

THE MOLECULAR BASIS OF GLYCOPEPTIDE RESISTANCE IN TWO CLINICAL  
ISOLATES: *BACILLUS LENTUS* RSA1208 AND *PAENIBACILLUS*  
*THIAMINOLYTICUS* RSA1221

Arshnee Moodley



A dissertation submitted to the Faculty of Health Sciences, University of the  
Witwatersrand, in fulfilment of the requirements for the Degree of Master of Science in  
Medicine

Johannesburg, 2005

## **DECLARATION**

I, Arshnee Moodley, declare that this dissertation is my own work. In compiling this dissertation, no form of copyright has been infringed. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at this or any University.

-----

Arshnee Moodley

B. Sc, B. Sc (Hons)

21<sup>st</sup> day of July, 2005

## **DEDICATION**

To my family and to Krzysztof Blaski, “kocham cie”.

And in special memory of my dearest grandmother.

## **PRESENTATION/S**

The following presentation/s arose from work done towards this dissertation.

### **Oral presentation**

Moodley A. “**The molecular basis of glycopeptide resistance in two clinical isolates: *Bacillus lentus* RSA1208 and *Paenibacillus thiaminolyticus* RSA1221**” presented at the Molecular and Cell Biology Group Annual Symposium, 6<sup>th</sup> October 2004, Wits Medical School, Johannesburg, South Africa

## ABSTRACT

The molecular mechanisms of glycopeptide resistance in two Gram-positive clinical isolates, *Bacillus lentus* RSA1208 and *Paenibacillus thiaminolyticus* RSA1221 were investigated. The glycopeptide resistance genotypes were determined by PCR. If *van* genes were detected, recombinant DNA techniques and sequencing were used to determine the gene sequence. The location of the resistance determinant was investigated using Southern hybridization techniques. To determine the 5' and 3' ends flanking the resistance operon, sub-genomic libraries were constructed. Transmission electron microscopy was used to assess possible structural changes of the *B. lentus* RSA1208 cell wall.

*B. lentus* RSA1208 exhibits inducible, high-level resistance to both glycopeptides, but does not possess any known *van* resistance genes. Electron micrographs showed a visible increase in cell wall thickness in *B. lentus* RSA1208 grown in vancomycin compared to the isolate grown in vancomycin-free media. However, it remains to be confirmed as to whether this resistance is solely responsible for the high-level resistance phenotype.

*P. thiaminolyticus* RSA1221 exhibits constitutive, high-level resistance to vancomycin only. It was found to possess a chromosomally-borne, *vanA* gene cassette. The *vanA* gene showed the highest amino acid identity to the *vanA*-like D-ala: D-lac gene found in *P. thiaminolyticus* PT-2B1 and *Enterococcus faecium* BM4147. All five genes of the *vanA* gene cluster (*vanR*, *vanS*, *vanH*, *vanX*, *vanY*) were amplified and sequenced. No *vanZ* gene was detected. The *vanA* operon in *P. thiaminolyticus* RSA1221 was found not to be associated with any known mobile elements. The observed constitutive expression of resistance maybe due to a two amino acid insertion in the VanSB<sub>pt1221</sub> protein.

## ACKNOWLEDGEMENTS

This research was performed in the Division of Clinical Microbiology and Infectious Diseases, School of Pathology at the University of the Witwatersrand, Johannesburg, South Africa.

I would like to thank my supervisors Dr. E Marais and Prof. A.G Duse' for facilitating this project. I would also especially like to take this opportunity to express my sincere gratitude to Dr. Marais for her endless help in the laboratory, invaluable advice and support.

I would like to acknowledge:

- Medical Research Council (MRC), National Health Laboratory Service (NHLS) and the Andrew Mellon Postgraduate Mentoring Programme for their financial assistance
- Respiratory and Meningeal Pathogens MRC Research Unit- Dr. A Smith, Dr. M du Plessis and Mr. G Coulsen for use of their automated Gene sequencer
- Transmission electron microscopy- Mrs. L van der Walt, Electron Microscope Department, Anatomical Pathology, NHLS
- The staff at Infection Control, Wits Medical School, Johannesburg, South Africa

## TABLE OF CONTENTS

Declaration.....	ii
Dedication.....	iii
Presentation/s.....	iv
Abstract.....	v
Acknowledgements.....	vi
Table of contents.....	vii
List of figures.....	xiii
List of tables.....	xv
Abbreviations.....	xvii

## CHAPTER ONE

### **LITERATURE REVIEW**

1.1 Antimicrobial chemotherapy.....	1
1.1.1 Mode of action of antibiotics.....	1
1.1.1.a Inhibition of nucleic acid synthesis.....	1
1.1.1.b Inhibition of protein synthesis.....	2
1.1.1.c Disruption of metabolic pathways .....	2
1.1.1.d Disruption of the plasma membrane .....	3
1.1.1.e Inhibition of cell wall synthesis.....	3

1.1.2 Mechanisms of antimicrobial resistance .....	8
1.1.2.a Alterations of the drug target in the bacterium.....	8
1.1.2.b Decreasing accessibility of the drug target molecule.....	8
1.1.2.c Inactivation or destruction of the drug.....	9
1.1.3 Emergence of resistance.....	9
1.2 Glycopeptide antibiotics.....	11
1.2.1 Teicoplanin.....	11
1.2.2 Vancomycin.....	12
1.3 Enterococci.....	17
1.4 Glycopeptide resistant enterococci.....	18
1.5 Vancomycin resistance.....	19
1.6 Mechanisms of glycopeptide resistance.....	23
1.6.1 vanA resistance phenotype.....	23
1.6.2 vanB resistance phenotype.....	28
1.6.3 vanC resistance phenotype.....	29
1.6.5 vanE resistance phenotype.....	31
1.6.6 vanF resistance phenotype.....	32
1.6.7 vanG resistance phenotype.....	33
1.6.8 vanB phenotype- <i>vanA</i> genotype.....	34
1.7 Glycopeptide resistance in non-enterococcal species.....	36
1.7.1 Glycopeptide resistance determinants in clinically relevant Gram-positive bacteria.....	36
1.7.2 Glycopeptide resistance determinants in other non-enterococcal clinical isolates and environmental organisms.....	37
1.8 Vancomycin resistance in <i>Staphylococcus aureus</i> .....	40



1.8.1 Vancomycin intermediate resistant <i>Staphylococcus aureus</i> .....	40
1.8.2 Vancomycin resistant <i>Staphylococcus aureus</i> .....	42
1.9 Origins of the vancomycin resistance genes.....	43

## CHAPTER TWO

### **AIMS OF THE STUDY**

Study A: Glycopeptide resistance characterization of human clinical isolate

<i>Bacillus lentus</i> RSA1208.....	47
-------------------------------------	----

Study B: Characterization of glycopeptide resistance of human clinical isolate

<i>Paenibacillus thiaminolyticus</i> RSA1221.....	49
---	----

## CHAPTER THREE

### **MATERIALS AND METHODS**

3.1 Chemical and reagents, media and kits.....	51
3.2 Strains, plasmids and growth conditions.....	51
3.3 Phenotypic characterization of <i>B. lentus</i> RSA1208 and <i>P. thiaminolyticus</i> RSA1221.....	54
3.4 Antibiotic susceptibility tests.....	56
3.4.1 Broth microdilution for determining MIC using the microtitre plate method.....	56
3.4.2 E-test for MIC determination of vancomycin and teicoplanin.....	57
3.4.3 Kirby-Bauer disc diffusion antibiotic susceptibility method.....	57
3.5 Expression of vancomycin resistance.....	58

3.6 DNA preparation.....	59
3.6.1 Plasmid DNA preparations.....	59
3.6.2 Total genomic DNA preparations.....	59
3.7 DNA amplification and electrophoresis.....	60
3.8 Nucleotide sequencing.....	67
3.9 Recombinant DNA techniques and nucleotide sequencing.....	67
3.10 DNA-DNA hybridization.....	69
3.10.1 Restriction enzyme digest and Southern blots.....	69
3.10.2 DIG-labelled probe synthesis and hybridization.....	70
3.10.3 Chemiluminescent detection of hybridized DIG-labelled probe.....	71
3.11 Contour clamp homogenous electric field gel electrophoresis and Southern hybridization to determine the location of the resistance determinant .....	73
3.11.1 Agarose plugs .....	73
3.11.2 Digestion of DNA with restriction endonucleases.....	74
3.11.3 Pulse field gel electrophoresis.....	75
3.11.4 Southern hybridization with a DIG-labelled <i>vanA</i> and 16S rRNA probe.....	75
3.11.5 Stripping membrane of DIG-labelled probed after chemiluminescent detection.....	76
3.12 Sub-genomic library to determine the 5' and 3' end upstream of the vancomycin resistance cassette.....	77
3.12.1 Pre-enrichment of the sequence of interest.....	77
3.12.2 Colony hybridization.....	77
3.12.3 Hybridization and chemiluminescent detection of hybridized DIG- labelled probe.....	78

3.13 Transmission electron microscopy.....	79
3.14 List of Genbank accession numbers used in this study.....	81
3.15 Reagent buffers, solutions and restriction endonucleases used in this study.....	82

## CHAPTER FOUR-STUDY A

### ***BACILLUS LENTUS* RSA1208**

4.1. Results.....	88
4.1.1 Phenotypic characterization of <i>B. lentus</i> RSA1208.....	89
4.1.2 Antibiotic susceptibility testing.....	91
4.1.3 Glycopeptide resistance expression.....	93
4.1.4 PCR detection of the <i>van</i> ligase gene.....	94
4.1.5 PCR detection of the D-specific $\alpha$ -ketoacid dehydrogenase.....	94
4.1.6 Cell wall morphology analysis.....	95
4.2. Discussion.....	99

## CHAPTER FIVE- STUDY B

### ***P. THIAMINOLYTICUS* RSA1221**

5.1. Results.....	102
5.1.1 Phenotypic characterization of <i>P. thiaminolyticus</i> RSA1221.....	103
5.1.2 Antibiotic susceptibility testing.....	105
5.1.3 Glycopeptide resistance expression.....	107
5.1.4 PCR detection of the <i>van</i> ligase gene.....	108

5.1.5 Location of the <i>vanA</i> resistance determinant.....	110
5.1.6 <i>vanA</i> nucleotide and sequence similarity analysis.....	111
5.1.7 Investigation of other genes found in the RSA1221 <i>vanA</i> gene cluster.....	115
5.1.8 5' and 3' of the <i>van</i> cassette.....	132
5.2. Discussion.....	133
5.3. Conclusion.....	146

## CHAPTER SIX

<b>GENERAL CONCLUSIONS.....</b>	<b>147</b>
---------------------------------	------------

## CHAPTER SEVEN

<b>REFERENCES.....</b>	<b>151</b>
------------------------	------------

## CHAPTER EIGHT

### **APPENDICES**

Appendix I: NCBI Genbank Flat File of <i>P. thiaminolyticus</i> RSA1221 <i>vanA</i> glycopeptide resistance gene cluster.....	167
Appendix II: Table of chemicals and reagents and their sources.....	171
Appendix III: Media.....	173
Appendix IV: List of kits used in this study.....	176
Appendix V: The NCCLS guidelines for zone diameter interpretive standards and MIC breakpoints for <i>Staphylococcus</i> spp.....	177

## LIST OF FIGURES

<b>Figure 1.1:</b> Gram-positive cell wall.....	4
<b>Figure 1.2:</b> Gram-negative cell wall.....	5
<b>Figure 1.3:</b> Peptidoglycan subunit of <i>Bacillus megaterium</i> .....	6
<b>Figure 1.4:</b> Cross-linking of peptidoglycan subunits .....	7
<b>Figure 1.5:</b> Chemical structure of teicoplanin.....	12
<b>Figure 1.6:</b> Chemical structure of vancomycin.....	13
<b>Figure 1.7:</b> Mechanism of action of vancomycin.....	14
<b>Figure 1.8:</b> Mechanism of resistance to vancomycin.....	20
<b>Figure 1.9:</b> Schematic representation of the glycopeptide resistance transposon Tn1546 from <i>E. faecium</i> BM4147.....	24
<b>Figure 1.10:</b> The induction of vancomycin resistance expression.....	25
<b>Figure 1.11:</b> Schematic representation of the <i>vanB</i> gene cluster.....	29
<b>Figure 1.12:</b> Schematic representation of the genetic arrangement of the <i>vanC1</i> and <i>C2/C3</i> gene clusters.....	30
<b>Figure 1.13:</b> Schematic representation of the <i>vanD</i> gene cluster.....	31
<b>Figure 1.14:</b> Schematic representation of the <i>vanE</i> gene cluster.....	31
<b>Figure 1.15:</b> Schematic representation of the <i>vanF</i> gene cluster.....	32
<b>Figure 1.16:</b> Schematic representation of the <i>vanG</i> gene cluster from <i>E. faecalis</i> WCH9 and BM4518.....	34
<b>Figure 1.17:</b> Transmission electron microscopy of Mu50 and H1.....	42
<b>Figure 3.1:</b> Schematic diagram of the downward capillary southern hybridization.....	70
<b>Figure 4.1:</b> Streak plate of <i>B. lentus</i> RSA1208.....	89
<b>Figure 4.2:</b> <i>B. lentus</i> RSA1208 vancomycin (VA) and teicoplanin (TP) E-test.....	92
<b>Figure 4.3:</b> Glycopeptide resistance expression of <i>B.lentus</i> RSA1208. ....	93

<b>Figure 4.4:</b> Transmission electron micrograph of <i>B. lentus</i> RSA1208 grown with antibiotic.....	97
<b>Figure 4.5:</b> Transmission electron micrograph of <i>B. lentus</i> RSA1208 grown without antibiotic.....	98
<b>Figure 5.1:</b> Streak plate of <i>P. thiaminolyticus</i> RSA1221.....	103
<b>Figure 5.2:</b> <i>P. thiaminolyticus</i> RSA1221 vancomycin and teicoplanin E-test.....	106
<b>Figure 5.3:</b> Glycopeptide resistance expression of <i>P. thiaminolyticus</i> RSA1221.....	107
<b>Figure 5.4:</b> PCR analysis of <i>van</i> ligase resistance gene in <i>P. thiaminolyticus</i> RSA1221.....	109
<b>Figure 5.5:</b> Phenogram of VanA proteins.....	114
<b>Figure 5.6</b> Summary of the glycopeptide resistance genes found in <i>E. faecium</i> BM4147, <i>P. thiaminolyticus</i> RSA1221 and <i>P. thiaminolyticus</i> PT2B1.....	115
<b>Figure 5.7:</b> Deduced nucleotide and amino acid sequence of the <i>vanR</i> <sub>p.t1221</sub> .....	118-119
<b>Figure 5.8:</b> Deduced nucleotide and amino acid sequence of the <i>vanS</i> <sub>p.t1221</sub> .....	123-124
<b>Figure 5.9:</b> CLUSTAL W amino acid sequence alignment of the VanS sensor protein from <i>P. thiaminolyticus</i> RSA1221, <i>E. faecium</i> BM4147 and <i>B. circulans</i> VRO709.....	125-126
<b>Figure 5.10:</b> Kyte-Doolittle hydrophobicity plot of VanS <sub>p.t1221</sub> .....	127

## LIST OF TABLES

Table 1.1: Summary of the defining characteristics of the different vancomycin resistance phenotypes.....	22
Table 1.2: Summary of <i>vanA</i> resistance genes, their respective gene products and the function of the proteins encoded by the resistance operon.....	27
Table 1.3: Nucleotide identity (%) between <i>vanHAX</i> -like genes in <i>P. thiaminolyticus</i> PT-2B1, <i>P. apiarius</i> PA-B2B and <i>vanA E. faecium</i> BM4147.....	39
Table 3.1: Bacterial strains used in this study.....	52
Table 3.2: Bacterial plasmids used in this study.....	54
Table 3.3: Biochemical characteristics of <i>B. lentus</i> and <i>P. thiaminolyticus</i> .....	55
Table 3.4: Oligonucleotide primers sequences used for the PCR detection of <i>van</i> ligase genes.....	62
Table 3.5: Oligonucleotide primers sequences used for the PCR detection of other <i>van</i> genes.....	63
Table 3.6: Degenerate primers used in the <i>B. lentus</i> RSA1208 study.....	64
Table 3.7: Oligonucleotide primers sequences used for the PCR detection of the 5'end and the open reading frames of the <i>Tn1546</i> .....	65
Table 3.8: Sequence of plasmid M13 universal primers.....	68
Table 3.9: Sequence of the 16S rRNA primers.....	76
Table 3.10: Stock solutions used in this study.....	82
Table 3.11: Buffers used in the alkaline lysis plasmid preparation method.....	84
Table 3.12: Buffers used for total genomic DNA preparation.....	84
Table 3.13: Buffers used in DNA-DNA hybridization experiments.....	85
Table 3.14: Buffers used in CHEF gel electrophoresis experiments.....	86

Table 3.15: List of enzymes used in this study.....	87
Table 4.1: Phenotypic characterization of <i>B. lentus</i> RSA1208 using conventional microbiological methods.....	90
Table 4.2: <i>B. lentus</i> RSA1208 MIC determination using two different methods: broth microdilution and E-test method.....	91
Table 4.3: <i>B. lentus</i> disk diffusion antibiotic susceptibility testing.....	92
Table 4.4: Cell wall diameter of <i>B. lentus</i> RSA1208 was grown in BHI containing 32µg/mL of vancomycin and <i>B. lentus</i> RSA1208 was grown in antibiotic- free BHI.....	96
Table 5.1: Phenotypic characterization of <i>P. thiaminolyticus</i> RSA1221.....	104
Table 5.2: <i>P. thiaminolyticus</i> RSA1221 MIC determination using two different Methods: broth microdilution and E-test method.....	105
Table 5.3: Comparison of the nucleotide and deduced amino acid sequence of <i>vanA</i> <sub>p,t1221</sub> with other documented <i>vanA</i> genes, the D-ala:D-ala ligase gene.....	113
Table 5.4: A comparison of level of identity between the nucleotide and deduced amino acid sequence of <i>vanR</i> <sub>p,t1221</sub> with other documented <i>vanR</i> genes.....	117
Table 5.5: A comparison of level of identity between the nucleotide and deduced amino acid sequence of <i>vanS</i> <sub>p,t1221</sub> with other documented <i>vanS</i> genes.....	122
Table 5.6: A comparison of level of identity between the nucleotide and deduced amino acid sequence of <i>vanH</i> <sub>p,t1221</sub> with other documented <i>vanH</i> genes.....	130
Table 5.7: A comparison of level of identity between the nucleotide and deduced amino acid sequence of <i>vanX</i> <sub>p,t1221</sub> with other documented <i>vanX</i> genes.....	131



## ABBREVIATIONS

ADP	adenosine diphosphate
AIDS	acquired immunodeficiency syndrome
Anti-DIG-AP	anti-DIG-alkaline phosphatase
Arg (R)	arginine
Asn (N)	asparagine
Asp (D)	aspartic acid
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BHI	brain heart infusion
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	Bovine Serum Albumin
C	cytosine
C-terminus	carboxy terminus
CAMHB	cation-adjusted Mueller-Hinton broth
cfu/mL	colony forming units per milliliter
CHEF	contour clamp homogenous electric field
CODEHOP	COnsensus DEgenerate Hybrid Oligonucleotide Primer
CSPD	disodium 3-(4-meth-oxyspiro {1,2-dioxetane-3, 2'-(5'-chloro) tricyclo [3.3.1.1 <sup>3,7</sup> ] decan}-4-yl) phenyl phosphate
d. H <sub>2</sub> O	distilled water
D-ala	D-alanine
D-lac	D-lactate
D-ser	D-serine
D-ala-D-ala	D-alanyl-D-alanine
D-ala-D-lac	D-alanyl-D-lactate
D-ala-D-ser	D-alanyl-D-serine
<i>ddl</i>	D:ala:D:ala ligase
DIG	digoxigenin
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
g	relative centrifugal force
G	guanine
Glu (E)	glutamic acid

Gly (G)	glycine
GRE	glycopeptide resistant enterococci
GRAF	glycopeptide-resistant <i>E. faecium</i>
H- bond	hydrogen bond
His (H)	histidine
hr	hour
HPLC	high performance liquid chromatography
IR	inverted repeat
IR <sub>L</sub>	inverted repeat left
IR <sub>R</sub>	inverted repeat right
kb	kilo base
kDa	kilo Dalton
LB	Luria Bertani
LPS	lipopolysaccharide
Lys (K)	lysine
M	molar
<i>mecA</i>	PBP2a
Met (M)	methionine
MH	Mueller-Hinton
MIC	minimum inhibitory concentration
min	minute
mRNA	messenger ribonucleic acid
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
N-terminus	amino terminus
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NCBI	National Center for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
NHLS	National Health Laboratory Service
ORF	Open reading frame
P <sub>H</sub>	<i>vanH</i> promoter region
P <sub>R</sub>	<i>vanR</i> promoter region
PABA	<i>p</i> -aminobenzoic acid
PBP	penicillin-binding protein

PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulse field gel electrophoresis
PG	peptidoglycan
Phos-VanR	phosphorylated VanR
PMSF	phenylmethylsulfonyl fluoride
Pro (P)	proline
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
<i>rrs</i>	16S rRNA gene
SDS	sodium dodecyl sulfate
sec	second
spp	species
SSC	saline sodium citrate
T	thymine
TAE	Tris-acetate EDTA buffer
TE	Tris EDTA buffer
TEM	transmission electron microscopy
TP	teicoplanin
tRNA	aminoacyl transfer RNA
U	units
USA	United States of America
UV	ultraviolet
VA	vancomycin
Van <sup>R</sup>	vancomycin resistance genes
VanA	D-ala: D-lac ligase
VanH	D-specific $\alpha$ -ketoacid dehydrogenase
VanR	Transcriptional response regulator
VanS	Membrane bound histidine sensor kinase
VanT	Serine racemase
VanX	D, D-dipeptidase
VanY	D, D-carboxypeptidase
VISA	vancomycin intermediate resistant <i>Staphylococcus aureus</i>
VRE	vancomycin resistant enterococci
VRSA	vancomycin resistant <i>Staphylococcus aureus</i>