotide and amino substitution that result in impaired resistance to teicoplanin (Hashimoto *et al*., 2000). The lysine in parentheses show the nucleotide and amino acid sequence of *E. faecium* BM4147. Conserved motifs H, N, G1, F and G2 are indicated above the nucleotide sequence by dash lines. The clusters of hydrophobic amino acids predicted to represent transmembrane regions are shown in black, boldface lettering.

```
B.circulans             ----MKNKNKKNDYSKLERKLYMYIVAIVVVAVIFVLYIRSMIRGKLGEWIVRFLENEYG 56
E. faecium              --MVIKLKNKKNDYSKLERKLYMYIVAIVVVAIVFVLYIRSMIRGKLGDWILSILENKYD 58
P.thiaminolyticus       LAIKLKNKNKKTDYSKLKRKLYQYIVVIVMAAVVFVLFLRLFIKGTLGEWIVRFLENSYH 60
                             :*.****.*****:**** ***.**:.*::***::* :*:*.**:**:.:***.*

B.circulans             LNQLDAMKLYHYSIRNNIEIFIYVAIAISILILCRVMLSKFAKYFDEINTGIDVLIQNED 116
E. faecium              LNHLDAMKLYQYSIRNNIDIFIYVAIVISILILCRVMLSKFAKYFDEINTGIDVLIQNED 118
P.thiaminolyticus       LDRLDAMKLYQYSIRNNIDIFIYVAIVISILILCRVMLSKFAKYFDEINTGIDVLIQNED 120
                        *::*******:*******:*******.**********************************

                                                          Catalytic centre
                                                          dimerization domain

B.circulans             KQIELSAEMDVMEHKLNTLKRTLEKREQDAKLAEQRKNDVVMYLAHDIKTPLTSIIGYLS 176
E. faecium              KQIELSAEMDVMEQKLNTLKRTLEKREQDAKLAEQRKNDVVMYLAHDIKTPLTSIIGYLS 178
P.thiaminolyticus       KQIELSAEMDVMEQKLNTLKRTLEKREQDAKLAEQRKNDVVMYLAHDIKTPLTSIIGYLS 180
                        ************:*********************************************

B.circulans             LLDEAPDMPVDQKAKYVHITLDKAYRLEQLIDEFFEITRYNLQTITLTKTHIDLYYMLVQ 236
E. faecium              LLDEAPDMPVDQKAKYVHITLDKAYRLEQLIDEFFEITRYNLQTITLTKTHIDLYYMLVQ 238
P.thiaminolyticus       LLDEAPDMPVDQKAKYVHITLDKAYRLEQLIDEFFEITRYNLQTITLTKTHIDLYYMLVQ 240
                        ************************************************************

B.circulans             MTDEFYPQLSAHGKQAIIHASVDLTVSGDPDKLARVFNNILKNAAAYSEDNSIIDITAGL 296
E. faecium              MTDEFYPQLSAHGKQAVIHAPEDLTVSGDPDKLARVFNNILKNAAAYSEDNSIIDITAGL 298
P.thiaminolyticus       MTDEFYPQLSAHGKQAVIHAPEDLTVSGDPDKLARVFNNILENAAAYSEDNSVIDITAGL 300
                        ****************:***..******************:**********:*******
```

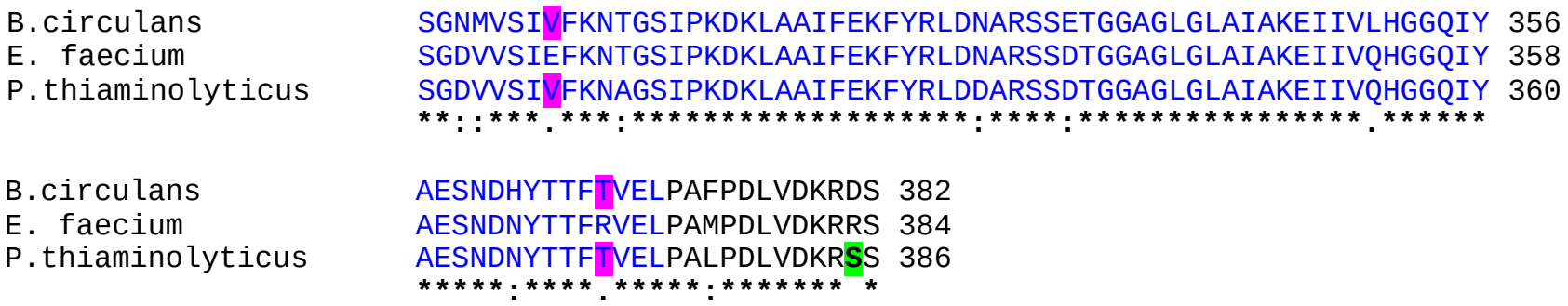


Figure 5.9: CLUSTAL W amino acid sequence alignment of the VanS sensor protein from *P. thiaminolyticus* RSA1221, *E. faecium* BM4147 and *B. circulans* VR0709. The numbers on the right refer to the last amino acid in the corresponding sequence. Identical amino acids are indicated by "*****", conserved substitutions "**:**" and semi-conserved substitutions "**.**". The open-end bracket indicates the catalytic centre dimerization domain of VanS and it includes His_{164} (black, boldface letter), which is the putative autophosphorylation site in *E. faecium* BM4147. Highlighted in red is the conserved domain of His Kinase A (dimerization/phosphoacceptor). Highlighted in blue is the conserved domain of HATPase (His kinase-like ATPase). Highlighted in green are the amino acid variations in *P. thiaminolyticus* RSA1221 compared to the other sequences. Highlighted in pink are the amino acid substitutions present in *B. circulans* VR0709 and *P. thiaminolyticus* RSA1221 relative to *E. faecium* BM4147. Highlighted in yellow in the two amino acid insertion in *P. thiaminolyticus* RSA1221.

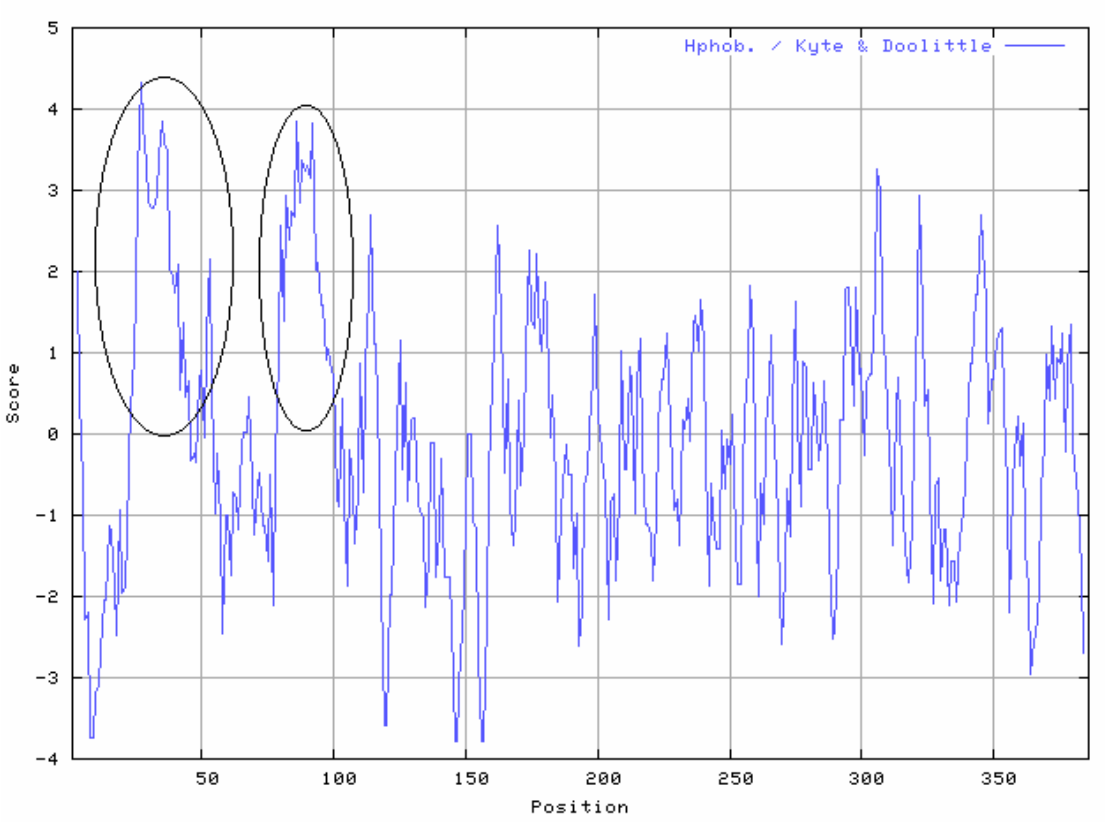


Figure 5.10: Kyte-Doolittle hydrophobicity plot of $VanS_{p.t1221}$using the Expasy Protscale primary sequence analysis programme. A score > 0 indicates hydrophobic amino acids. The two circles indicate putative transmembrane regions in the N-terminus of $VanS_{p.t1221}$.

A3. *vanH, vanX, vanY* and *vanZ* genes

The presence of other genes in the *vanA* cluster was investigated by PCR using primers specific for *vanH, vanX, vanY and vanZ* (Table 3.5).

The $vanH_{p.t1221}$ and the *E. faecium* BM4147 *vanH* had the highest nucleotide and amino acid sequence identity of 93% and 95%, respectively. The $vanH_{p.t1221}$ and the *B. circulans vanH* ($vanH_{b.c}$) had a 91% nucleotide and 92% amino acid sequence identity (Table 5.6). The three conserved residues, arginine (Arg/ R) $_{232}$, Glu_{260}, His_{292}, which are postulated to participate in substrate binding and catalysis, were present in $vanH_{p.t1221}$ (data not shown) (Casadewall *et al*., 1999).

VanX is a zinc-containing D-ala-D-ala dipeptidase and is one of the five essential proteins required for the glycopeptide resistance phenotype. The consensus sequence, described by McCafferty *et al*. (1997), (SxHxxGxAxD) was present in $VanX_{p.t1221}$. His_{116} and Asp_{123} are the putative zinc binding residues and Glu_{181}, the putative catalytic residue, was also present in $VanX_{p.t1221}$ (McCafferty *et al*., 1997). The $vanX_{p.t1221}$ and the *vanX* gene from *E. faecium* BM4147 had the highest nucleotide identity (92%) but the $VanX_{p.t1221}$ and the *B. circulans* VanX had the highest amino acid sequence identity of 95% (Table 5.7).

Partial sequence analysis of the $vanY_{p.t1221}$ gene showed a three nucleotide insertion (GGG) at the 5' end of the gene resulting in a Gly $(G)_{10}$ amino acid insertion at the N-terminus of the $VanY_{p.t1221}$ protein. This insertion causes a frameshift mutation and the introduction of a premature STOP codon and presumably a truncated $VanY_{p.t1221}$ protein (data not shown).

Based on this result, it can be postulated that VanY protein may be non-functional in *P. thiaminolyticus* RSA1221.

Two primer sets used to amplify the *vanZ* gene in *P. thiaminolyticus* RSA1221 are shown in Table 3.5. The primers bind at different positions in the *E. faecium* BM4147 *vanZ* gene. No PCR product was amplified from *P. thiaminolyticus* RSA1221 with any of the primer pairs (data not shown), which would suggest that the gene is not present in this isolate, since it is unlikely that the binding sites for both primer sets has been altered. Southern hybridization, with an enterococcal *vanZ* DIG-labelled probe, confirmed that *P. thiaminolyticus* RSA1221 does not contain the *vanZ* gene.

Table 5.6: A comparison of level of identity between the nucleotide and deduced amino acid sequence of $vanH_{p.t1221}$ with other documented *vanH* genes and D-specific α-ketoacid dehydrogenase from the glycopeptide-producing organisms *A. orientalis* and *S. toyocaensis*

	P. thiaminolyticus RSA1221	*E. faecium* BM4147 (*vanA*)	*B. circulans* VR0709 (*vanA*)	*S. aureus* (*vanA* type VRSA, Michigan)	*E. faecalis* Tn*1549* (*vanB*)	*E. faecium* N03-0072 (*vanD*)	*E. faecalis* N00-410 (*vanE*)	*P. popilliae* (*vanF*)	*E. faecalis* BM4518 (*vanG*)	*A. orientalis*	*S. toyocaensis*
Length (bp)	968	969	969	969	972	972	NP	969	NP	1047	993
% Nucleotide identity	**---**	**93%**[a]	91%	**93%**[a]	---	---	NP	33%	NP	---	---
% Amino acid identity	**---**	**95%**[a]	92%	**95%**[a]	83%	73%	NP	83%	NP	79%	72%
G+C content	46%	46%	44%	46%	51%	46%	NP	48%	NP	60%	65%

a. The numbers in boldface indicate the highest percentage of identity with *P. thiaminolyticus* RSA1221

NP. Not present

Note that *vanH* data for *P. thiaminolyticus* PT-2B1and *P. apiarius* PA-B2B is not available

Table 5.7: A comparison of level of identity between the nucleotide and deduced amino acid sequence of $vanX_{p.tl221}$ with other documented *vanX* genes and D,D-dipeptidases from the glycopeptide-producing organisms *A. orientalis* and *S. toyocaensis*

	P. thiaminolyticus RSA1221	*E. faecium* BM4147 (*vanA*)	*B. circulans* VR0709 (*vanA*)	*S. aureus* (*vanA* type VRSA, Michigan)	*E. faecalis* Tn*1549* (*vanB*)	*E. faecium* N03-0072 (*vanD*)	*E. faecalis* N00-410 (*vanE*)	*P. popilliae* (*vanF*)	*E. faecalis* BM4518 (*vanG*)	*A. orientalis*	*S. toyocaensis*
Length (bp)	609	609	609	609	609	609	NP	672	NP	609	627
% Nucleotide identity	**---**	**92%**[a]	90%	**92%**[a]	---	---	NP	80%	NP	---	---
% Amino acid identity	**---**	94%	**95%**[a]	94%	85%	83%	NP	92%	NP	75%	79%
% G+C content	45%	44%	43%	44%	47%	47%	NP	46%	NP	65%	66%

a. The numbers in boldface indicate the highest percentage of identity with *P. thiaminolyticus* RSA1221

NP. Not present

Note that *vanS* data for *P. thiaminolyticus* PT-2B1and *P. apiarius* PA-B2B is not available

5.1.8 5’ and 3’ ends of *van* cassette

PCR analysis revealed the absence of *orf*1 and *orf* 2, which have been described as part of Tn*1546*, and is commonly associated with the *vanA* genotype. The *orf* described in the clinical isolate *B. circulans* VR0709 was also found to be absent.

Hybridization experiments using a transposon probe (Tn*1546*) obtained from amplification of *E. faecium* BM4147 DNA, showed no hybridization with *P. thiaminolyticus* DNA, further supporting the notion that this resistance cassette is part of a novel transferable element, or are not associated with a mobile element.

To determine the 5’ and 3’ ends beyond the *vanA* gene complex identifiable using PCR techniques, sub-genomic libraries of *P. thiaminolyticus* RSA1221 were constructed. Sequencing of the 5’ and 3’ containing clones revealed no Tn-related *orfs*.

5.2 Discussion

P. thiaminolyticus RSA1221 properties

Clinical isolate RSA1221 was provisionally identified as *Paenibacillus alvei* but due to limited typing resources the isolate was sent to a reference laboratory at the Glasgow Caledonian University for further characterization. The final identification was *Paenibacillus thiaminolyticus* (von Gottberg *et al*., 1999). The results of additional RSA1221 biochemical assays are consistent with the *P. thiaminolyticus* description given by Parry *et al*. (1984) and Bergey's Manual of Systematic Bacteriology (1984).

vanB phenotype- *vanA* genotype incongruence

P. thiaminolyticus RSA1221 expresses high-level resistance to vancomycin and intermediate resistance to teicoplanin. This glycopeptide resistance phenotype is consistent with the vanB phenotype in terms of levels of glycopeptide resistance. However, *P. thiaminolyticus* RSA1221 harbours a *vanA* resistance operon. Similar vanB phenotype-*vanA* genotype incongruence was previously reported by Hashimoto and co-workers in Japan (2000) and Lauderdale and colleagues in Taiwan (2002).

One of the three amino acid substitutions shown to be responsible for the vanB- *vanA* incongruence by Hashimoto *et al*. (2000) is present in the $vanS_{p.t1221}$ gene. This group reported that these amino acid substitutions occur in the putative glycopeptide sensor-domain of VanS (Hashimoto *et al*., 2000). Lauderdale and co-workers (2002) showed that

the vanB phenotype- *vanA* genotype incongruence could also result when only two of the three mutations are present. It is possible that the vanB phenotype- *vanA* genotype incongruence observed in *P. thiaminolyticus* RSA1221 is a result of the critical $Lys_{50} \rightarrow Val_{50}$ substitution that was found, and that this is sufficient for the phenotype observed.

Identification and localization of the vancomycin resistance determinant of *P. thiaminolyticus* RSA1221

To date all described *vanA* resistance operons have been associated with Tn*1546* or other mobile elements. This transposon-borne resistance operon is located either on plasmids or on the bacterial chromosome. The location of the *vanA* gene cluster in *P. thiaminolyticus* RSA1221 was determined by Southern hybridization experiments. The results indicate that the *vanA* resistance cluster is chromosomally-borne and not found on a plasmid.

Characterization of the *vanA* gene of *P. thiaminolyticus* RSA1221

Phylogenetic analysis was done using the World Wide Web-based CLUSTAL W computer programme. It can be noted from the phenogram that the *vanA* gene in *P. thiaminolyticus* RSA1221 (Genbank accession number AY926880) is likely to be closely related to the *vanA* gene found in *E. faecium* BM4147 and *vanA*-like genes found in *P. thiaminolyticus* PT-2B1 and *P. apiarius* PA-B2B.

The same % G+C content and the high nucleotide and amino acid sequence similarity of the *vanA*$_{p.t1221}$ and the *vanA*-like gene in *P. thiaminolyticus* PT-2B1 would suggest that the two genes originate from a common ancestor. The *vanA*$_{p.t1221}$ gene was also closely related to the *E. faecium* BM4147 *vanA* gene, with a 95% amino acid identity.

Constitutive expression of resistance in *P. thiaminolyticus* RSA1221 and the role of *vanS*

The vanA phenotype is characterized by high-level, inducible glycopeptide resistance. Glycopeptide resistance expression experiments showed that resistance expression is constitutive in *P. thiaminolyticus* RSA1221. To date, the only documented classic enterococcal van phenotype expressing constitutive resistance is vanD.

vanR and *vanS* encode a two-component regulatory system that is commonly found in bacteria used to sense and respond to the external environment (Wright *et al*., 1993). Constitutive expression could be a result of defects in the two-component regulatory system. The kinase and phosphatase properties of VanS regulate the levels of Phos-VanR. In the absence of a stimulus, VanS acts as a phosphatase preventing the accumulation of Phos-VanR. Mutations near the putative autophosphorylation site in VanS may alter the phosphatase activity required for the negative regulation of Phos-VanR. Alternatively, mutations in the signal recognition domain of VanS may lead to the phosphorylation of VanR even in the absence of an inducer (Casadewall *et al*., 1999 and Depardieu *et al*., 2003a).

In 1998, Ligozzi and co-workers reported the isolation of *B. circulans* VR0709 with constitutive vanA- type glycopeptide resistance. The researchers did not speculate on possible reasons for this constitutive resistance (Ligozzi *et al*., 1998). Sequence analysis of the VanS proteins from *B. circulans* VR0709 ($_{bc}$), *E. faecium* BM4147 and *P. thiaminolyticus* RSA1221 revealed two amino acid changes present only in *B. circulans* VR0709 and *P. thiaminolyticus* RSA1221, viz. $(Glu/E)_{bc304/pt308} \rightarrow$(Val/V) and $(Arg/R)_{bc367/pt371} \rightarrow$ threonine (Thr/T). These amino acid substitutions, relative to *E. faecium* BM4147, occur in the HATPase conserved domain and may impair the autophosphorylation activity of VanS.

The ATPase domain reduces ATP to adenosine diphosphate (ADP) by removing one phosphoryl group. In response to a stimulus, VanS undergoes ATP-dependent autophosphorylation on the His_{164} in *E. faecium* BM4147, and subsequently transfers that phosphate group to VanR (Wright *et al*., 1993). A characteristic structural feature of His protein kinases is that they are able to catalyze the transfer of the phosphoryl group from ATP to the autophosphorylation site (Dutta *et al*., 1999). Based on the strong sequence similarity between VanS and characterized His protein kinases, it is can be proposed that the same process occurs during VanS autophosphorylation.

The $E_{bc304}/E_{pt308} \rightarrow$V substitution is in the G1 ATP-binding and hydrolysis signature motif of the HATPase domain. All His protein kinases have a conserved ATP-binding domain which is required for kinase activity (Wolanin *et al*., 2002). The ATP-binding catalytic domain together with the dimerization domain, which includes the conserved H- box motif, forms the kinase core. The catalytic domain contains the four other conserved motifs; N, G1, F, G2, which are involved in the binding and catalysis of ATP, and the subsequent

phosphotransfer to the autophosphorylation His residue (Wolanin *et al*., 2002). Studies by Ellefson and co-workers (1997) on the catalytic domain of CheA, a His protein kinase associated with chemotaxis, showed that a mutation in either G1- or F- domains might cause a change in the orientation of ATP, limiting the ability of the H-domain to dock and accept the phosphoryl group from ATP at the site of autophosphorylation (Ellefson *et al*., 1997). Yang and Inouye (1993) showed that full kinase activity requires functional and intact N-, G1- and G2 motifs and phosphatase activity requires the G2 motif (Yang and Inouye, 1993).

The His protein kinase binds to the ATP molecule by forming H-bonds between the adenine ring of ATP and the amino acid residues in the N and G1 domains (Wolanin *et al*., 2002). The $E_{bc304}/E_{pt308} \rightarrow V$ substitution in the G1- ATP binding domain of *B. circulans* VR0709 and *P. thiaminolyticus* RSA1221 may alter the binding of ATP to VanS since glutamate a polar, hydrophilic, negatively charged amino acid is substituted by valine, an aliphatic, hydrophobic, neutral amino acid.

With the second substitution in the ATPase conserved domain ($R_{bc367}/_{pt371} \rightarrow T$), arginine a polar, hydrophilic positively charged amino acid is substituted by threonine, which is a polar, hydrophilic neutral amino acid. This amino acid change and the $E_{bc304}/E_{pt308} \rightarrow V$ substitution may impair the autophosphorylation pathway and lead to VanS being in a state of constant activation or the inability of VanS to undergo autophosphorylation. The inability of VanS to undergo autophosphorylation would suggest even in the absence of the VanS kinase activity, Phos-VanR is generated, possibly by a host protein kinase, at sufficient levels that results in the constitutive, high-level resistance phenotype observed in *P. thiaminolyticus* RSA1221. To determine whether these amino acid substitutions are

significant, protein functional studies need to be performed, which should determine if the substitution affects autophosphorylation, phospho-transfer or phosphatase activity.

In addition to the above mentioned substitutions, other amino acid changes present only in *B. circulans* VR0709 and *P. thiaminolyticus* RSA1221 were noted in the N-terminus of the VanS protein sequences. These amino acid substitutions could occur in the sensing domain and may lead to inefficient signal transduction. Furthermore, additional amino acid variations (~20%) between *P. thiaminolyticus* RSA1221, *B. circulans* VR0709 and *E. faecium* BM4147 is found in the N-terminus of $VanS_{p.t1221}$. The N -terminus contains the transmembrane- and sensory- domains, which is believed to respond to the presence of glycopeptides in the environment (Arthur *et al*., 2001). The amino acid substitutions in *P. thiaminolyticus* RSA1221 and *B. circulans* VR0709 alone or the additional amino acid changes in *P. thiaminolyticus* RSA1221 in the N-terminus, may result in mutations in the signal recognition domain leading to the phosphorylation of VanR even in the absence of a stimulus.

Analysis of the nucleotide and deduced amino acid sequence of $vanS_{p.t1221}$ showed a 6bp nucleotide insertion resulting in a two amino acid insertion at the 5' end of the gene. The two amino acid insertion does not alter the protein translation frame and no premature stop codons are introduced by the insertion. This amino acid insertion may result in altered protein folding. The function of the VanS protein may be altered and the insertion could be responsible for the observed constitutive expression of resistance in *P. thiaminolyticus* RSA1221. We were unable to determine if the 6bp nucleotide insertion would have an effect on the protein, as mutation studies would need to be conducted to establish the significance of the insertion.

The two amino acid insertion occurs in the N-terminus of the $VanS_{p.t1221}$ protein, which is postulated to contain the sensory and transmembrane domains. The insertions occur prior to the putative transmembrane domains of *P. thiaminolyticus* RSA1221. The general view is that the N-terminus of $VanS_B$ is in the cytoplasm (Baptista *et al*., 1997). This has not been confirmed nor has the exact location of the sensing domain been defined. It is possible that the N-terminus, which contains hydrophilic amino acid residues, may be the extra-cellular sensing domain and the two amino acid insertion in *P. thiaminolyticus* RSA1221 may impair signal recognition.

The mutations reported by Baptista and colleagues (1997), viz. a $T_{237}\rightarrow K$ substitution in $VanS_B$, which is located in the vicinity of the putative autophosphorylation site of $VanS_B$ (H_{233}) and results in impaired phosphatase activity leading to constitutive expression of the resistance genes (Baptista *et al*., 1997), was not present in $VanS_{p.t1221}$.

Constitutive expression of resistance in *P. thiaminolyticus* RSA1221 and the role of *vanR*

In the presence of an external stimulus (glycopeptides or related antibiotics) VanS undergoes autophosphorylation. Phosphorylated VanS transfers the phosphoryl group to VanR. Phos-VanR binds to the putative P_H and P_R promoter regions and activates transcription of the *van* genes.

VanR can undergo phosphorylation *in vitro* in the absence of a kinase, by using phosphate substrate donors such as acetyl-phosphate (Holman *et al*., 1994). Studies have also shown that in the absence of VanS, VanR can be phosphorylated by other protein kinases encoded

by the host chromosome (Arthur *et al*., 1997), which may provide an alternate pathway for Phos-VanR synthesis and leading to constitutive transcriptional activation of the *van* resistance genes (Wright *et al*., 1993 and Arthur *et al*., 1999).

Arthur and colleagues (1997) demonstrated *in vitro* that in the absence of VanS phosphatase activity and the slow dephosphorylation of Phos-VanR, produced by an alternate pathway, lead to accumulated levels of Phos-VanR, which resulted in constitutive activation of P_H and P_R promoter regions.

Spontaneous dephosphorylation of Phos-VanR is very slow, and the protein has a half-life of 10-12 hrs, which is relatively long in comparison to other response regulators. Therefore the prolonged levels of circulating Phos-VanR allow the protein to exert its transcriptional activation of the *van* resistance genes in the absence of functional VanS (Arthur *et al*., 1997).

vanR$_{p.t1221}$ and *vanS*$_{p.t1221}$ and their role in constitutive expression

To summarize the possible causes associated with the VanR and VanS proteins for the constitutive expression of the resistance genes in *P. thiaminolyticus* RSA1221:

1. **Impaired signal recognition properties of VanS$_{p.t1221}$**. The amino acid substitutions found in *P. thiaminolyticus* RSA1221 in the N-terminus of the protein may result in the inability of VanS$_{p.t1221}$ to sense the presence or absence of

glycopeptides, leading to phosphorylation of VanR even in the absence of an inducer.

2. **Altered kinase activity of $VanS_{p.t1221}$**. The $E_{bc304}/E_{pt308} \rightarrow V$ amino acid substitution in the G1-ATP binding domain may alter the kinase activity of $VanS_{p.t1221}$, resulting in VanS being in a constant state of activation.

3. **Non functional $VanS_{p.t1221}$ and an alternate pathway for Phos-VanR synthesis**. The two amino acid insertions at the N-terminus of $VanS_{p.t1221}$ could alter protein folding resulting in the synthesis of non-functional VanS proteins. The $E_{bc304}/E_{pt308} \rightarrow V$ amino acid substitution in the G1-ATP binding domain may impair the kinase activity of $VanS_{p.t1221}$, resulting in inefficient autophosphorylation of $His_{166p.t1221}$. Studies have shown that VanR can be phosphorylated by host protein kinases in the absence of VanS, to produce sufficient levels of Phos-VanR that result in high-level, constitutive activation of the *van* resistance genes (Arthur *et al*., 1997). The Phos-VanR binds to the promoter regions and forms a positive-feedback loop with increased expression of *vanR*. Phos-VanR has a long half-life and undergoes slow dephosphorylation therefore sustained high-levels of circulating Phos-VanR, produced by the alternate pathway, may contribute to the constitutive expression of the $vanA_{p.t1221}$ operon.

Characterization of the *vanH*, *vanX*, *vanY* and *vanZ* genes of *P. thiaminolyticus* RSA1221

P. thiaminolyticus RSA1221 possesses the essential *van* resistance genes *vanH* and *vanX* (Genbank accession number AY926880). Sequence analyses of these genes showed no changes relative to *E. faecium* BM4147 genes suggesting that they are functional. The signature overlap of the 3' end of *vanH* and the 5' end of *vanA*, as described by Marshall and colleagues (1998b), is present in the RSA1221 *vanA* operon.

Analysis of the $vanY_{p.t1221}$ gene and its translated protein indicated that the deduced polypeptide would terminate prematurely after amino acid 153 and as a result would lack the active sites for zinc-dependent D, D-carboxypeptidase activity. The *vanY* gene in *E. faecium* BM4147 results in the formation of the tetrapeptide PG precursors as a result of hydrolysis of terminal amino acids of cytoplasmic PG precursors. VanY contributes to glycopeptide resistance; however the contribution to high-level resistance mediated by the *vanH, vanA,* and *vanX* genes is moderate because hydrolysis of D-ala-D-ala dipeptides by VanX efficiently prevents synthesis of the pentapeptide PG precursors. Therefore, VanY is an accessory protein not required for the expression of high-level glycopeptide resistance phenotype.

No *vanZ* gene was detected in *P. thiaminolyticus* RSA1221 by PCR or Southern hybridization experiments. This may explain the reduced teicoplanin resistance observed in this isolate, since the VanZ protein contributes to low-level teicoplanin resistance.

Homology of the *van* gene cluster of *P. thiaminolyticus* RSA1221

To determine the similarity of the entire *vanA*$_{p.t1221}$ cluster (*vanR, vanS, vanH, vanA, vanX*) in *P. thiaminolyticus* RSA1221 to other *vanA* clusters, homology analysis using the NCBI BLAST tools were used. The results show that the nucleotide and predicted amino acid sequences have a high degree of identity to the *E. faecium* BM4147 *vanA* cluster associated with Tn*1546*.

The classic *orfs* associated with Tn*1546* were not detected in *P. thiaminolyticus* RSA1221. The *orf* described in *B. circulans* VR0709 was also absent. The absence of known transposon *orfs* suggests that the resistance cassette in *P. thiaminolyticus* RSA1221 is not part of a mobile genetic element or might be associated with a novel transferable element. Southern hybridization experiments show that the resistance determinant is chromosomal.

Approximately 500bp of the 5' upstream region of the *vanR*$_{p.t1221}$ gene was sequenced. The results from the sub-genomic library clone sequences showed no nucleotide homology with any sequence on the Genbank database suggesting that sequence is part of the *P. thiaminolyticus* RSA1221 chromosomal DNA, which has not been systematically sequenced.

Evolutionary origins of the *van* resistance genes found in *P. thiaminolyticus* RSA1221and enterococci

Marshall *et al*. (1998b) hypothesized that the glycopeptide-producing bacteria are potential reservoirs for *van* resistance genes (Marshall *et al*., 1998b). The low amino acid sequence identity (59-64%) and the G+C content of the *vanA* (45%), *vanB* (49%) and the *vanA* homologue (66%) in *A. orientalis* suggest that the *van* genes did not transfer directly from the glycopeptide- producing organisms to enterococci. It is possible that other microorganisms may have been the source of the *van* resistance genes found in enterococci or that they may have been intermediates between the glycopeptide-producing organisms and enterococci.

To date three *Paenibacillus* spp. (the biopesticide *P. popilliae* reported by Rippere *et al*. (1998) and two recently described environmental isolates *P. thiaminolyticus* PT-2B1 and *P. apiarius* PA-B2B reported by Guardabassi *et al*. (2004) as well as one *Bacillus* spp. (clinical isolate *B. circulans* VR0709 reported by Ligozzi *et al*. (1998)) have been documented as harbouring *van* genes, suggesting that members of the family *Bacillaceae* and *Paenibacillaceae* could have played a role in the evolution of glycopeptide resistance genes in enterococci. *P. thiaminolyticus* RSA1221 is the fourth member of the *Paenibacillaceae* family that exhibits high-level glycopeptide resistance, as a result of the presence of a *vanA* gene cluster. The similar G+C content of the *vanA*-like genes in the *Paenibacillus* species including *P. thiaminolyticus* RSA1221 and enterococcal *vanA* gene would suggest that these genes might have had a common ancestor.

Given the sequence similarity of the *van* cassettes, a series of transfer events and evolutionary changes can be postulated. The glycopeptide-producing organisms may naturally transfer their glycopeptide resistance genes to surrounding bacteria found in the environment e.g. soil. A *Paenibacillus* predecessor species in the environment may have acquired these genes and became vancomycin resistant. The genes in this species may have transferred to unknown intermediates and/or through evolution diverged into two lineages observed today, the *P. popilliae vanF* resistance cluster and the *vanA*-like resistance cluster found in *P. thiaminolyticus* and *P. apiarius*.

Studies by Patel *et al*. (2000) found that the *vanF* gene cluster in *P. popilliae* was not associated with a mobile genetic element (Patel *et al*., 2000) suggesting that this operon may be part of the *P. popilliae* genome. The glycopeptide resistant *Paenibacillus* species described by Guardabassi and colleagues (2004) were not characterized fully in terms of location and all the genes associated with the *vanA*-like gene. In the present study, the *vanA* gene cluster in *P. thiaminolyticus* RSA1221 was not found to be associated with any mobile genetic element, suggesting that this gene cluster is intrinsic to the bacterium. As a result of evolutionary events the vancomycin resistance cluster in the *P. thiaminolyticus* or ancestor could have become associated with a transposon and transferred to enterococci.

Enterococci and *Paenibacillus* species are able to survive long periods in the environment. *Paenibacillus* species produces spores that would facilitate their widespread distribution and contact with enterococci in the environment, allowing transfer of glycopeptide resistance determinants. The increased use of vancomycin in the 1970s for the treatment of staphylococcal and *Clostridium* infections and the use of glycopeptides in agriculture, could have selected for the survival of glycopeptide resistant bacteria.

5.3 Conclusion

At the outset of this study *P. thiaminolyticus* RSA1221 was the second *Paenibacillus* spp. known that exhibited high-level glycopeptide resistance; the other was the biopesticide *P. popilliae* described by Rippere *et al*. (1998). During the final stages of the study two additional *Paenibacillus* spp. were described by Guardabassi *et al*. (2004), as being glycopeptide resistant. In the present study, it was observed that *P. thiaminolyticus* RSA1221 harbours a similar *vanA* gene cassette to that found in *vanA*-carrying *E. faecium* BM4147. All the genes associated with the *vanA* resistance operon (*vanR, vanS, vanH, vanA, vanX* and *vanY*) were detected, sequenced and characterized. The absence of *vanZ* gene was noted and confirmed by Southern hybridization experiments. The $vanA_{p.t1221}$ resistance operon is chromosomally-borne and appears not to be associated with a genetic mobile element. To the best of our knowledge this is the first *vanA* gene cluster not associated with a mobile genetic cluster.

Resistance in *P. thiaminolyticus* RSA1221 is expressed constitutively. It is the second clinical isolate described that possesses a *vanA* gene cluster and shows constitutive glycopeptide resistance.

CHAPTER 6

GENERAL CONCLUSIONS

The isolation of Gram-positive bacteria from patient blood cultures is often disregarded since it is considered to be either a contaminant, because of their ubiquitous presence in the laboratory environment, or clinically irrelevant (Frankard *et al*., 2004). On the contrary, the study of these less pathogenic organisms is important as potential, emerging opportunistic pathogens of an increasing population of immunocompromised people (the elderly and HIV infected individuals), which may additionally have reduced antibiotic susceptibilities, that might delay and influence the appropriate antimicrobial therapy.

The genus *Bacillus* is a large, diverse group of microorganisms that are ubiquitous in the environment. The majority of *Bacillus* spp. appear to have little or no pathogenic potential and are rarely associated with disease in humans and animals. The exceptions to this are *B. anthracis*, the organism that causes anthrax, and *B. cereus*, which has been implicated in food poisoning, and other human and animal infections. However, various opportunistic infections caused by *Bacillus* spp. other than *B. anthracis* and *B. cereus* have been reported timeously and may highlight the clinical importance of these organisms which are thought to be "clinically insignificant" (Murray *et al*., 2003).

These seemingly low virulent *Bacillus* spp. have been shown to cause local, deep tissue and systemic infections primarily in individuals that are immunocompromised and intravenous drug users (Rowan *et al*., 2001). A case study of an immunocompetent, non

intravenous drug user presenting with recurrent bacteraemia was associated with presence of *B. licheniformis*, *B. pumilus*, and *P. polymyxa*, which was isolated from repeat blood cultures. The patient was later diagnosed with having Munchausen's syndrome and it was determined that the bacteraemia was a result of self-injection of *Bacillus* spores (Galanos *et al*., 2003). *B. licheniformis* associated infection has previously been reported in an immunocompetent individual having an invasive device (Blue *et al*., 1995), illustrating that this organism is capable of causing disease in "healthy" individuals.

In 1982, Holm had already reported a change in the microbial population of septicaemia associated pathogens. The author described how microorganisms previously thought to be irrelevant have acquired clinical significance and attributes this to changes in host-pathogen interactions as a result of invasive therapy, antibiotic prescription practices and improving microbiological detection methods (Holm, 1982).

Vancomycin is empirically used in the treatment of severe Gram-positive infections, post surgery cover for *S. aureus* and to treat *C. difficile*-associated diarrhoea. Most Gram-positive bacteria are susceptible to glycopeptides but an increasing number of non-enterococcal species are found to have acquired the *van* gene cluster first described in enterococci, both *in vitro* and under natural conditions. This is a serious public health issue.

In this project, two unusual Gram-positive bacteria were identified from clinical specimens that may have been involved in a disease process, and were found to be glycopeptide resistant.

The glycopeptide resistance determinant in *B. lentus* RSA1208 (Study A) is yet to be fully elucidated. We have confirmed here that glycopeptide resistance is not a result of the classic enterococcal *van* resistance gene and preliminary TEM results indicate that structural changes in the cell wall do occur as observed in Mu50 but it remains to be confirmed if this resistance mechanism is primarily responsible for the high-level glycopeptide resistance phenotype. An additional novel mechanism of resistance may be responsible for the reduced susceptibility of this isolate to glycopeptides. Complementary cell wall composition analysis and gene expression studies need to be conducted to fully elucidate the glycopeptide resistance mechanisms in *B. lentus* RSA1208.

Study B provides a molecular analysis of an enterococcal-like, chromosomally-borne *vanA* resistance operon in a non- enterococcal clinical bacterial isolate. The results from study B show that the glycopeptide resistance gene cluster in *P. thiaminolyticus* RSA1221 displays a high-level of homology to the *E. faecium* Tn*1546* associated *vanA* gene cluster. The arrangement of the essential genes in the RSA1221 operon (*vanH, vanA, vanX*) is similar to the gene arrangement found in the other *vanA* operons and in the glycopeptide resistance gene cluster in the glycopeptide-producing organisms *A. orientalis* and *S. toyocaensis*. The considerable amino acid similarity of the Van proteins, the orientation of the genes in the operons, and the finding that the vancomycin resistance operon in *P. thiaminolyticus* RSA1221 is not associated with a transposon associated, would suggest that the vancomycin resistance operon in *P. thiaminolyticus* RSA1221 is the precursor to the operon found in enterococci *vanA* operon.

To the best of our knowledge this is the first clinical isolate of *P. thiaminolyticus* investigated due to glycopeptide resistance. Results from this study show that *P.*

thiaminolyticus harbours a *vanA* gene cluster with a high degree of homology to the enterococcal *vanA* gene cluster. This is also the first account of the full genomic structure of the van_{pt1221} genes as well their location. The *vanA* gene cluster is chromosomally-borne and appears not to be associated with any known mobile genetic element, which has not been previously described with *vanA* gene clusters. Here we document the first time mutations in the *vanS* gene associated with a *vanA* operon that may be linked with constitutive glycopeptide resistance observed in this isolate. Additional protein functional assays of the Van resistance proteins need to be performed to assess their contribution to the resistance phenotype observed in *P. thiaminolyticus* RSA1221.

Further molecular analysis of the genes conferring glycopeptide resistance will help to elucidate the evolutionary processes of glycopeptide resistance and enhance the understanding of the dissemination and selection of resistant organisms. This will impact both the knowledge of the global epidemiology of glycopeptide-resistant organisms, and aid in the prudent prescription of antibiotics and the design of novel glycopeptide derivatives.

CHAPTER 7

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www.stripe.colorado.edu/~kocht/Home.html

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www.uic.edu/pharmacy/courses/pmpr342/itokazu/glycopeptide.html

University of Illinois

Glycopeptide

G. Itokazu

www.omedon.co.uk/ vrsa/vancomycin/

Vancomycin resistant *Staphylococcus aureus*

Tim Smith

www.aic.cuhk.edu.hk/web8/antibacterials.htm#killing_characteristics

Department of Anaesthesia and Intensive Care

The Chinese University of Hong Kong

Charles Gomerall

www.cat.cc.md.us/courses/bio141/lecguide/unit1/prostruct/cw.html

The prokaryotic cell: Bacteria

Prokaryotic cell structure

The Peptidoglycan Cell Wall

Gary E. Kaiser

CHAPTER 8

APPENDICES

Appendix I: NCBI Genbank Flat File of *P. thiaminolyticus* RSA1221 *vanA* glycopeptide resistance gene cluster

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DEFINITION Paenibacillus thiaminolyticus RSA1221 vanA resistance gene cluster,
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ACCESSION AY926880
SOURCE Paenibacillus thiaminolyticus
ORGANISM Paenibacillus thiaminolyticus
           Bacteria; Firmicutes; Bacillales; Paenibacillaceae; Paenibacillus.
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  AUTHORS Moodley,A., van Nierop,W. and Marais,E.
  TITLE Paenibacillus thiaminolyticus RSA1221 vanA resistance gene cluster
  JOURNAL Unpublished
REFERENCE 2 (bases 1 to 4654)
  AUTHORS Moodley,A., van Nierop,W. and Marais,E.
  TITLE Direct Submission
  JOURNAL Submitted (10-FEB-2005) Clinical Microbiology and Infectious
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Appendix II: Table of chemicals and reagents and their sources

All chemicals and reagents used in this study were of analytical quality.

Chemicals and reagents	**Source**
Agarose	Whithead Scientific-Brakenfell, South Africa
Ampicillin powder	Sigma-Aldrich- St. Louis, MO, USA.
Antibiotic discs	Oxoid-Basingstoke, Hampshire, UK.
Anti DIG-AP	Roche Applied Science, Indianapolis, IN, USA
Boric acid	Sigma-Aldrich
Bovine serum albumin	Amersham Biosciences- Uppsala, Sweden
Brij 58	Sigma-Aldrich
Chloroform	Sigma-Aldrich
CSPD	Roche Applied Science
Deoxyadenosine triphosphate (dATP)	Promega- Madison, WI, USA
Deoxycholic acid	USB, Cleveland, OH, USA
Deoxycytosine triphosphate (dCTP)	Promega
Deoxyribonucleotide triphosphates(dNTP)	Promega
DIG Blocking reagent	Roche Applied Science
Dimethylsulfoxide (DMSO)	Merck KGaA, Darmstadt, Germany.
Distilled water (5ml)	Sabax
Distilled water pour bottles (1L)	Sabax
DNA molecular weight marker II, DIG-labelled	Roche Applied Science
DNA 100bp molecular weight marker	Promega
DNA 1kb molecular weight marker	Promega
EDTA	Sigma-Aldrich
Ethanol (99.7-100%)	Analar- Midrand, South Africa
Glacial acetic acid	Merck
Hydrochloric acid	Merck
Isoamyl alcohol	Sigma-Aldrich
Isopropyl alcohol	Sigma-Aldrich

LB agar tablets	Sigma-Aldrich
LB broth tablets	Sigma-Aldrich
Maleic acid	Roche Applied Science
Magnesium chloride	Merck
Mineral oil	Sigma-Aldrich
PCR buffer without $MgCl_2$	Roche Applied Science
PCR grade water	Roche Applied Science
PCR Master Mix	Roche Applied Science
Phase lock gel	Eppendorf AG-Hamburg, Germany.
Phenol	Sigma-Aldrich
Phenol-chloroform-isoamyl alcohol	Sigma-Aldrich
PMSF	Roche Applied Science
Polyethylene Glycol 6000 (PEG)	Fermentas Life Science- Hanover, MD. USA
Potassium acetate	Analar- Midrand, South Africa
Pulsed field gel electrophoresis agarose	Bio-Rad Laboratories- Hercules, CA,USA
Pulsed field gel electrophoresis marker I,	Bio-Rad Laboratories
λ marker	Amersham Biosciences
Sodium acetate	Analar
Sodium chloride	Merck
Sodium citrate	SMM Chemicals, Vorna Valley, South Africa
Sodium dodecyl sulphate (SDS)	Merck
Sodium hydroxide	Merck
Sodium lauryl sarcosine	Sigma-Aldrich
Sucrose	Merck
Teicoplanin E-test	AB Biodisk, Solna, Sweden
Template suppression buffer	Applied Biosystems
Tris	Roche Applied Science
Vancomycin E-test	AB Biodisk, Solna, Sweden

Appendix III: Media (Diagnostics Media, NHLS, Rietfontein, South Africa)

1. Phosphate buffered saline (PBS)

Na_2HPO_4	10.7g
KH_2PO_4	2.72g
NaCl	8.5g
Deionized water	make up to 1L

2. 5% blood agar plate

Columbia agar (Oxoid CM0331)	39g
Horse blood (SAVP O52)	50mL
Deionized water	make up to 1L

3. Brain heart infusion broth

Brain heart infusion (Difco 0037-17)	37 g
Deionized water	make up to 1L
pH 7.4∀0.2	

4. Semi solid agar vials

Nutrient broth N2 (Oxoid CM67)	25g
Bitek agar (Difco 214530)	9g
Deionized water	make up to 1L

5. Mueller-Hinton agar plate

Mueller Hinton Agar (Oxoid CM337)	38 g
Deionized water	make up to 1L
pH 7.4±0.2	

6. Mueller- Hinton agar plate, supplemented with sheep's blood

Mueller Hinton Agar (Oxoid CM337)	38 g
Sheep blood	50mL
Deionized water	make up to 1L

7. Mueller-Hinton Broth (Oxoid)

Beef, dehydrated infusion	300.0g
Casein hydrolysate	17.5g
Starch	1.5g
Deionized water	make up to 1L
pH 7.4±0.2	

8. Cation-adjusted Mueller Hinton Broth

0.5mL calcium chloride

0.25mL magnesium chloride

100mL Mueller Hinton Broth

9. Luria Bertani agar plates

1 tablet (Sigma-Aldrich) [9.14g/L enzymatic digest of casein, 4.57g/L yeast extract, 4.57g/L sodium chloride, 13.72g/L bacteriological agar, 1.6g/L inert tableting aids]

Add 2 tablets to 100mL d. H_2O and autoclave at 121^oC for 20min.Allow molten agar to cool in a water bath at 55^oC. Add 1mL ampicillin (5mg/mL) to 100mL of LB agar and pour into sterile, plastic petri dishes. Allow plates to set at room temperature and store at 4^oC.

10. Luria Bertani broth

1 tablet (Sigma-Aldrich) [10g/L enzymatic digest of casein, 5g/L yeast extract, 5g/L sodium chloride, 2g/L inert tableting aids]

Add 2 tablets to 100mL d. H_2O and autoclave at 121^oC for 20min. Store broth at 4^oC.

Appendix IV: List of kits used in this study

Kit name	Company	Catalogue number
Big-dye terminator cycling sequencing kit	Applied Biosystems	4336917
DyeEx Big-dye terminator removal kit	Qiagen	63204
DIG Easy Hyb buffer	Roche Applied Science	1 603 558
DIG Wash and block buffer set	Roche Applied Science	1 585 762
DIG nucleic acid detection kit	Roche Applied Science	1 175 041
DIG probe synthesis kit	Roche Applied Science	1 636 090
Expand long template system	Roche Applied Science	1 681 834
High pure PCR template preparation kit	Roche Applied Science	1 796 828
QIAquick Gel extraction Kit	Qiagen	28704
QIAprep Miniprep Plasmid purification Kit	Qiagen	27104

Appendix V: NCCLS guidelines for zone diameter interpretive standards and MIC breakpoints for *Staphylococcus* spp.

Antibiotic	Disk concentration	Zone diameter to the nearest whole cm			MIC breakpoints (μg/mL)	
		R	I	S	R	S
Gentamicin (Aminoglycoside)	10μg	≤1.2	1.3 – 1.4	≥1.5	≥8	≤4
Rifampicin (Ansamycin)	5μg	≤1.6	1.7 – 1.9	≥2.0	≥4	≤1
Penicillin (β-lactam)	10U	≤2.8	--	≥2.9	B-lactamase production	≤0.1
Imipenen (Carbapenem)	10μg	≤1.3	1.4 - 1.5	≥1.6	≥16	≤4
Ceftazidime (Cephem)	30μg	≤1.4	1.5 – 1.7	≥1.8	≥32	≤8
Ofloxacin (Fluoroquinolone)	5μg	≤1.2	1.3 – 1.5	≥1.8	≥8	≤2
Clindamycin (Lincosamide)	2μg	≤1.4	1.5 – 2.0	≥2.1	≥4	≤0.5
Erythromycin (Macrolide)	15μg	≤1.3	1.4 – 2.2	≥2.3	≥8	≤0.5

R=resistant, I= intermediate resistance, S=susceptible

NCCLS guidelines (2003b)