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Mutational analysis of the rifampicin glycosyl-transferase (rgt) inactivation protein from Nocardia brasiliensis and its relationship to the vancomycin resistance of this organism

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1 Introduction

1.1 Nocardioform bacteria

The nocardioforms are a group of bacteria classified under the larger bacterial group, the actinomycetes. The actinomycetes are gram-positive bacteria containing DNA with a high GC composition. The actinomycetes are distinctive from other bacteria as they produce asexual spores and have filamentous hyphae that do not normally undergo fragmentation. Although they are true bacteria, their overall morphology superficially resembles that of fungi (Prescott *et al*, 1999). The actinomycetes bacteria are further classified according to cell wall type and other morphological features. The cell wall composition of the actinomycetes varies greatly among these different groups and thus plays a valuable role in the taxonomy (Prescott *et al*., 1999).

Nocardioforms are distinguished from other bacteria by the presence of mycolic acids in their cell walls. This results in them resisting the decolourization of the acid-alcohol mixture in the acid-fast stain procedure. These mycolic acids are also partially responsible for the inherent high level of tolerance that the nocardioforms have, to many potentially toxic chemicals since they impede the entry of such chemicals (Kaiser, 2001). Mycolic acids are high molecular weight, long chain, 3-hydroxy fatty acids with a long alkyl branch in the 2-position (Chun and Goodfellow, 1995).

The taxonomy of the nocardioforms is complex and constantly evolving (Huggins, 2003). The *Mycobacteriaceae* and *Nocardiaceae* are families currently classified under the actinomycetes. The family *Mycobacteriaceae* contains the genus *Mycobacterium*. These slow growing aerobic bacteria may be free-living saprophytes although the mycobacteria are better known as pathogens causing disease such as tuberculosis (*M. tuberculosis*) and leprosy *(M. leprae*). The family *Nocardiaceae* currently contains four genera; *Gordona, Nocardia, Rhodococcus* and *Tsukamurella* (Salinas-Carmona, 2000). Two of these genera, the *Nocardia* and *Rhodococcus,* share such similarities that they are generally termed the "nocardioforms" due to the fact that they all resemble members of the *Nocardia* (Prescott *et al*., 1999).

1.1.1 The genus *Nocardia*

The genus *Nocardia*, named after the French veterinarian, Edmond Nocard, who, in 1888, first described a bovine disease caused by an aerobic actinomycete (Salinas-Carmona, 2000), contains a group of aerobic, gram-positive, partially acid-fast, non-motile filamentous bacteria, which have DNA with a high GC content.

The *Nocardia* contain two rRNA subgroups, which are based on rRNA sequence similarities. One of these subgroups corresponds to *N. otitidiscaviarum* and the other corresponds to *N. asteroids*. The latter which consists of six species namely;

N. asteroides, N. brevicatena, N. carnea, N. brasiliensis, N. farcinica and *N. transvalensis. N. brasiliensis*. All species within this subgroup have either been implicated or classified as human pathogens (Chun and Goodfellow, 1995).

1.1.2 The species *Nocardia brasiliensis*

Nocardia brasiliensis, classified as an aerobic actinomycete, is a non-motile gram-positive bacteria. It most commonly lives as a saprophyte in soil although it has also been recovered from fresh and salt water as well as animal matter (Salinas-Carmona, 2000). Growth is indicated by salmon-coloured, shiny colonies.

1.1.3 Pathogenicity of *Nocardia brasiliensis*

Nocardia brasiliensis is the causative agent of actinomycotic mycetoma. This is a chronic disease characterized by slow-progressing tumefaction with nodules, abscessation and draining sinuses. *N. brasiliensis* enters the skin through traumatic inoculation with thorns or splinters, and thus the mycetomas resulting from this sort of infection are located primarily on the hands, feet and arms although there have been reports of other infection sites (Salinas-Carmona, 2000).

N. brasiliensis is responsible for five classes of nocardiosis, namely; pulmonary, central nervous system, systemic, extra-pulmonary as well as cutaneous,

subcutaneous and lymphocutaneous. Pulmonary infection most frequently develops after the inhalation of *N. brasiliensis* residing on air-borne dust particles. The likelihood of an infection developing is dependent on the bacterial cell number inhaled as well as the growth state thereof and the virulence of the individual nocardial cells inhaled (Jawetz *et al.,* 1982). Infection usually originates in the lungs and tends to migrate to other regions of the body such as the eyes, kidney or adrenals although it most commonly migrates to the brain or skin (Kotton, 2002). Central nervous system infection results from the spread of the infection to the cerebrum and about a quarter of pulmonary infections also involve the central nervous system. Further there is conclusive evidence of a hematogenous spread which results in, what is referred to as, a systemic nocardiosis (Beaman, 2000). Reports have also shown that four out of six patients with nocardiosis have had extensive soil exposure prior to disease development (Huggins, 2003). These patients develop mycetomas on the feet, hands and arms, although other locations have been reported. *N. brasiliensis* enters through the skin after a traumatic inoculation with thorns or splinters. The infection is characterised by slow progressing tumefaction with nodules, abscess and draining sinuses (Salinas-Carmona, 2000). *N. brasiliensis* is an opportunistic pathogen and thus cases of infection are rather rare in healthy individuals with most cases developing in individuals with a weakened or compromised immune system (Kotton, 2002). Previously, opportunistic pathogens were not a focus of medical or scientific research. However, the HIV/AIDS pandemic has changed the approach taken towards these bacterial infections. It has created an awareness of

the importance of obtaining as much information as possible about these pathogens, and their resistance to antibiotics.

There has been a rise in the number of incidences of nocardiosis. This is due to a number of factors. These include; an increase in the number of diabetic cases and leukaemia patients, along with an increase in the use of antibiotics. In addition there have been rises in the number of organ transplants and the percentage of HIV infected people. All these factors affect the immuno-competence of an individual and thus play a major role in the ability of *N. brasiliensis* to manifest disease (Kotton, 2002).

1.2 The rifamycins

The rifamycins are natural products of the actinomycete *Amycolatopsis mediterranei* (previously classified as *Nocardia mediterranei*). The biosynthesis of the rifamycins involves the synthesis of a polyketide through chain extension (Floss and Yu, 1999) and they are classified under the group of antibiotics known as the naphthalenic ansamycins (Yazawa *et al*., 1993). The ansamycins are a group of macrocyclic antibiotics, which possess an ansa-bridge (A on figure 1) connecting two separate positions of an aromatic nucleus at non-adjacent positions. The rifamycins contain a naphthalene nucleus (B on figure 1) whilst other ansamycin antibiotics contain a benzene aromatic nucleus (Lephoto, 2002).

The rifamycins first showed antimicrobial potential in 1957 when five active compounds were isolated from a fermentation broth of *A. mediterranei*. Unfortunately, of the five compounds isolated namely; rifamycin A, B, C, D and E, only one, rifamycin B, was stable and yet this compound was also the least active. Studies were continued and it soon became evident that rifamycin B spontaneously converts into an activated state, rifamycin S. This activated form was then be reduced to produce a compound, rifamycin SV, which exhibited a high level of activity against gram-positive bacteria. Unfortunately though, this compound did not display desirable activity during the clinical trials, since it showed; low levels of absorption in the gastrointestinal tract, low blood levels after oral administration and rapid excretion after parenteral administration. Thus, as part of a widespread chemical modification program, Dow-Lepetit Research Laboratories, in Italy, modified the compound chemically to reduce these shortcomings. A semi-synthetic derivative was born. This compound, a hydrozone of 3-formyl rifamycin SV with N-amino-N'-methylpiperazine, became known as rifampicin, and exhibited improved gastrointestinal absorption as well as prolonged and higher antibacterial activity (Sensi, 1983).

1.3 Rifampicin

Rifampicin (Figure 1) is a semi-synthetic derivative of the natural metabolite, rifamycin. It is an odourless, red-brown crystalline powder that is freely soluble in dimethyl sulfoxide and chloromethane, soluble in ethyl acetate, methanol and tetrahydrofuran and slightly soluble in water and acetone (Merck index, 11th Ed).

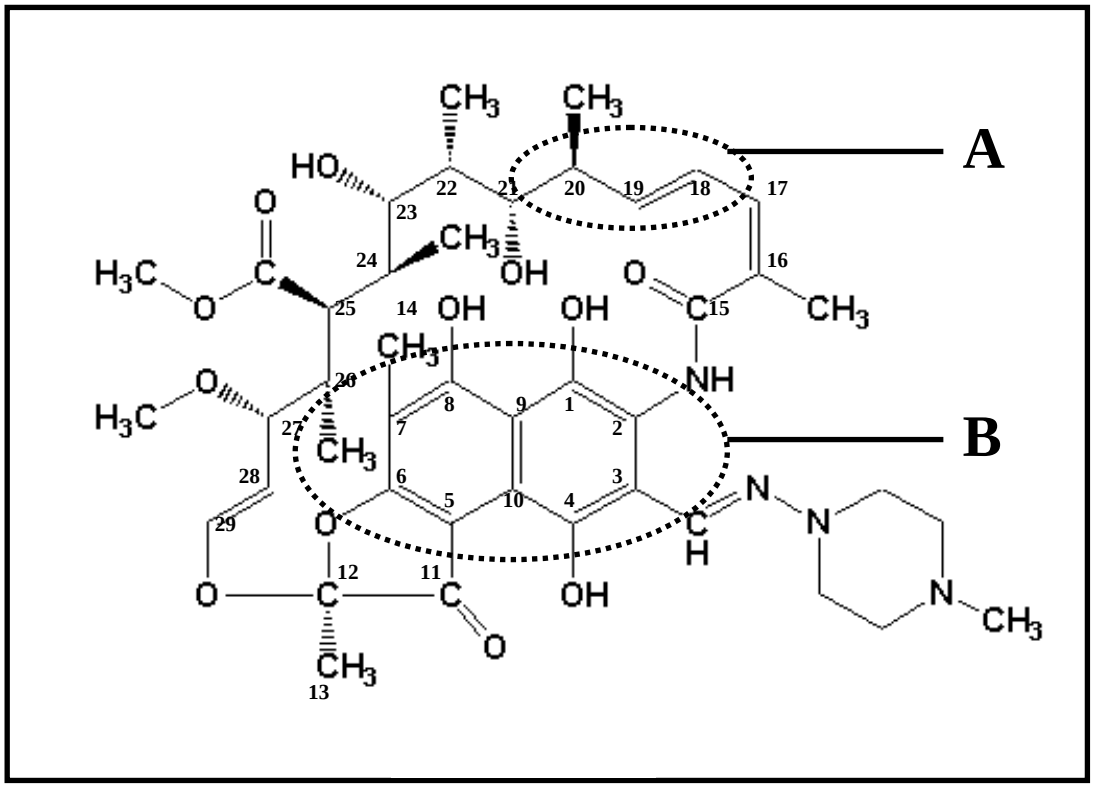


Figure 1: The structure of the semi-synthetic antibiotic rifampicin. The antibiotic is classified under the large class of antibiotics known as the ansamycins as a result of the ansa-bridge (A). The distinguishing feature of the rifamycins is the naphthalene nucleus (B).

Rifampicin is active against eukaryotes, viruses and bacteria (Pukrittayakamee *et al*., 1994). It has been shown to exhibit high level activity against mycobacteria, gram-positive and gram-negative cocci, many gram-negative bacilli, most anaerobes and cellular parasites. It exerts it bactericidal action against micro-organisms inside the leukocytes and macrophages. Furthermore, the frequency of resistant mutants developing in most bacterial species has been shown to be 10^{-8} (Sensi, 1983).

1.3.1 Mode of action

The mode of action of rifampicin and the other rifamycins is unique from all other nucleic acid synthesis inhibitors. Instead of inhibiting the synthesis of DNA, they specifically inhibit the RNA synthesis. This inhibition occurs through the inhibition of the bacterial DNA-dependent RNA polymerase (Yazawa *et al*., 1993). RNA polymerase is a multi-subunit enzyme that exists in two forms, the core enzyme and the holo enzyme. The core enzyme consists of two α-subunits, one β-subunit and a single β'-subunit and when this is joined with another protein, the σ-subunit, it is known as the holo enzyme. The r*poA* gene encodes the α-subunits, the *rpoB* gene the β-subunit, the *rpoC* gene the β'-subunit, and the *rpoD* gene the σ-subunit (Burgess *et al*., 1987). Rifampicin inhibits the activity of RNA polymerase, by binding to the β-subunit of the enzyme and thus prevents the elongation of the initiated RNA chains. This causes an accumulation of the initiation products in the cell (Heym and Cole, 1997).

It was found that the antimicrobial activity of rifampicin results from the hydroxy groups present on C-21 and C-23 as well as the two polar groups on C-1 and C-8. Therefore, any modification occurring at one of these critical sites may reduce or inhibit the activity of the compound or render it inactive (Sensi, 1983).

1.4 Mechanisms of antibiotic resistance

There are four different mechanisms whereby an organism may develop a resistance to an antibiotic. Firstly, the drug may be inactivated, prior to it reaching the target, by the organism. This is known as 'drug inactivation'. Secondly, the target site for the drug within the organism may be altered such that the drug can no longer bind or at least, has a reduced affinity for the target. This is named 'target alteration'. Thirdly, the organism may develop a new or modify an existing mechanism to bind to the drug and thereby reduce its permeability into the cell. Finally the organism may develop a resistance by creating or modifying an efflux pump to extrude the drug (Figure 2) (Putman *et al*, 2000).

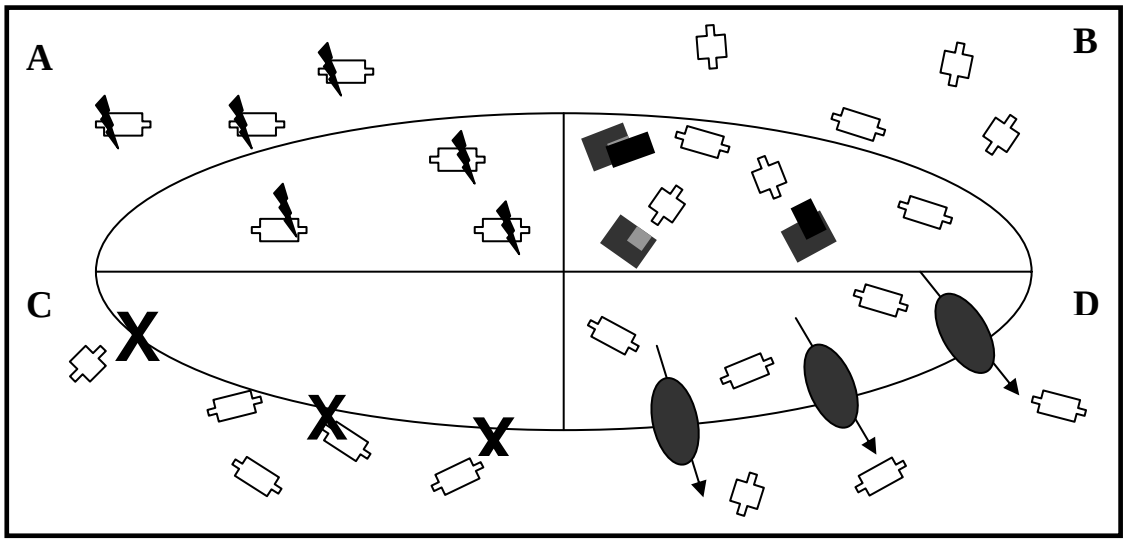


Figure 2: Mechanisms by which bacteria can develop resistance. (A) drug inactivation, (B) target alteration, (C) reduced permeability, (D) active transport of the drug out of the cell (adapted from Putman *et al.,* 2000).

1.5 Resistance to rifampicin

Nocardia and *mycobacterium* infections are treated with various antibiotics. *Mycobacteria* are highly resistant bacteria, possibly due to their highly hydrophobic cell wall (Heym and Cole, 1997) and thus infections are treated with a combination of several different antibiotics. Rifampicin has had a major impact on the efficacy and duration of this treatment regime (Cole, 1995). However, due to the widespread use of rifampicin to combat these infections rifampicin resistant strains have developed (Williams *et al*., 1994).

1.5.1 Target modifications

The classical form of endogenous resistance to rifampicin is by mutation to the target site. Extensive studies of the development of resistance by *M. tuberculosis* to rifampicin revealed that in most cases it develops as a result of mutations occurring in a region of the *rpoB* gene, thereby modifying the β-subunit of the RNA polymerase such that it is no longer recognisable by the antibiotic. Nevertheless, the enzyme does maintain its binding and functional properties (Heym and Cole, 1997). Several studies conducted on rifampicin resistant clinical isolates and multi-drug resistant isolates have shown that these mutations are missense mutations and occur in a 69 bp conserved region encoding 23 amino acids (Musser, 1995).

1.5.2 Reduced permeability

Resistance to rifampicin, through the prevention of drug influx by modifications affecting the permeability of the cell wall has been observed in *Neisseria meningitis* (Abadi *et al*., 1996). Two rifampicin resistant strains were identified and both were shown to harbour the same point mutation in the *rpoB* gene, however it was only after experiments were conducted in the presence of Tween 80, a substance which alters the permeability of the cellular membrane, that only one of these strains was found to be resistant as a result of prevention of drug influx. In the case of the other identified strain, it was concluded that another mechanism must also be involved (Hui *et al*., 1977).

1.5.3 Active efflux

Studies conducted on *Haemophilus influenzae* revealed that resistance to rifampicin arose as a result of efflux pumps actively transporting the drug out of the cell. A three-gene complex, homologous to the *arcRAB* cluster in *Escherichia coli*, was found to encode the proteins involved in the efflux system. These efflux pumps also played a major role in the extrusion of toxic dyes and chemicals from the cell (Sánchez *et al*., 1997).

1.5.4 Modification enzymes

More recently it has become evident that resistance to rifampicin developed in yet another way. Nocardioforms, and other closely related species, are shown to become resistant to the antibiotic through inactivation of the drug itself (Andersen *et al*., 1996). Since first suggested (Dabbs, 1987), four inactivation mechanisms have been identified. These mechanisms appear to be specie-specific among the mycolic acid-containing bacteria (Andersen *et al*., 1996; Tanaka *et al*., 1996) and are indicated on the table below.

Table 1: The four different enzymatic mechanisms which inactivate rifampicin. ADP-ribosylation, phosphorylation, decomposition and glucosylation.

Mechanism of inactivation	**Gene responsible**	**Organism in which mechanism occurs**
ADP-ribosylation	ADP-ribosyl transferase *(iri)*	*Mycobacteria, Gordona, Tsukamurella*
Phosphorylation	Yet unknown	*Bacillus*
Decomposition	Monooxygenase *(adr)*	*Nocardia, Rhodococcus, Mycobacteria, Bacillus*
Glucosylation	Glycosyltransferase *(rgt)*	*Nocardia*

1.5.4.1 ADP-ribosylation

Rifampicin resistance can develop as a result of the transfer of an ADP-ribose group to the C-23 of rifampicin. This mechanism of inactivation was first identified in 1995 when the inactivated compounds were isolated during studies conducted on fast-growing strains of *mycobacteria.* After chromatographic and spectrometric analysis these compounds were identified as the ribosylated and inactivated forms of rifampicin. These structures were granted the names RIP-Ma and RIP-Mb and the structures were shown to be those of 3-formyl-23-(*O*-(α-$_D$-ribofuranosyl))rifampin and 23-(*O*-(α-$_D$-ribofuranosyl))rifampin respectively (Dabbs *et al*., 1995). The DNA responsible for the inactivation of rifampicin by the enzyme, ADP-ribosyltransferase, was cloned and characterised (Quan, 1997). Functional studies were further conducted through the production and characterisation of mutants. This study produced only one mutant, which had a modification in the open reading frame. This sequence mutation resulted in a single amino acid change that affected the morphology of the *E. coli* cell harbouring the plasmid (Puhača, 2003).

1.5.4.2 Phosphorylation

Studies conducted on the inactivation of rifampicin by *Nocardia otitidiscaviarum* revealed that two products are produced. These compounds were designated RIP-3 and RIP-4 and after analysis were identified as 21-(O-phosphoryl)rifampicin

and 3- formyl-21-(O-phosphoryl)rifamycin SV (Yazawa *et al*., 1994). Rifampicin is thus also inactivated through the transfer of a phosphate group to the hydroxy group of C-21 (Morisaki *et al*., 2000). The DNA responsible for this inactivation mechanism is yet to be cloned and characterized.

1.5.4.3 Decomposition

The decomposition of rifampicin results from the transfer of a single oxygen atom to the naphthalene moiety of the drug causing it to literally break apart (Anderson, 1996). This rifampicin decomposition gene was cloned and shown to yield a high sequence similarity to the genes encoding the monoxygenases (hydroxylases), which act on phenolic compounds and may be involved in the biosynthesis of polyketides (Andersen *et al*., 1996).

1.5.4.4 Glucosylation

The transfer of a single glucose moiety to C-23 of rifampicin has been shown to render the drug inactive (Yazawa et al., 1993). Glucosylation is simply defined as the transfer of a glucose moiety from a nucleoside diphosphate glucose to an acceptor molecule. The enzymes that catalyse these transfers are called glycosyl-transferases (Cundliffe, 1992). Detailed studies of *Nocardia* spp. revealed that many *Nocardia* spp. were inherently resistant to rifampicin and this was reported to be as a result of an inactivation enzyme mechanism that was present in these

bacteria (Yazawa *et al*., 1993). The chromatographic analysis of the inactive forms of rifampicin in *N. brasiliensis* revealed two compounds, RIP-1 and RIP-2. These compounds were identified using NMR Spectroscopy to be 3-formyl-23-(*O*-(β-$_D$-glucopyranosyl))rifamycin SV and 23-(*O*-(β-$_D$-glucopyranosyl))rifamycin respectively, the glucosylation products of rifampicin. The downfield shifts of the H-23 and C-23 signals indicated that a $_D$-glucose moeity was transferred to the hydroxy group of C-23 (Yazawa *et al*., 1993), resulting in the inactivated states of the antibiotic.

In 2002, the DNA responsible for glucosylation in *Nocardia brasiliensis* IFM 0236 was cloned and characterised. It was found to exist in a *Pst* I fragment 1.8 kb in size. This fragment contains two putative converging ORF's. One of these ORF's is predicted to code for a 406 amino acid protein, which has been shown to be capable of utilizing both UDP-glucose and UDP-galactose as a cofactor and thus the enzyme is referred to as a glycosyl-transferase as opposed to a glucosyl-transferase (Lephoto, 2002).

1.6 The glycopeptides

Glycopeptide antimicrobial agents are distinguished by a peptide core consisting of seven amino acids, of which five are aromatic. These molecules also contain a triphenyl-ether molecule and a diphenyl group (Heydorn *et al*, 2000). This basic structure is referred to as an aglycone and one or more sugars are linked to the

aglycone at different positions via glycosidic bonds. Glycopeptide antibiotics are natural products synthesized by various species of soil-dwelling actinomycetes and are large, complex structures (Fischetti *et al*, 2000). As of yet, over 200 different glycopeptides have been isolated. Only vancomycin and teicoplanin have however, successfully been developed and approved of for human use.

Glycopeptides act on prokaryotic cells and inhibit late cell wall synthesis. They are thus only effective against gram-positive bacteria. Due to the large, rigid and hydrophobic structure of glycopeptides they cannot penetrate bacterial cell membranes nor exert their activity by binding directly to the cell wall; instead they complex with carboxy-terminal alanine residues on peptidoglycan precursors (Fischetti *et al*, 2000).

1.7 Vancomycin

Vancomycin (Figure 3) is a valuable glycopeptide that occupies a niche in the treatment of severe, life-threatening infections caused by gram-positive pathogens, especially *Staphylococcus aureus* and *Enterococcus faecalis*. It is commonly referred to as "the drug of last resort" since it used in cases where multi-drug resistance has developed in a pathogenic organism and all other treatments are failing as a result of the organism not being susceptible to other antibiotics (Brickner, 1997).

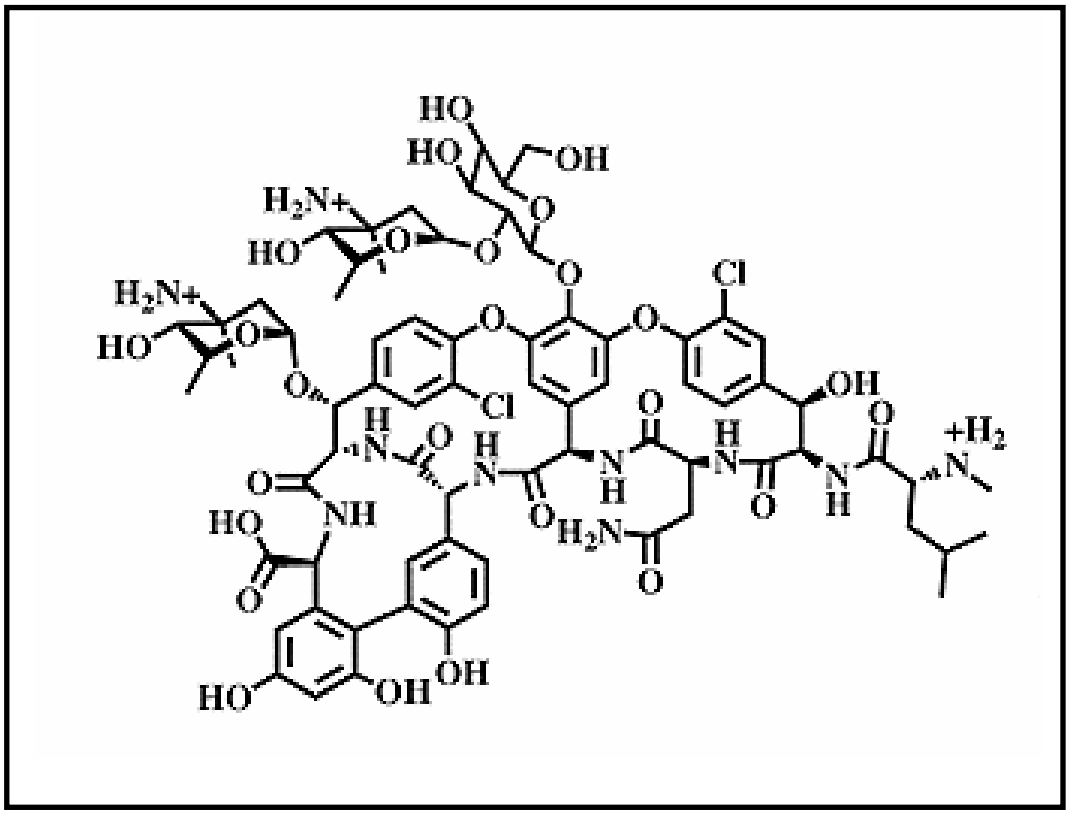


Figure 3: The structure of the glycopeptide antibiotic vancomycin. The antibiotic binds to the $_{D}$-ala-$_{D}$-ala terminal dipeptide preventing the completion of the peptidoglycan biosynthesis and thereby compromising the development of the bacterial cell wall.

Vancomycin was discovered in 1956 (Thomas *et al*. 1987). It is a natural product of *Amycolatopsis orientalis* and contains a deoxysugar moiety which is essential for its biological activity. It is a broad spectrum antibiotic and exists as a white powder which is soluble in water, moderately soluble in dilute methanol and insoluble in higher alcohols, acetone and ether (Merck Index, 11th Ed.).

1.7.1 Mode of action

Vancomycin exerts it antimicrobial activity by inhibiting the peptidoglycan biosynthesis in gram-positive bacteria. There are two main steps involved in the synthesis of the gram-positve bacterial cell wall: transglycosylation and transpeptidation (Walsh, 1999). Compromising either of these steps leads to the formation of walls with weakened structural integrity, leaving the cell vulnerable to lysis in a hypo-osmotic environment (Walsh, 1999; Roper *et al*., 2000). Peptidoglycan biosynthesis involves the binding of five amino acid residues to the carboxy group of a muramic acid. The first amino acid to attach is $_{L}$-alanine and the second and third, respectively, are $_{D}$-glutamic acid and $_{L}$-lysine, finally a dipeptide, $_{D}$-alanyl-$_{D}$-alanine, binds and completes the biosynthesis of the pentapeptide. Vancomycin affects biosynthesis of the peptidoglycan by binding specifically to the $_{D}$-alanine-$_{D}$-alanine group on the peptide side chain of one of the membrane-bound intermediates. This prohibits the formation of peptide cross-linkages between adjacent strands, the transpeptidation step. Thus, a weakened cell wall is synthesized (Walsh, 1999; Roper *et al*., 2000).

1.8 Resistance to vancomycin

Resistance to vancomycin can be categorized into the four general mechanisms as previously described for rifampicin. However, the acquisition of external genetic elements, plasmids and transposons, carrying resistance genes is central to

bacterial resistance to vancomycin. In addition to this, the most widely known mechanism of resistance to vancomycin is modification to the target site. This modification does not however, result from mutations to the target, but rather from modification enzymes that are coded for by a transposon. Resistance of *Enterococcus* spp. to vancomycin was found to be due to a transposon, Tn*1546* (Uttley et al., 1988). A gene cluster from this was cloned and shown to be sufficient for the induction of glycopeptide resistance in *E. faecium* BM4147. This resistance determinant consisted of a five-gene cluster: *vanR, vanS, vanH, vanA* and *vanX*. *VanR* and *vanS* encoded for a regulatory system, VanR-VanS, which initiates the transcription of the other genes in response to vancomycin in the medium (Leclercq *et al*., 1998). The *vanA* gene encodes a $_D$-alanine:$_D$-alanine ligase with altered specificity, catalyzing the addition of $_D$-hydroxy acids as well as $_D$-amino acids to the $_D$-alanine (Bugg *et al*., 1991). In the presence of this gene cluster vancomycin resistant *Enterococcus* manufacture altered peptidoglycan precursors, which terminate in $_D$-lactate instead of $_D$-alanine (Billot-Klein *et al*., 1992; Handwerger *et al*., 1992). Since the C-terminal $_D$-alanine is required for the formation of a stable complex with vancomycin (Nieto and Perkins, 1971) the negation of the use of $_D$-alanine terminating precursors by the action of these genes ensures the production of $_D$-alanyl-$_D$-lactate terminating peptidoglycan chains which are bound very weakly to the drug, thus rendering the bacteria resistant (Roper *et al*., 2000).

1.9 Relationship between the *rgt* gene and vancomycin resistance

It has been shown that the glycosyl-transferase gene sequence confers a small, yet reproducible resistance to vancomycin (Lephoto, 2002). There were three hypothesis established to explain this.

1. The enzyme may remove a critical glucose moiety from the glycopeptide and thus render it inactive.
2. Rifampicin glycosyl-transferase may add a glucose motif to the vancomycin molecule thereby reducing its effectivity.
3. There exists the possibility that the enzyme has no effect on the antibiotic, but instead alters the target in such a way that the organism becomes less susceptible to vancomycin and thus develops a low level of resistance. This hypothesis is the most likely and is supported by the knowledge that glycosyl-transferases are involved in peptidoglycan biosynthesis (Lephoto, 2002).

The organism responsible for the biosynthesis of rifamycins, *Amycolatopsis mediterranei*, is closely related to and shares many biochemical and physiological similarities with, the *Nocardia* spp. This relationship partially explains the reason why so many of the nocardioforms have an inherent ability to inactivate rifampicin enzymatically since, often antibiotic resistance genes originated from antibiotic producing organisms (Yazawa *et al*., 1993). Furthermore, the glycosyl-transferase gene has been shown to have a large sequence similarity to those genes

encoding the enzymes involved in the biosynthesis of other antibiotics (Lephoto, 2002). One of the genes contained in the *Pst* I fragment encodes a protein, having a high percentage of sequence similarity to the balhimycin glycosyl-transferase from *A. mediterranei* DSM 5908 and the glycosyl-transferase in *A. orientalis* C329.4, that biosynthesises vancomycin (Lephoto, 2002). This indicates that these 'inactivation' genes may be responsible for the biosynthesis of a yet unidentified antibiotic by *N. brasisliensis.*

Thus, a complete screening of the genomic DNA of *N. brasiliensis* may result in the identification of other DNA regions involved in the resistance of this organism to vancomycin and possibly the relationship between the rifampicin glycosyl-transferase inactivation gene and vancomycin resistance. In addition to this a mutational analysis of the rifampicin glycosyl-transferase inactivation gene may allow for further characterisation of the inactivation mechanisms involved in rifampicin resistance.

1.10 Aims of this project

The HIV/AIDS pandemic has created a necessity to understand, characterise and combat secondary infections caused by opportunistic pathogens. *Nocardia brasiliensis*, one such pathogen, has an inherently high level resistance to both rifampicin and vancomycin (Lephoto, 2002). The gene responsible for the inactivation of rifampicin has previously been cloned and characterised whilst

little is known regarding the DNA involved in the development of vancomycin resistance. The *rgt* gene encodes a rifampicin inactivating enzyme and has been shown to not only inactivate rifampicin but also to confer a small, yet reproducible increase to the MIC of vancomycin. Thus, there exists a hypothesis that this *rgt* gene is involved somewhat in the resistance to vancomycin.

The aims of this study were two-fold:

A: To further understand the rifampicin glycosyl-transferase inactivation mechanism through the development and characterisation of spontaneously arising mutants.

B: To screen the genome of *N. brasiliensis* to identify other regions of DNA that play a role in the development of resistance to vancomycin

2 Materials and Methods

2.1 Bacterial strains

The bacterial strains and plasmids used in this work are listed in Table 2 and Table 3 respectively.

Table 2: Bacterial strains used in this work.

Strain	Characteristics	Source
Escherichia coli MM294-4	*end*A1, *hsd*R17, *gyr*A	E. Dabbs
Rhodococcus rhodochrous Ri8R	Highly transformable, rifampicin resistant	E. Dabbs

2.2 Media and growth conditions

Bacterial strains were stored in 33 % glycerol solutions at -70° C until required. They were recovered by streaking onto Luria Agar (LA) plates and could be kept at 4° C for short-term storage. Cultures for *Escherichia coli* were grown overnight in Luria-Bertani (LB) medium at 37° C. *Rhodococcus rhodochrous* cultures were grown in LB, supplemented with iso-osmotic sucrose concentrations and glycine, at 30° C for two days. Both *E. coli* and *R. rhodochrous* strains were kept in liquid culture on a wheel at room temperature for short-term storage.

2.3 Determination of the minimal inhibitory concentration

The minimal inhibitory concentration (MIC) of all relevant antibiotics for *E. coli* and *R. rhodochrous* was determined by the agar dilution method. Freshly grown colonies were individually inoculated into 200 µl of sdH_2O and this was replica plated onto LA plates containing appropriate antibiotic dilutions. Once the samples had been absorbed into the medium, plates were incubated at 30° C for 72 hrs for *Rhodococcus* strains and 37° C for 24 hrs for *E. coli* strains. Results were analyzed after incubation, and the antibiotic concentration at which there was no confluent growth was taken as the MIC.

2.4 DNA Preparations

2.4.1 *E. coli* bulk plasmid preparation

A single colony was used as the inoculum in 100 ml of LB medium, supplemented with the appropriate selective agent, ampicillin in the case of pDA71 and pACYC177 transformants but chloramphenicol for pACYC184 transformants. The culture was grown up overnight, at 37° C with high aeration. After which, the cells were pelleted through centrifugation at 25º C for 10 min in a JA-10 Beckman rotor at 6 000 rpm. The supernatant was discarded, and the pellet re-suspended in 5 ml room temperature solution I (Appendix B). 11 ml of room temperature solution II (Appendix B) was added and the tube gently mixed through inversion. The tube was left to stand at 37° C for 30 min. After which, 8

ml of chilled solution III (Appendix B) was added and the tube shaken vigorously for 20 sec. Immediately, the tube was placed in an ice-slurry for 10 min. Cellular debris was removed through centrifugation in a pre-chilled Beckman JA-20 rotor at 15 000 rpm for 10 min. The supernatant was transferred to a fresh tube and warmed to 37° C in a water bath, the pellet discarded. 12 ml of isopropanol was added to the tube which was then mixed gently though inversion and left to stand at room temperature for 5 min.

DNA was precipitated by centrifugation in a JA-20 Beckman rotor at 15 000 rpm for 10 min. The supernatant was discarded and the pellet washed carefully with 2 ml ethanol and then briefly centrifuged in a JA-20 Beckman rotor at room temperature. The DNA pellet was dried in a Savant Speed Vac Concentrator for 20 min, and then re-suspended, with gentle agitation, in 4 ml TE buffer for a minimum of 2 hrs.

Thereafter, 400 µl of a 1 % EtBr and 4.1 g CsCl were added and the refractive index adjusted to a value between 1.388 and 1.389. The solution was then loaded into a Beckman Quick-seal tube and centrifuged for 16 hours at 45 000 rpm in a Beckman vertical VTi 65.2 rotor. The lower DNA band was carefully removed from the CsCl gradient using a hypodermic syringe and transferred to an Eppendorf tube. The EtBr was removed and the DNA purified by means of a butanol extraction and a DNA precipitation as described in 2.5.1.

2.4.2 *E. coli* mini plasmid preparation

Single transformant colonies were used to individually inoculate 1 ml LB supplemented with an appropriate antibiotic, usually ampicillin at a concentration of 100 μg/ml. These mini-cultures were incubated at 37° C with high aeration overnight. Cells were harvested through micro-centrifugation at room temperature for 30 sec. The supernatant was discarded and the pellet re-suspended in 80 μl of ice cold solution I (Appendix B). 160 μl of solution II (Appendix B) was added and the preparation was left standing at room temperature for 15 min. 120 μl of chilled Solution III (Appendix B) was added and the solutions mixed thoroughly by vigorous shaking. Immediately, preparations were placed in an ice-slurry for 5 min. Samples were micro-centrifuged at 4° C for 10 min, the supernatant transferred to a new eppendorf tube and the pellet discarded. The supernatant was then warmed in a 42° C water bath after which, 220 μl of isopropanol was added and the preparations mixed through gentle inversions. Thereafter, tubes were left to stand for 5 min at room temperature to allow precipitation to proceed. The supernatant was discarded and the DNA pellet washed in 150 μl of 96 % ethanol. Tubes were then micro-centrifuged at room temperature for 1 min. The ethanol was discarded and the pellet dried in a Savant Speed Vac Concentrator for 20 min. The isolated DNA was re-suspended in 150 μl of sdH_2O or 150 μl 4 mM Tris.HCl (pH 8.0) containing freshly boiled RNase (10 μg/ml). A small aliquot of the DNA was then analyzed by electrophoresis on a 0.8 % agarose gel.

Table 3: Plasmids used in this work.

Plasmid	Relevant characteristics	Source
pDA71	*E. coli-Rhodococcus* shuttle vector 8800 bp; Amp^R, Cm^R Functional suicide gene	S. Quan and E. Dabbs (1993)
pDA71*	Functional suicide gene removed from pDA71	E. Dabbs
pACYC177	Amp^R, $Cm^{R;}$; 3940 bp	E. Dabbs
pACYC184	Cm^R, Tc^R ; 4245 bp	E. Dabbs
pCL1	Rif^R clone isolated from *N. brasiliensis* IFM 0236 *Pst* I library	C. Lephoto (2002)
pCL2	Reverse orientation of insert in pCL1	C. Lephoto (2002)

2.5 DNA Manipulations

DNA extracts prepared as described previously were subjected to several manipulation protocols: phenol-chloroform extraction, precipitation, preparation from CsCl gradients, restriction enzyme digestions, ligations, alkaline phosphatase treatment and agarose gel electrophoresis.

2.5.1 DNA extraction from CsCl gradient

EtBr was removed from DNA solutions by thorough mixing with 0.1 vol of butanol. The upper layer was removed and discarded and the procedure repeated until the EtBr was completely removed and DNA in CsCl solution remained. The DNA was obtained by addition of 2 vol of sdH_2O and 3 vol of ethanol. The sample was micro-centrifuged at 4° C for 20 min, and the DNA pellet dried in a Savant Speed Vac Concentrator for 20 min. Thereafter the DNA was re-suspended in sdH_2O or 4 mM Tris.HCl.

2.5.2 Restriction enzyme digestions

Restriction enzyme digestion is a one of molecular biology's greatest tools. The specificity of these enzymes enables template DNA to be cut at distinct sites. Many factors affect the performance of restriction enzymes, including template purity, reaction buffer components, temperature, time and the unique kinetics of each enzyme (Williams et al., 1996). These bacterial endonucleases recognize specific DNA sequences, four to eight base pairs in length, and cleave both strands of the DNA generating either blunt ends or sticky overhangs. All enzymes utilized in this research were obtained from Boehringer Mannheim, New England Biolabs or Amersham and used according to the instructions given by the manufacturer. The total volume of digestions was 20 µl (18 µl DNA + 2 µl 10X buffer). The volume of enzyme used in each digestion was 0.4 µl and the reaction

mixtures were incubated at the optimal temperature for the particular enzyme for a time period of between 4–16 hrs.

2.5.3 Phenol-chloroform extraction

DNA was extracted and all enzymes removed from aqueous solution through the addition of phenol and chloroform. Phenol is an organic compound which removes proteins and other substances from solution. However, the presence of even trace amounts of phenol in the DNA solution may interfere with other manipulation techniques and thus it is vital to completely remove the phenol. This is done through the addition of the organic solvent chloroform.

Small volumes of aqueous DNA, to be subjected to a phenol-chloroform extraction were adjusted to 300 µl with TE buffer. 100 µl of TE-saturated phenol was then added and the solution mixed through inversion and micro-centrifuged at room temperature for 5 min. The upper aqueous layer was transferred to a sterile Eppendorf tube containing 133 µl of chloroform. The solution was once again mixed through inversion and then micro-centrifuged for 30 sec. The upper aqueous layer was transferred to a sterile Eppendorf tube and the DNA precipitated with salt and ethanol.

2.5.4 DNA precipitation with salt and ethanol

DNA was precipitated out of aqueous solution using 0.1 vol of 1 M NaCl and 2 vol of 96 % EtOH. The mixture was micro-centrifuged at 4° C for 20 min. The supernatant was discarded and the DNA pellet dried completely in a Savant Speed Vac Concentrator for 20 min. The DNA was re-suspended in the appropriate vol of sdH_2O or 4 mM Tris.HCl.

2.5.5 Alkaline phosphatase treatment

Following digestion by restriction endonucleases, vectors were treated with alkaline phosphatase in order to prevent the vector from re-ligating on itself. Alkaline phosphatase removes the 5'-phosphates that are necessary for ligation by DNA ligase and although the vector can no longer re-ligate onto itself, it is capable of ligating to the insert DNA that retains the 5'-phosphates (Weaver, 1999).

1 µl of calf intestinal alkaline phosphatase, from Boehringer Mannheim, was added to 20 µl of reaction mixtures following restriction enzyme digestions. This was then incubated at 37° C for 1 hr and immediately after incubation all enzymes were extracted with phenol.

2.5.6 DNA ligations

Digested DNA fragments can be joined, by ligation, to other fragments provided that either compatible sticky ends or blunt ends are created. DNA ligases are enzymes which catalyze the formation of phosphodiester bonds by joining the 3'-hydroxyl and the 5'-phosphate ends of dsDNA. Bacteriophage T4 DNA ligase was used in all ligations performed in this study. It utilizes energy from ATP hydrolysis and is capable of successfully ligating both sticky and blunt ends.

T4 DNA ligase, from Boehringer Mannheim, was used to join DNA fragments. The total vol for ligations was kept minimal at 20 µl and a constant vol of 2 µl of 10 X ligation buffer was used. Ligation buffer and the appropriate vol of sdH_2O were added to the DNA sample, mixed by tapping and then micro-centrifuged briefly for 1 sec. Following this, 1 µl of ligase was added, re-mixed and then micro-centrifuged. Ligations were performed at 14° C for 22 hrs.

2.5.7 Klenow enzyme treatment

In order to ligate DNA with non-compatible sticky ends it is necessary to first create blunts ends by 'filling in' the overhang. The Klenow fragment of *E. coli* DNA polymerase I, large fragment, was used to fill in the overhangs and create blunt ends. 2 µl of hexanucleotide mix, 2 µl of dNTPs and 1 µl of Klenow enzyme were added to 20 µl reaction mixtures. This was mixed by tapping and then briefly micro-centrifuged. It was then incubated at 37° C for 1 hr, after which the

enzymes and other contaminants were removed with phenol and the DNA transferred in the aqueous phase to a clean Eppendorf tube. The DNA was precipitated with NaCl and EtOH and then re-suspended in 18 µl of sdH_2O and re-ligated. All the components used were supplied by Boehringer Mannheim.

2.6 Transformations

Transformation is the uptake of free DNA by a bacterial cell of another genotype and is incorporated into the recipient's cell genome. It is only genetically noticeable if it leads to the modification of at least one of the characteristics of the recipient cell. Various methods exist to produce cells which are "competent" and thus capable of DNA-uptake, for both gram-positive and gram-negative organisms. However it has been shown that recombination of the DNA strand is somewhat slower in gram-positive organisms (Dubnau, 1999).

2.6.1 $CaCl_2$-mediated transformation of *E. coli*

E. coli MM294-4 pre-cultures were grown up in 5 ml LB at 37 ° C with high aeration. The culture was grown using a 0.01 vol of the pre-culture as the inoculum, in LB containing 0.5 % glucose. The culture was grown up until an OD_{590} of 0.2–0.4 had been reached. Growth was then stopped by placing the culture immediately onto an ice-slurry and swirling the flask for at least 5 min. The cells were then centrifuged in a pre-chilled JA-20 Beckman rotor at 10 000 rpm for 5 min at 4 ° C. The supernatant was discarded and the pellet re-suspended

in half the original vol of ice-cold transformation buffer (Appendix B) and then left on ice for at least 15 min. Thereafter, it was centrifuged in a pre-chilled JA-20 Beckman rotor at 10 000 rpm for 5 min. The supernatant was once again discarded and the pellet carefully re-suspended in one fifteenth of the original vol of ice-cold transformation buffer, and then left on ice for a time period of between 2 and 24 hours, since during this time the cells are competent for transformation.

100 µl aliquots of the competent cells were placed in Eppendorf tubes and 4 µl of chilled DNA added and mixed gently by tapping. The DNA-cell mixtures were left on ice for at least 10 min to allow diffusion of DNA. The cells were then heat shocked by placing them in a 44° C water bath for 90 sec. The cell-DNA mixture was then incubated at 37° C for 60 min following the addition of 500 µl of pre-warmed LB to each tube. This time period allowed for phenotypic expression of the selective genes. The transformed cells were then carefully spread onto LA plates containing the appropriate selective agent and incubated at 37° C overnight.

2.6.2 PEG-mediated transformation of *R. rhodochrous*

R. rhodochrous Ri8R was grown up for two days in 5 ml LBSG (2 % glycine) at room temperature with gentle agitation. 1.2 ml of this culture (50–100 µl per transformation) was micro-centrifuged for 30 sec. The pellet was washed in 1 ml of basal buffer (Appendix B). The suspension was micro-centrifuged for 30 sec, and the supernatant discarded. 5 µg lysozyme was added, using a sterile toothpick, to the pellet, which was then re-suspended in 1 ml of basal buffer. The cell

suspension was incubated in a 37° C water bath for 60 min, and the suspension mixed by inversion every 10 min.

Whilst the sample was incubating, protoplast buffer (Appendix B) was freshly prepared and used to prepare a PEG solution. 0.5 g PEG, which had prior to this been sterilized under UV light for 5 min, was dissolved in 1 ml of protoplast buffer by vigorous vortexing. After incubation the resultant protoplast suspension was micro-centrifuged for 10 sec, washed in basal buffer and then re-suspended in 500 μl of protoplast buffer. Aliquots (100-200 μl) of the protoplast suspension were transferred to chilled Eppendorf tubes, which were placed on ice for 10 min. An appropriate vol of plasmid DNA was added to each tube and the contents carefully mixed by bubbling air through. Tubes were then left to stand at room temperature for 10 min. Following this, an equal vol of PEG solution was added to each tube and the two phases were mixed carefully by once again gently blowing bubbles through. The contents of the tube were spread carefully onto chilled regeneration plates. These plates were incubated at 30° C for 10 hrs to allow effective phenotypic expression of the appropriate genes, after which chloramphenicol, to a final concentration of 40 μg/ml, was used to underlay each plate. Plates were then incubated at 30° C until colonies were evident. Colonies were usually visible after three to four days.

2.6.3 Electroporation of *R. rhodochrous*

Single Ri8R colonies were used to inoculate 10 ml LBSG (2 % glycine), supplemented with 50 μg/ml rifampicin, and cultured at 30° C with vigorous aeration until the OD_{600} reached 0.2-0.4. 1 ml of cells was then transferred into each of a series of Eppendorf tubes. These were left on ice for 15 min. The cells were then pelleted by micro-centrifuging at 4° C for 5 min. The supernatant was discarded and the cells washed four times with ice-cold sdH_2O. The cells were then re-suspended in 100 μl of ice-cold sdH_2O, and plasmid DNA was added to each Eppendorf. The contents were mixed by bubbling air through and then incubated on ice for 10 min. The cell-DNA mix was transferred to a pre-chilled Bio-Rad 0.2 cm electroporation cuvette. Electroporation was conducted using a Biozyme Remediation-Rad Pulser I set at a voltage of 2.5 kV, a capacitance of 25 μF and a resistance of 400 Ω. Following electroporation the cells were transferred to sterile Eppendorf tubes containing 1 ml of warm LB, which were then incubated for 8 hours at 30° C to allow for phenotypic expression of the Cm-R gene. The contents of the tube were then plated onto LA plates containing 20 μg/ml chloramphenicol, and incubated at 30° C until colonies were evident. Colonies had usually developed after two to three days.

2.7 Gel electrophoresis

Agarose gel electrophoresis is a common method used to separate DNA molecules according to size. This technique was applied to this work both analytically and

preparatively. DNA is negatively charged at a neutral pH and thus samples are loaded into wells at the cathode end and move toward the anode. Separation of DNA molecules occurs as a result of the resistance incurred by molecules due to the agarose matrix. Larger molecules encounter a greater resistance and thus have the most difficulty in passing through the gel pores. All gels prepared in this work contained 0.8 % agarose and are ideal to separate DNA molecules ranging from 0.5 to 10 kb. The size of unknown fragments is easily determined by running a standard molecular weight ladder of known molecular weight size markers on the same gel.

2.7.1 Agarose gel electrophoresis

Stock solutions of 0.8 % Agarose (SeaKem®) were prepared in 0.5 X TBE buffer (Appendix B) and sterilized at 121° C for 20 min. When needed, gels were prepared by melting the agarose stock, and transferring 25 ml to a flask, to which 2.5 µl of EtBr (10 µg/ml) was added. The gel was then poured into a plastic cast and wells were created by placing a 12-tooth well forming comb into the poured gel solution. It was left standing at 4° C for 20 min to allow for polymerization. The comb was removed once the gel had set and the gel was then placed in an electrophoretic tank and covered with 0.5 X TBE containing 1 µg/ml EtBr.

Samples were prepared by adding bromophenol blue and glycerol to them and then these were loaded individually into the wells. Generally, electrophoresis was conducted at 100 V and a current of 20-25 mA until the bromophenol blue front

was < 1 cm from the bottom edge of the gel. DNA sizes were determined using a standard curve calibration generated from the electrophoresis of known molecular weight markers, usually λII, from Boehringer Mannheim, on the same gel as the samples.

2.7.2 Low gelling agarose gel electrophoresis

Low gelling agarose gels were used as a preparative tool prior to ligations. 1 % low gelling agarose gels (FMC, Seaplaque) were prepared in 0.5 X TBE and sterilized at 121° C for 20 min. The gels were prepared as discussed in 2.7.1 however, an 8-tooth well forming comb was used. The gel was then electrophoresed at 4° C and 100 V until the bromophenol blue front was < 1 cm from the bottom edge of the gel.

Separation and identification of desired fragments was confirmed by viewing the gel under a long wavelength UV light (366nm). The necessary band was excised from the gel using a sterile scalpel and was transferred to a sterile Eppendorf tube. The agarose was melted at 60° C for 30 min. TE buffer was added to a final vol of 300 µl, after which three phenol extractions were conducted followed by a chloroform extraction to remove the phenol. 20 µl of 3 mM Tris.HCl was added after each phenol extraction to stabilize the pH. Finally, the DNA was precipitated with NaCl and EtOH.

2.7.3 Viewing and photography of gels

The EtBr, added to both the gels and electrophoresis buffer, serves as the stain for DNA molecules. It is an intercalating agent that binds between the stacked base-pairs of the DNA and thus concentrates at sites where DNA is located. Under UV light the DNA bands fluoresce orange-red. Gels were viewed and photographed using a 2.UV transilluminator, UVPBioDock-It^{TM}system.

3 Results

3.1 Screening for vancomycin resistance

3.1.1 Recovery of the genomic library of *N. brasiliensis*

A genomic library of the DNA from *Nocardia brasiliensis* IFM 0236 was prepared by partial digestion with *Pst* I. This library was constructed in *E. coli* MM294-4, a highly transformable bacterial strain, using the *E. coli-Rhodococcus* shuttle vector pDA71 (Lephoto, 2002). Initially 100 µl of this library was used to inoculate 1000 ml of LB in order to conduct a large scale plasmid preparation. However, after incubation at 37° C, for two days with good aeration, no growth had occurred. So, for large scale preparation of the plasmid DNA contained in the genomic library, it was essential to re-generate the library.

The frozen genomic library was melted and used directly in a large scale plasmid preparation, because, although the *E. coli* cells are dead some DNA is still intact and can be recovered. The plasmid DNA was then purified from a CsCl gradient and used to transform *E. coli* MM294-4 cells. Following the transformation it was necessary to determine the minimum number of clones required to represent the genomic DNA, and thus record the completeness of the library.

3.1.1.1 Determination of the minimum number of clones required

Individual transformant colonies were randomly selected from several of the LA plates containing *E. coli* MM294-4 transformed with *Pst* I fragments of genomic DNA from *N. brasiliensis* IFM 0326. Following mini-plasmid DNA preparations conducted on these colonies, the precipitated DNA was subjected to digestion by *Pst* I. Since this is the only restriction endonuclease used in the preparation of the library it will excise the insert from the plasmid. After digestion, samples were loaded into the wells of a 0.8 % agarose gel and electrophoresed to obtain separation (Figure 1). An agarose concentration of 0.8 % was utilized because it is ideal for the separation of fragments ranging from 0.5 to 10 kb. The genomic library was prepared in pDA71, an 8.8 kb plasmid and the average insert size was shown to be 1.9 kb (Figure 4). Thus the pore size created by a 0.8 % agarose gel is ideal.

A calibration curve of the molecular weight marker (λII) was prepared and used to determine the average insert size. After conducting several repeated experiments, this was found to be 1.9 kb whilst the average insert size per clone was 1.4 kb. This was due to the fact that some of the clones did not contain inserts. It was shown that 60 % of the transformants contained a detectible DNA insert. According to the formula of Clarke and Carbon (1976) (Appendix D) and an assumed genome size of 4.5 x 10^6 bp's ~10 000 clones are required to ensure a

probability > 95 % that any fragment of DNA within the genome will be represented at least once.

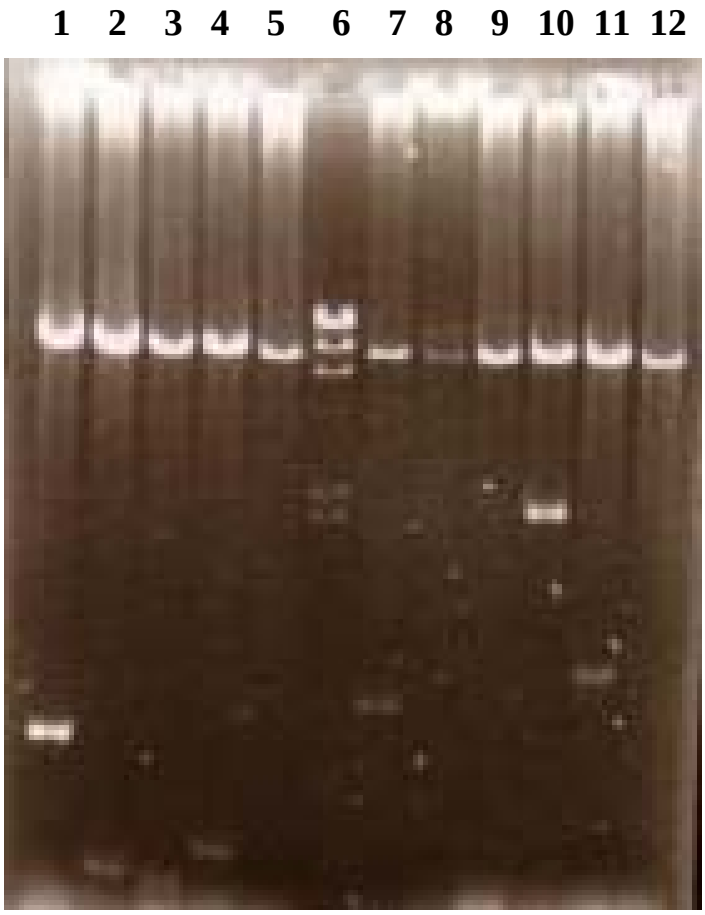


Figure 4: Electrophoresis of digested plasmid DNA from selected *E. coli* clones to determine the average insert size. (Lane 1-5) *Pst* I digestion of selected *E.coli* MM294-4 clones transformed with genomic DNA from *N. brasiliensis* IFM 0236, (Lane 6) λII molecular marker DNA, (Lane 7-12) *Pst* I digestion of selected *E.coli* MM294-4 clones transformed with genomic DNA from *N. brasiliensis* IFM 0236

3.1.1.2 Transformation into *E. coli* MM294-4

Following several $CaCl_2$-mediated transformations of *E. coli* MM294-4 with DNA from the genomic library of *N. brasiliensis* IFM 0236, ~13 000 clones were obtained. This number, according to Clarke and Carbon (1976) (Appendix D) ensured a probability greater than 98 % that each fragment will be represented in the library at least once.

Transformants were washed off the plates, using 1-5 ml of LB, and pooled together. In order to homogenise the suspension, it was incubated at 35 rpm at 37° C for 1 hr. Following incubation, 100 µl of the suspension was used as the inoculum in order to amplify the library and subsequently purify the DNA from a CsCl gradient. The remaining suspension was stored at -70° C. DNA was successfully isolated from a CsCl gradient and this DNA was then transformed into a gram-positive host in order to screen for high level and low level vancomycin resistant clones.

3.1.2 Optimization of the transformation of *R. rhodochrous*

In order to screen for an increased MIC to vancomycin the genomic DNA must be transformed into a gram-positive host. Vancomycin interferes with the transpeptidation step of peptidoglycan synthesis and therefore is not effective against gram-negative bacteria. Thus the detection of clones with increased resistance to vancomycin will be far easier in a gram-positive strain. The host

selected was a highly transformable strain of *Rhodococcus rhodochrous*, Ri8R, which is inherently resistant to high levels of rifampicin. During this study, two different transformation techniques, one chemical and mediated by PEG, and the other physical, mediated by an electro-pulse, were optimized. The average insert size, for the genomic DNA of *N. brasiliensis* represented in pDA71, was shown to be 1.9 kb whilst the average insert size per clone was 1.4 kb.

3.1.2.1 PEG-mediated transformation

Polyethylene glycol-mediated transformation is a chemical means of transformation which exploits iso-osmotic sucrose concentrations, enzymatic degradation of peptidoglycan and polyethylene glycol to transform cells through the creation of protoplasts.

Ri8R, a highly transformable strain of *Rhodococcus rhodochrous*, was cultured in LB supplemented with an iso-osmotic concentration of sucrose as well as 2 % glycine. The sucrose in the medium causes the cell walls to weaken because it creates an increase in the osmotic pressure, and thus compromises in the turgidity of the cell wall. The glycine also weakens the cell walls. Lysozyme is added to these harvested cells and is an enzyme which specifically degrades peptidoglycan and thus removes the cellular wall. This aids further in the creation of protoplasts. These protoplasts are then exposed to the PEG and it is the re-swelling of the cell that occurs once osmotic pressure has been normalized that specifically mediates the DNA entry into the protoplasts.

In order to optimize the transformation of Ri8R cells, several parameters were varied including; lysozyme concentration, duration of exposure to lysozyme, concentration of the PEG solution and the time allowed for the regeneration of the cell wall and the phenotypic expression of selective genes (Table 4). Various concentrations of glycine were added to the LB but this showed little variation on the growth of the cells although transformation occurred more frequently after growth in LBSG containing 2 % glycine. The most critical element in obtaining results from PEG-mediated transformation is optimizing the amount of lysozyme required to sufficiently degrade the peptidoglycan of the cell walls such that protoplasts are created yet not kill the cell and enable the cell wall to fully recover. It was found that 5 μg/ml of lysozyme degraded the cell walls whilst still enabling complete regeneration. Thereafter, the other parameter which was altered was the concentration of the PEG solution used to mediate the transfer of 10 μl of DNA into the protoplast. It has previously been shown that the higher concentrations of PEG result in the greatest transformation efficiency. This study showed that a 60 % PEG solution was suitable for mediating the entry of DNA into the cells. The ratio of protoplasts to DNA was also optimized and it was found that a 10:1 ratio was most suitable and resulted in a two-fold increase in the number of transformants produced (Figure 5). Another critical and variable parameter is the incubation period to allow for regeneration and the phenotypic expression. Incubation times of between 7 and 13 hrs were tested, and it was found that the most optimal incubation time was that of 10 hrs. Following

incubation, the selective agent, chloramphenicol, was carefully added to each plate by means of the underlay technique.

Table 4: Optimization of PEG-mediated transformation of *R. rhodochrous* Ri8R. Significant improvements to the transformation procedure were made by increasing the concentration of lysozyme, PEG and increasing the duration allowed for regeneration and phenotypic expression.

Lysozyme (mg/ml)	**Lysozyme incubation time (min)**	**PEG (%)**	**Incubation time prior to underlay (hr)**	**Vol ratio of protoplasts:DNA (μl)**	**Average no. of transformant colonies/plate**
1	120	50	8	200:10	0
1	120	50	10	200:10	<10
5	120	50	10	200:10	25
5	60	50	10	200:10	38
5	60	60	10	200:10	73
5	60	60	10	200:20	116

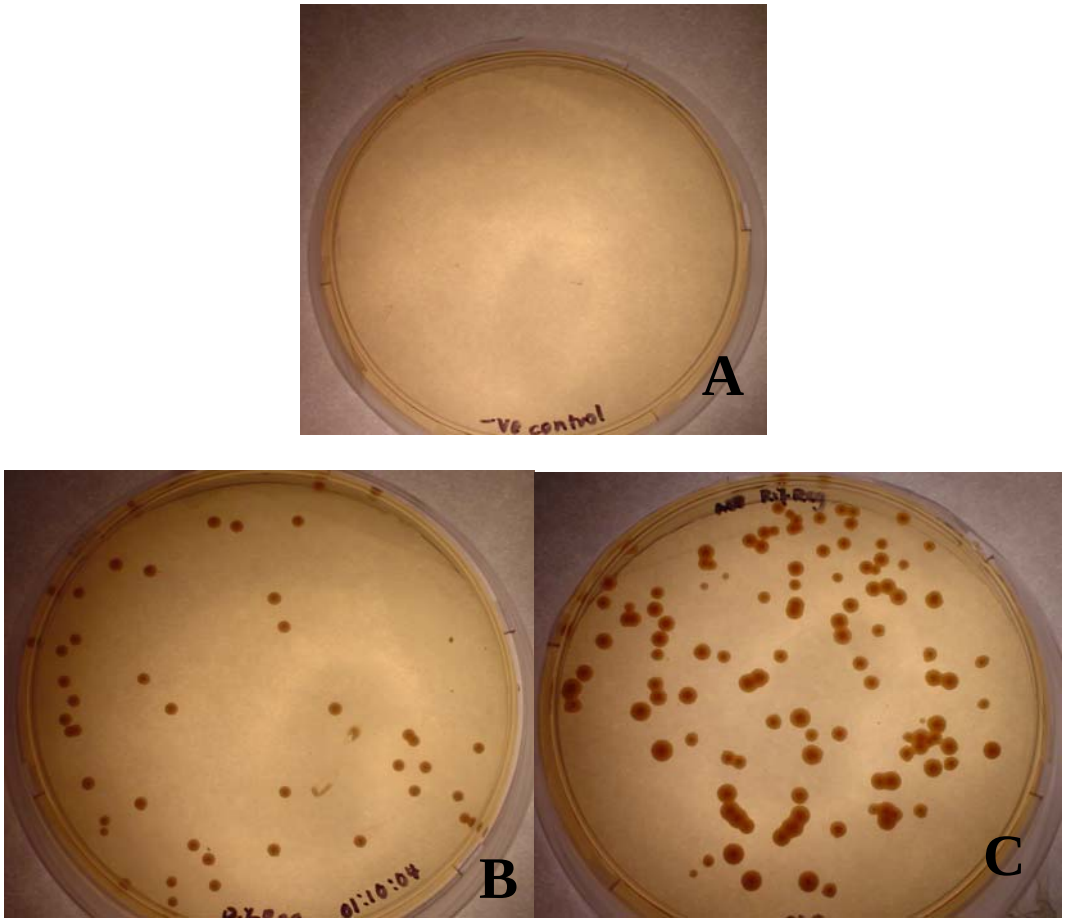


Figure 5: Transformant colonies after optimization of PEG-mediated transformation. (A) indicates the negative control, (B) indicates the improvement to transformation after a higher PEG concentration was used and (C) indicates the improvement in efficiency after the vol of DNA added was double.

3.1.2.2 Electroporation

The second approach taken to transform the *Rhodococcus rhodochrous* Ri8R with DNA from the genomic library of *Nocardia brasiliensis* IFM 0236 was electroporation. Electroporation is a physical means of transformation in which a direct current is applied for a short duration and causes pores to form through which foreign DNA can enter. A genomic library of *N. brasiliensis* IFM 0236,

was purified from CsCl by ultra-centrifugation (2.5.1). The electroporation procedure needed first to be optimized to ensure efficient transformation, thus enabling the maximum number of clones to be obtained. The parameter which was varied was the ratio of cells to DNA. The voltage, capacitance and resistance had been optimized in previous studies, and were shown to be suitable for this study.

It was found that the transformation efficiency increased with the increases to amount of DNA added to a constant volume of cells (Table 5). The cell-DNA mixture was incubated for 10 hours prior to plating. It was found, however, that this time allowed for background growth, arising from spontaneous mutants, to develop and it was thus necessary to re-evaluate and reduce the incubation time allowed for phenotypic expression. An incubation time of 8 hrs was shown to be suitable; eliminating all background growth whilst still enabling phenotypic expression of the necessary selective genes, in this case Cm^R, and hence successful selection of transformants (Figure 6). The increase in the number of transformant colonies present is a direct result of reducing the background growth, since the transformants are no longer competing for nutrients present in the media. Thus it is essential to eliminate all possibilities of contamination and ensure sterility, for even if background growth is relatively insignificant it may still hinder the growth of the desired organism by competing for nutrients and possibly depleting an essential growth requirement of the desired organism.

Table 5: Optimization of electroporation of *R. rhodochrous* Ri8R Modifications to the amount of DNA relative to cells, as well as a reduction to the incubation time prior to plating, resulted in significant improvements to the transformation efficiency.

Vol ratio of cells: DNA (µl)	Incubation time prior to plating (hr)	Average no. of transformant colonies/plate
100:1	1	66
100:1	1	483
50:1	2	818
50:1	5	975
20:1	5	1693

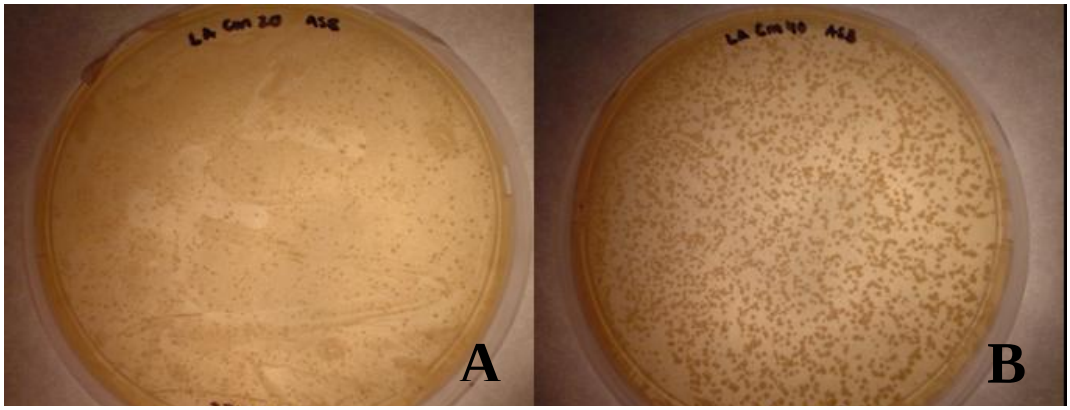


Figure 6: Transformant colonies after optimization of electroporation. (A) The high level of background growth occurring after cells and DNA were incubated for 10 hrs, prior to plating on selective media and (B) the resulting transformants after optimized electroporation and incubation for only 8 hrs, prior to plating on selective media.

3.1.3 Screening the genomic library for vancomycin resistant clones

Optimization of electroporation for the transformation for *R. rhodochrous* Ri8R resulted in a highly effective yet simple procedure for transformation of gram-positive bacteria. Utilizing electroporation to transform *R. rhodochrous* Ri8R approximately 14 500 independent clones were obtained, thus ensuring a probability greater than 98%, that every region of the genome is represented in the pool of clone, at least once.

These individual clones were pooled together in LB and screened for both high level and low level resistance to vancomycin. The MIC of *R. rhodochrous* Ri8R was shown to be 1.5 μg/ml. The negative control used in these tests was pDA71*, and thus no high level or low resistant clones are expected to arise from these transformants.

The pool of clones was sub-cultured onto LA plates containing rifampicin (100 μg/ml), chloramphenicol (40 μg/ml) and vancomycin (1 μg/ml – 100 μg/ml). The plates were incubated at 30° C until growth occurred. Visible growth occurred after three days of incubation at 30° C. Substantial colonies had developed on both the test plates and those of the negative control. Furthermore, no significant difference in the MIC of the genomic DNA transformants and the negative control transformants was noted nor was there a significant difference in the number of colonies per plate (Table 6).

From this it is clearly deducible that no high level or low level resistance clones were identified. Thus as of yet, no DNA fragment, other than the *Pst* I fragment used in pCL1 and pCL2, obtained from a genomic library of *N. brasiliensis* has been shown to confer a increase in the MIC of *R. rhodochrous* to vancomycin (Figure 7).

Table 6: Average number of colonies obtained after screening for vancomycin resistance. Growth occurred after three days incubation at 30°C. There was no significant difference in the number of colonies between (A) untransformed Ri8R, (B) Ri8R transformed with pDA71* and (C) Ri8R transformed with the *Pst* I library of *N. brasiliensis*.

	Vancomycin concentration (μg/ml)							
	1.0	**1.5**	**2.0**	**5.0**	**10**	**20**	**50**	**100**
A	172	192	23	5	0	0	0	0
B	156	164	12	11	0	0	0	0
C	196	180	8	5	0	0	0	0

3.1.4 MIC of vancomycin in *R. rhodochrous* Ri8R

The MIC of Ri8R to vancomycin was shown to be ~ 1.5 μg/ml (Figure 7). Furthermore, there was no significant difference in the MIC of untransformed Ri8R and those transformed with pCL1, pCL2 or the negative control vector,

pDA71*. These results confirm that neither clone of the *rgt* gene, when in a gram-positive background, confer a significantly different resistance to vancomycin.

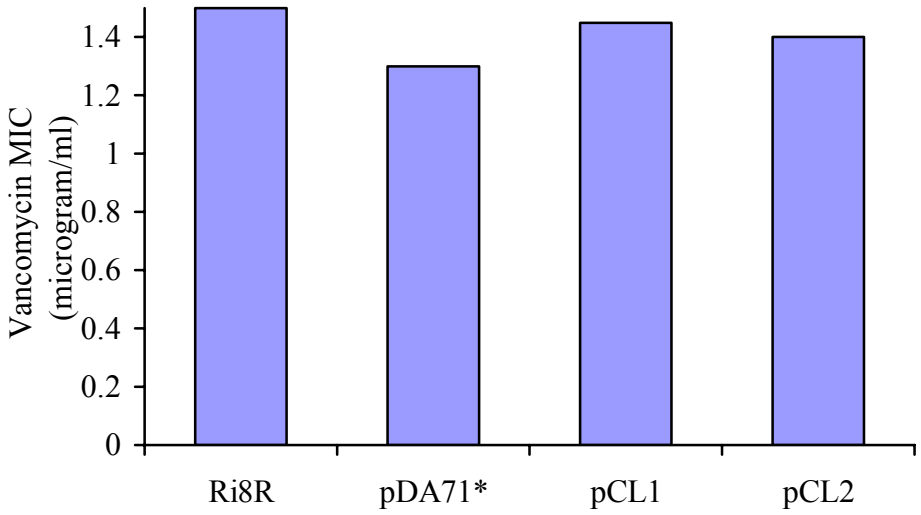


Figure 7: Vancomycin MIC of *R. rhodochrous* Ri8R due to pCL1 or pCL2. No significant difference was observed by any of the transformants and the MIC was shown to be ~ 1.5 μg/ml.

3.2 Mutational analysis of the rifampicin glycosyl-transferase inactivation protein

3.2.1 MIC of pCL1 and pCL2 to rifampicin

Prior to mutational analysis studies, it was necessary to confirm the phenotype of pCL1 and pCL2, when expressed in a gram-negative background. The phenotype of pCL1 and pCL2 was confirmed by transforming *E. coli* MM294-4 with each plasmid, and conducting a subsequent spot test on plates containing various concentration of rifampicin. Growth occurred after overnight incubation of the spot-test plates at 37° C, and it was found that the rifampicin MIC of pCL1 was

180 μg/ml whilst that of pCL2 was 160 μg/ml. *E. coli* MM294-4 transformed with pDA71*, the negative control, exhibited an MIC of 5 μg/ml (Figure 8).

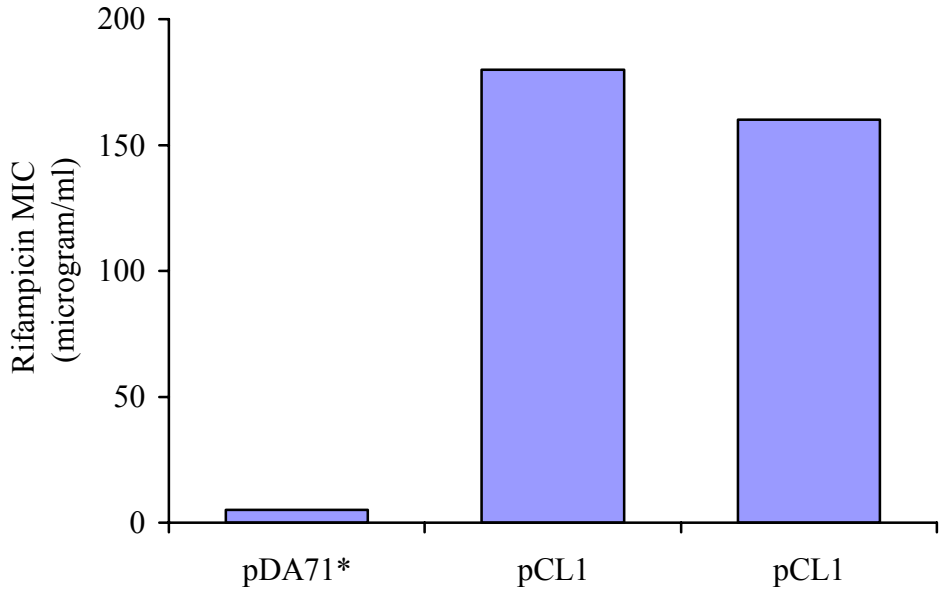


Figure 8: Rifampicin MIC of *E. coli* MM294-4 due to pCL1 or pCL2. Transformants containing pCL1 conferred an MIC of 180 μg/ml whilst those containing pCL2 conferred an MIC of 160 μg/ml.

From this, there is a 36-fold increase in the MIC of resistance upon transformation with pCL1 and a 32-fold increase upon the transformation with pCL2. Although previous data that has shown an 80-fold increase in resistance to rifampicin upon re-transformation with pCL1 (Lephoto, 2002).

3.2.2 Identification of a suitable host plasmid for the rifampicin glycosyl- transferase inactivation gene

In order to conduct a mutational analysis of the *rgt* gene and thus the protein encoded by this gene, the *Pst* I fragment on which the ORF lies, must be ligated

into a low copy number vector. This will ensure that increases in resistance which may occur, during spontaneous mutagenesis, are more likely to be due to mutations than due to a mere increase in the amount of inactivating protein present. Furthermore, once high level resistance clones are identified it will be necessary to confirm whether this resistance is either, the resulting effect of a spontaneously arising mutation that occurred in the ORF or alternatively, if the observed phenotype is merely the result of a mutation occurring in the plasmid itself or even in the chromosomal DNA of *E. coli*.

The objective of this study was to conduct a mutagenic analysis on *E. coli* MM294-4 transformed with the *Pst* I fragment of pCL1 in the hope of identifying a mutant with an increased resistance to rifampicin. The *Pst* I fragment, which contains the ORF for the rifampicin-glycosyl transferase inactivation protein, induces a phenotype with an 36-fold increase in the MIC in *E. coli* thus allowing growth to occur in the presence of up to 180 μg/ml of rifampicin. Therefore, screening should be conducted at level of rifampicin in excess of this.

Counter-selection is a procedure which is employed to select against transformant cells which exhibit an increased resistance to rifampicin as a result of unwanted mutations within the plasmid. The most suitable agent for counter-selection in a gram-negative background is the antibiotic ampicillin. Thus a plasmid with an ampicillin selective marker is not ideal since ampicillin could not longer be used. However, if a *Pst* I site is located within the ampicillin resistance gene, this would be ideal because the insertion of DNA into the *Pst* I site would disrupt the gene.

This would allow for easy identification of insert-containing plasmids by utilising ampicillin as the selective agent. The insert would disrupt the gene and thus the transformant would no longer exhibit a phenotype resistant to ampicillin.

3.2.2.1 Ligation into pACYC177

The plasmid of first choice was pACYC177 (Appendix E). This is a low copy number plasmid, of 3941 bp's, containing an ampicillin resistance gene and a *Pst* I restriction site within it. However, when preliminary digestions were conducted on this plasmid, which had been stored in CsCl at -70° C, using various enzymes with known recognition sites within the plasmid, the plasmid was not digesting the DNA as expected. This indicated that the enzymes were unable to recognise their restriction sites. Most critically, it was shown that *Pst* I was in fact digesting the enzyme twice, when there was only one *Pst* I site. The only reason to expect this would be if there was star activity, and thus the enzyme may cut non-specifically, but there is no star activity associated with *Pst* I or if there was another site *Pst* I recognised. The only *Pst* I isocaudamer is *Nsi* I but there are no *Nsi* I sites in pACYC177, furthermore when the specificity of *Pst* I was tested it was found that it was functioning normally. Thus, the stock of pACYC177, that was available in the lab, was unsuitable for this study.

3.2.2.2 Ligation into pACYC184

The plasmid of second choice was, pACYC184 (Appendix E). It is comprised of 4345 bp's and does not contain either an ampicillin resistance gene or a *Pst* I restriction site. In addition to this, there are no sites within the plasmid that produce compatible ends to those created by *Pst* I. Thus, it was necessary to linearize the plasmid using two alternative restriction enzymes, namely *Sal* I and *Bam* HI, and fill in the overhangs with Klenow fragment. This was conducted in conjunction with the digestion of pCL1 with *Pst* I and the subsequent generation of blunt ends using Klenow fragment. In order to prevent pDA71 from ligating onto itself, alkaline phosphatase was used to remove the 5' phosphates that are necessary for ligation by DNA ligase. The insert will still be able to ligate to the linearized pACYC184 by utilizing its 5' phosphates. Several attempts were made to ligate the insert into pACYC184 using bacteriophage T4 DNA ligase. The 'ligated DNA' was transformed into *E. coli* MM294-4, selecting for chloramphenicol resistance, in order to identify successful ligations. However, no transformants resulted. Colonies were however evident on the vector only control. This is expected, since the vector only control is linearlized and not subject to treatment with alkaline phosphatase, therefore it acts as a positive control.

In order to determine where the error lay, the process was analyzed carefully. All enzymes had been removed by adding phenol, saturated in TE buffer. The phenol was removed from the DNA solution through the addition of the organic solvent, chloroform. This step is one of the most critical elements in ensuring that the

ligation is successful. It is essential that all traces of phenol are removed, since phenol can severely jeopardise further DNA manipulations. The DNA is then precipitated out of solution with salt and ethanol, dried and re-suspended in sdH_2O. It was shown by agarose gel electrophoresis that there was a dramatic loss of insert DNA following the phenol-chloroform extraction. This may therefore have contributed to the inability to ligate successfully. Attempts were made to concentrate this insert DNA by excision of the insert from a low melting temperature agarose gel prior to ligations.

pCL1 was digested as before with *Pst* I to excise the insert . However, following extension of the insert DNA with Klenow fragment, the sample was electrophoresed on a low-temperature gelling agarose for four hours. The insert DNA was visible, under UV light (Figure 9), and was carefully cut from the gel. The gel was then melted, and the enzymes and excess gel removed by performing three careful phenol extractions.

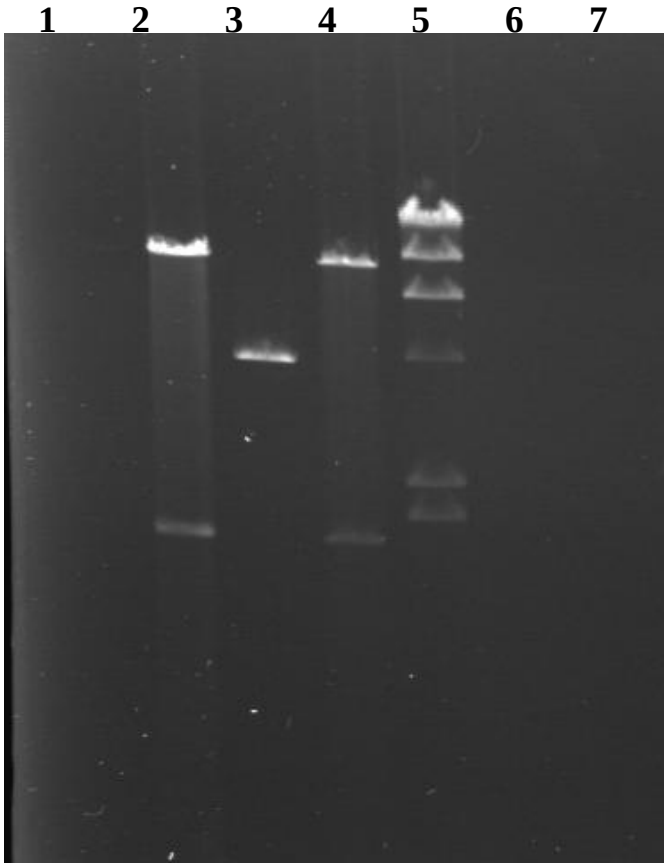


Figure 9: 0.8 % agarose gel of pCL1 digested with *Pst* I. Lane 2: *Pst I* digested pCL1, Lane 3: *Pst I* digested pACYC184, Lane 4: *Pst I* digested pCL2, Lane 5: λII M_W marker.

In order to confirm that the *Pst* I fragment was successfully excised from the gel, a small aliquot of DNA, following precipitation, was electrophoresed on a 0.8 % agarose gel. No DNA band was visible under UV light and thus no DNA or a very insignificant amount had been precipitated. It became clear that the failure to successfully ligate the *Pst* I fragment into pACYC184 was because such a high percentage of DNA was being lost during the phenol-chloroform extraction. Although excision from the gel was aimed at concentrating and purifying the insert DNA from vector DNA and other contaminants, a significant loss was occurring. This should not be the case however since the phenol is used to remove

proteins. It is hydrophobic in nature and thus denatures proteins but has no effect on DNA at pH 8.0, since DNA is hydrophilic in nature and remains in the upper aqueous layer. pH plays a key role in ensuring that the DNA remains in the aqueous phase whilst the contaminants remain in the organic phase. It was thus vital that all buffers used during phenol-chloroform extractions were set at a pH of 7.0 - 8.0.

Due to time constraints, the ligation process was not optimized and the *Pst* I fragment from pCL1 was not ligated into a low copy number plasmid. This prevented the screening of colonies transformed with this newly generated plasmid for spontaneous mutations causing high-level resistance to rifampicin.

4 Discussion

4.1 Screening of *R. rhodochrous* Ri8R for high level and low level resistance to vancomycin

The inherent MIC of *Nocardia brasiliensis* to vancomycin is very high at 100 μg/ml. It was shown however that although pCL1 and pCL2 confer an increase of ~ 50 % to the MIC of *Rhodococcus rhodochrous* Ri8R transformed with them, this is insignificant in comparison to the high MIC level which *N. brasiliensis* exhibits (Lephoto, 2002). Thus, it is clear that another region of DNA from the genome of *N. brasiliensis* is responsible either entirely, or in part, for the high level resistant phenotype.

This study aimed at identifying that DNA, but was unfortunately unsuccessful since no clones were obtained which were able to grow at higher levels of vancomycin than the negative control, which was Ri8R, transformed with pDA71*.

Screening for resistant clones was conducted at concentrations of vancomycin ranging from 1 μg/ml to 100 μg/ml. This broad spectrum ensured that any clones which may contain DNA involved in the resistant phenotype would have been identified. The negative control, as well as untransformed Ri8R, was able to grow at concentrations of 1 μg/ml and 1.5 μg/ml of vancomycin yet not at 2 μg/ml. Thus from this the MIC of Ri8R was shown to be 1.5 μg/ml.

Transformant clones containing *Pst* I fragments from *N. brasiliensis* genomic DNA did not grow on any media containing concentrations of vancomycin greater than 1.5 μg/ml, and thus behaved in exact accordance with the negative control. This was unexpected since from this no high level or low level resistant clones were identified. The phenotype of *N. brasiliensis* is most likely the result of the expression of multiple genes and the subsequent development and interaction of various proteins or enzymes. Thus, the expression of only a single gene may not have influenced the phenotype since all the necessary genes are required to produce a resistant strain. However, it was still hoped that a certain region of DNA would have played such an influential role in the resistance that an alternative phenotype would be observed.

There are three viable reasons for the inability to identify high level or even low level vancomycin resistant clones. Firstly, the average insert size of the genomic library has been shown to be 1.9 kb and it may be that all regions of the DNA involved in the high level resistance to vancomycin, which is seen in *N. brasiliensis*, are in fact larger than 1.9 kb. Or alternatively, the ORF may not be located across a single *Pst* I fragment, and therefore expression will not occur. Finally, the phenotype observed in *N. brasiliensis* may be the resultant effect of the interaction of various genes or proteins, and thus unless they are expressed together the phenotype remains unchanged.

It is however still very likely that the specific DNA involved in the phenotype can be identified, although it is unlikely that it will be found in the *Pst* I library of *N.*

brasiliensis. Thus in order to identify the DNA involved, a new genomic library must be prepared using a different restriction enzyme which would create different fragments since it will cut at different sites. Ultimately, this alternative enzyme must generate fragments in the range of between 2 and 4 kb's.

Enzymes, such as *Acc* I, *Cla* I, *Nar* I and *Pst* I create fragments which are too small since the recognition sites for these enzymes are located too frequently throughout the genome. *N. brasiliensis* is an organism with DNA of high GC content and most of the recognition sites for these enzymes are comprised mainly of G and C nucleotides. *Sfu* I produces suitably sized fragments but is not suitable for the construction of a genomic library in pDA71 since a recognition site exists too close to the 3' end of *EcoR* gene within this plasmid. Therefore, in order to produce an alternative genomic library a partial digestion will have to be performed using one of the afore-mentioned frequent cutters.

The transformation of this genomic library and the subsequent screening of the resultant transformants should, in essence, result in the identification of transformants expressing either a high level or low level resistance to vancomycin. Once high level or alternatively, low level clones have been identified, through growth on vancomycin-containing media, it will be necessary to identify whether the noted phenotype is due to the presence of the foreign plasmid DNA by performing a marker-rescue procedure. This procedure involves the screening of organisms on selective media that also contains ampicillin to ensure that only clones containing pDA71 are present, since this plasmid has an

ampicillin resistance marker gene. If this is the case then, more specifically it will have to be shown that it is the insert DNA from *N. brasiliensis* that confers the increased MIC observed in the phenotype. The minimum amount of DNA responsible for this phenotype can then be determined though subsequent restriction enzyme digestions, using various enzymes, and then re-transformation into *E. coli* MM294-4. Transformants containing the minimum DNA conferring and maintaining the phenotype can be identified through a series of spot-tests.

4.2 Mutational analysis of the rifampicin-glycosyl transferase inactivation protein

In order to conduct a mutational analysis on the rifampicin glycosyl-transferase inactivation protein from *N. brasiliensis* IFM 0236, it was necessary to clone the gene encoding this protein into a low copy number plasmid. This will ensure that any observed resistance increase is due to an alteration in the DNA and not merely the result of a high level of gene expression resulting from multiple copies of the plasmid. Following this, the plasmid was to be transformed into a gram-negative background of *E. coli* MM294-4, and transformant clones were to be spread onto LA plates containing rifampicin concentrations in excess of 180 μg/ml. Mutants were to be obtained *in vivo*, and this approach has previously been shown to be highly effective yet simple and elegant. The objective was then to characterize these mutants and identify those which had arisen as a result of plasmid-borne mutations. This was to be conducted through the utilization of the marker-rescue technique, since the restriction site into which the *N. brasiliensis* genomic DNA

was inserted was located in the Tc^R gene and thus all mutants without insert DNA will be able to grow in the presence of tetracycline whilst those with inserts would not. A spot test would be utilized to identify the mutants which contain genomic inserts from *N. brasiliensis*. Following identification of clones arising from plasmid-borne mutations it would have been necessary to distinguish between the three different categories of plasmid-borne mutations which could result in an increased MIC to rifampicin. Firstly, there are mutations which influence the copy number of the plasmid, and thus these mutations would not affect the rifampicin glycosyl-transferase inactivation gene, and thus would be of no value to this study. These mutations are easily identified since a small-scale preparation of the plasmid DNA followed by electrophoresis and viewing of an agarose gel would reveal a substantially larger amount of plasmid DNA relative to that of other high-level mutants. The second category of mutations that could arise includes mutations that occur in the promoter region of the plasmid. Mutations occurring in the promoter are useful in conducting direct studies on the protein, since these mutations stimulate increased expression of the protein itself. The third category of mutations includes those occurring in the ORF of the plasmid. It is this final category that is of interest to this study, since it involves taking a genetic approach to conduct a mutational study on the rifampicin glycosyl-transferase inactivation gene and the result these mutations may have on the encoded protein. Mutations occurring in ORF's are less frequent but can easily be distinguished from promoter-borne mutations through a technique known as fragment exchange.

However, due to the extensive problems arising during attempts to clone the *Pst* I fragment from pCL1 into the low copy number plasmid, pACYC184 no mutational analysis was conducted. There are two reasons most likely responsible for the inability to successfully ligate the *Pst* I fragment into pACYC184. Firstly, it may have been the result of the significantly low concentrations of insert DNA and the major loss that occurred during the purification of DNA with phenol. In addition to this, it has been shown that the ligation of blunt-ended DNA is far less successful than that of sticky-ended DNA, and this may have been the second possible reason. It is most likely, that it was a combination of these two factors that limited the effectiveness of a standard cloning procedure.

4.3 The relationship between expression of the *rgt* gene and high level resistance to vancomycin

The *rgt* gene isolated from *Nocardia brasiliensis* IFM 0236 and sub-cloned into pDA71 encodes an enzyme responsible for the inactivation of the antibiotic rifampicin through glucosylation. The ORF for this protein has been shown to exhibit high sequence similarity to glycosyl-transferases involved in the biosynthesis of vancomycin by *Amycolatopsis orientalis*. In addition to this the pCL1 and pCL2 clones conferred a small yet reproducible resistance to vancomycin. The organism from which the DNA used in this study was derived, *N. brasiliensis*, has an inherently high MIC to vancomycin of 100 μg/ml. From this it can be assumed that there are mechanisms, other that glucosylation involved in the resistance of *N. brasiliensis* to vancomycin. This study was aimed

at identifying the other regions of DNA involved in this phenotype though the screening of the *Pst* I genomic library in a gram-positive background. *Rhodococcus rhodochrous* was selected since it is closely related to *N. brasiliensis* and heterlogous gene expression should occur with ease.

Although no clones conferring either high level or low level resistance to vancomycin were identified, it cannot be concluded that they do not exist. Instead, it indicates that the DNA involved is larger than that represented by the *Pst* I fragments. Furthermore, it is likely that the enzymes involved in the high level resistance to vancomycin observed in *N. brasiliensis* have ORF's which do not lie entirely between *Pst* I sites. Therefore, it remains necessary to screen the genomic DNA.

Since no clones conferring increases to the MIC of *R. rhodochrous* to vancomycin were identified, no DNA sequences could be obtained and no significant data was obtained which related to the *rgt* gene of *N. brasiliensis* and thus no further information was obtained pertaining to the relationship between the *rgt* gene and the high level resistance *N. brasiliensis* exhibits towards vancomycin. However, it is assumed that the rifampicin glycosyl-transferase inactivation protein from *N. brasiliensis* may work in conjunction with other proteins to enable the high level resistance to vancomycin.

4.4 Future work

4.4.1 Screening for vancomycin resistance

As of yet, there are no known cases of vancomycin inactivation enzymes. The *rgt* gene does confer an increase to the MIC of vancomycin and this may be due to a similar mechanism to that observed in rifampicin. This work did not identify any other DNA regions from *N. brasiliensis* that may contribute to the development of vancomycin resistance and the possible inactivation of the antibiotic. It thus still remains necessary to continue to screen the genomic DNA of *N. brasiliensis* to identify the other regions involved. It is suggested that several genomic libraries be prepared, each using a different restriction enzyme, and a thorough screening is conducted of each of these. Once the DNA fragments are identified, it will be possible to clone the genes involved and conduct functional studies on the proteins themselves. This will assist in combating the escalating number of multi-drug resistant strains of bacteria.

4.4.2 Characterization of the *rgt* gene

A mutational analysis of the *rgt* gene from *N. brasiliensis* still remains to be done. It is suggested that in conjunction with the screening of spontaneous mutants, a chemical mutagenesis should be conducted in parallel. Identification and characterization of mutants will provide valuable insight into the inactivation mechanism involved in rifampicin inactivation, of which very little is known. It is

also suggested that the region of DNA upstream from the *rgt* gene be cloned, since most resistance genes exist as clusters and it has been suggested that the rifampicin inactivation genes form one such cluster (Quan, 1997). Following the identification of the DNA regions involved, it will be valuable to purify and characterize the expressed proteins.

5 Appendices

5.1 Appendix A: Growth Media

5.1.1 Luria Bertani Broth (LB)

1.0 % tryptone

0.5 % yeast extract

0.5 % NaCl

5.1.2 Luria Bertani Agar (LA)

1.0 % tryptone

0.5 % yeast extract

0.5 % NaCl

1.5 % technical agar

5.1.3 Luria Bertani supplemented with sucrose and glucose (LBSG)

1.0 % tryptone

0.5 % yeast extract

0.5 % NaCl

3.0 / 2.0 % glycine

10.3 % sucrose

5.1.4 Protoplast regeneration media

1 % tryptone

0.5 % yeast extract

0.3 % NaCl

10.3 % sucrose

0.33 % glucose

0.33 % $MgCl_2$

The compounds were mixed together in dH_2O. The sucrose was completely dissolved by micro-waving. The pH of the medium was set to 7.2 by the addition of NaOH. 1.83 % technical agar was then added and the medium autoclaved at 121° C for 20 min. The medium was then allowed to cool to 60° C before the following was added;

1 ml 0.5 % KH_2PO_4

2 ml 0.5 M $CaCl_2$

0.5 ml 10 mg/ml nystatin

0.5 ml 10 mg/ml rifampicin

Plates with a constant vol of 22 ml were poured and allowed to air dry for 2 days at 37° C.

5.2 Appendix B: Solutions

5.2.1 Solutions for plasmid preparations from *E. coli*

Solution I

50 mM glucose

25 mM Tris.HCl

10 mM EDTA

pH 8.0

Solution II

0.2 M NaOH

1.0 % SDS

Solution III

5 M KAc pH4.8

88.5 ml dH_2O

11.5 ml glacial acetic acid

5.2.2 Solutions for $CaCl_2$-mediated transformation

$CaCl_2$ transformation buffer

50 mM $CaCl_2$

10 mM Tris.HCl

pH 7.5

20 % glucose

4 g glucose

20 ml dH_2O

5.2.3 Solutions for PEG-mediated transformation

Basal buffer

10.3 g sucrose

25 mg K_2SO_4

202 mg $MgCl_2.6H_2O$

10 ml 0.25 M TES pH 7.

87.5 ml sdH_2O

Protoplast buffer

5 ml Basal buffer

50 10.5 % KH_2PO_4

125 ml 1 M $CaCl_2$

5.2.4 Solutions for agarose gel electrophoresis

Agarose gel

0.8 g (0.4 %), 1.6 g (0.8 %) or 2.4 g (1.2 %) agarose

20 ml 5 X TBE

180 ml dH_2O

Combine all and autoclave for 20 min at 121° C, store at 4° C until required. 20 ml of this was used per gel and 2 µl of EtBr (10 mg.ml) was added prior to use

TE buffer

0.2 ml 0.5 M EDTA pH 8.0

1.0 ml 1 M Tris-HCl pH 8.0

Buffer was made up to a total volume of 100 ml using dH_2O

5 X TBE

54.0 g Tris

27.5 g boric acid

20 ml 0.5 M EDTA pH 8.0

dH_2O to 1000 ml

Bromophenol blue tracking dye

30 % glycerol in TE

0.025 % bromophenol blue

Running buffer

25 ml 5 X TBE

225 ml sdH_2O

25 µl EtBr

5.2.5 Miscellaneous solutions

TE-saturated phenol

14 g phenol

10 ml TE buffer

0.5 M EDTA

18.6 g EDTA

dH_2O

Adjust pH to 8.0 with NaOH, and add dH_2O to a final vol of 1000 ml. Autoclave for 20 min at 121° C

1.0 M Tris-HCl

15.8 g Tris

dH_2O

Adjust pH to 8.00 with HCl and add dH_2O to a final vol of 100 ml. Autoclave solution for 20 min at 121° C

5.3 Appendix C: Antimicrobial and Antifungal Agents

Agent	Stock Concentration (mg/ml)	Solvent	Supplier
Ampicillin	100	3:7 dH_2O:ethanol	Boehringer Mannheim
Chloramphenicol	20	ethanol	Boehringer Mannheim
Kanamycin	10	H_2O	Boehringer Mannheim
Nalidixic Acid	10	3:7 dH_2O:ethanol	Sigma
Nystatin	10	methanol	Sigma
Rifampicin	10	methanol	Sigma
Streptomycin	20	H_2O	Boehringer Mannheim
Vancomycin	10	H_2O	Sigma

5.4 Appendix D: Formulae

Calculating the number of clones required for a genomic library

$$N = \frac{\ln (1 - P)}{\ln (1 - a/b)}$$

where;

N = number of clones required

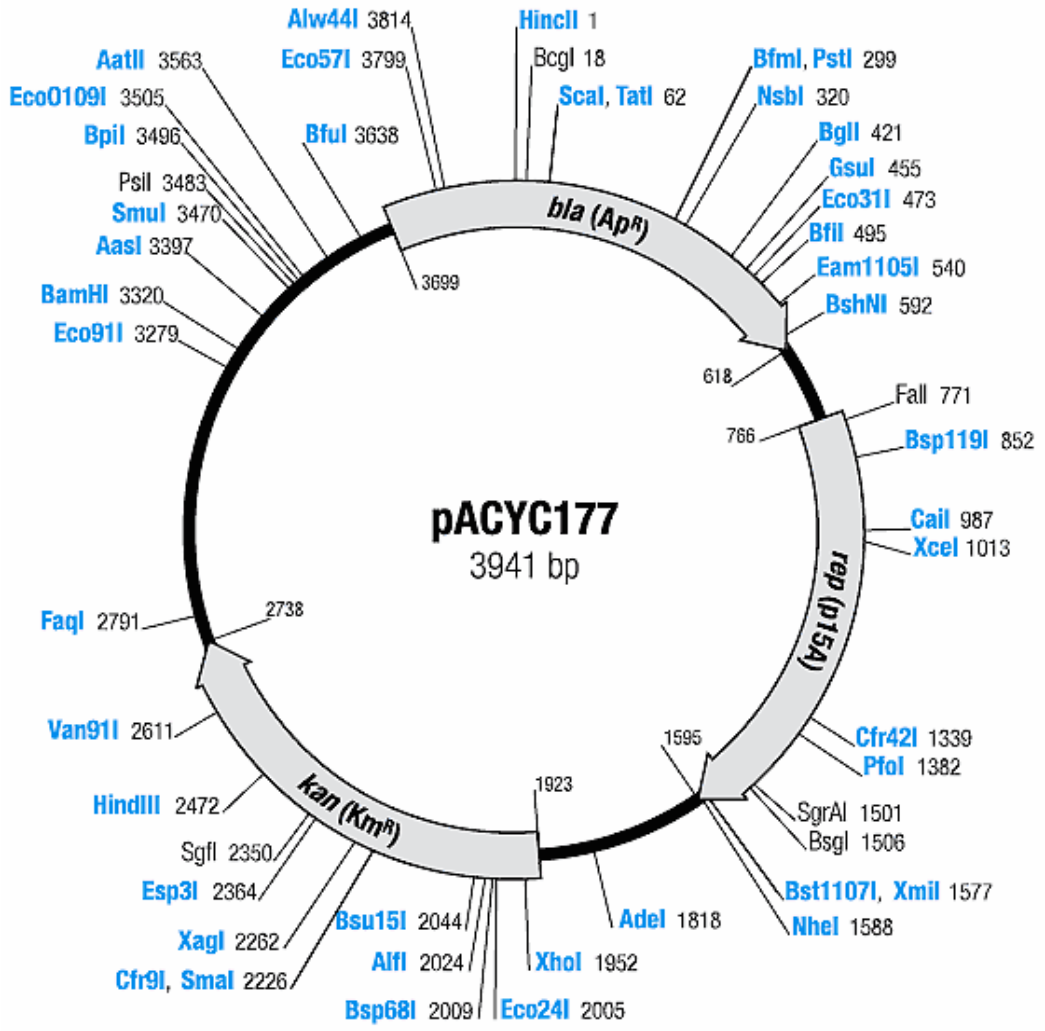
p = probability that any gene is present in library

a = average size of the DNA fragment inserted into the vector

b = total size of the genome

5.5 Appendix E: Restriction maps of the plasmids used in this work

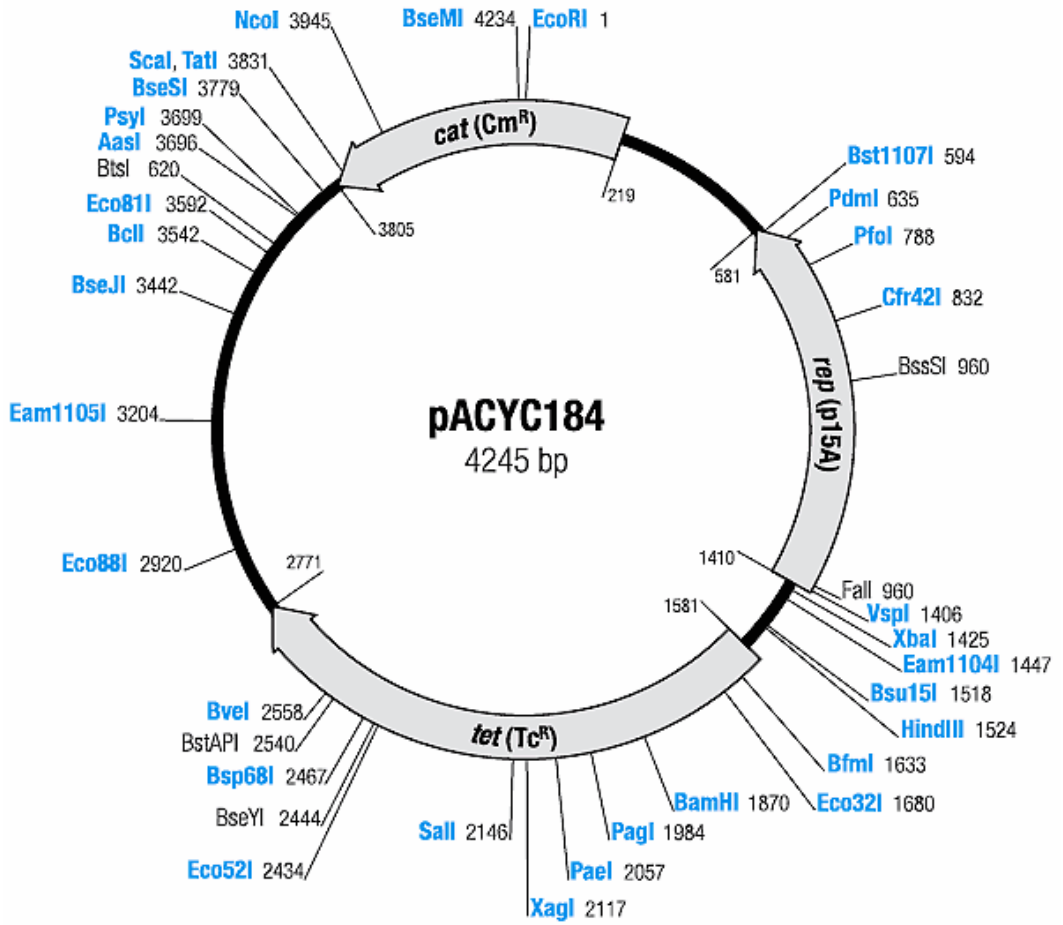
5.5.1 pACYC177



Restriction map of pACYC177, a low copy number cloning vector comprised of 3941 bp's. It contains a *Pst* I site within an Amp^R gene and does not contain an *Nsi* I site.

(http://www.fermentas.com/techinfo/nucleicacids/mappacyc177.htm)

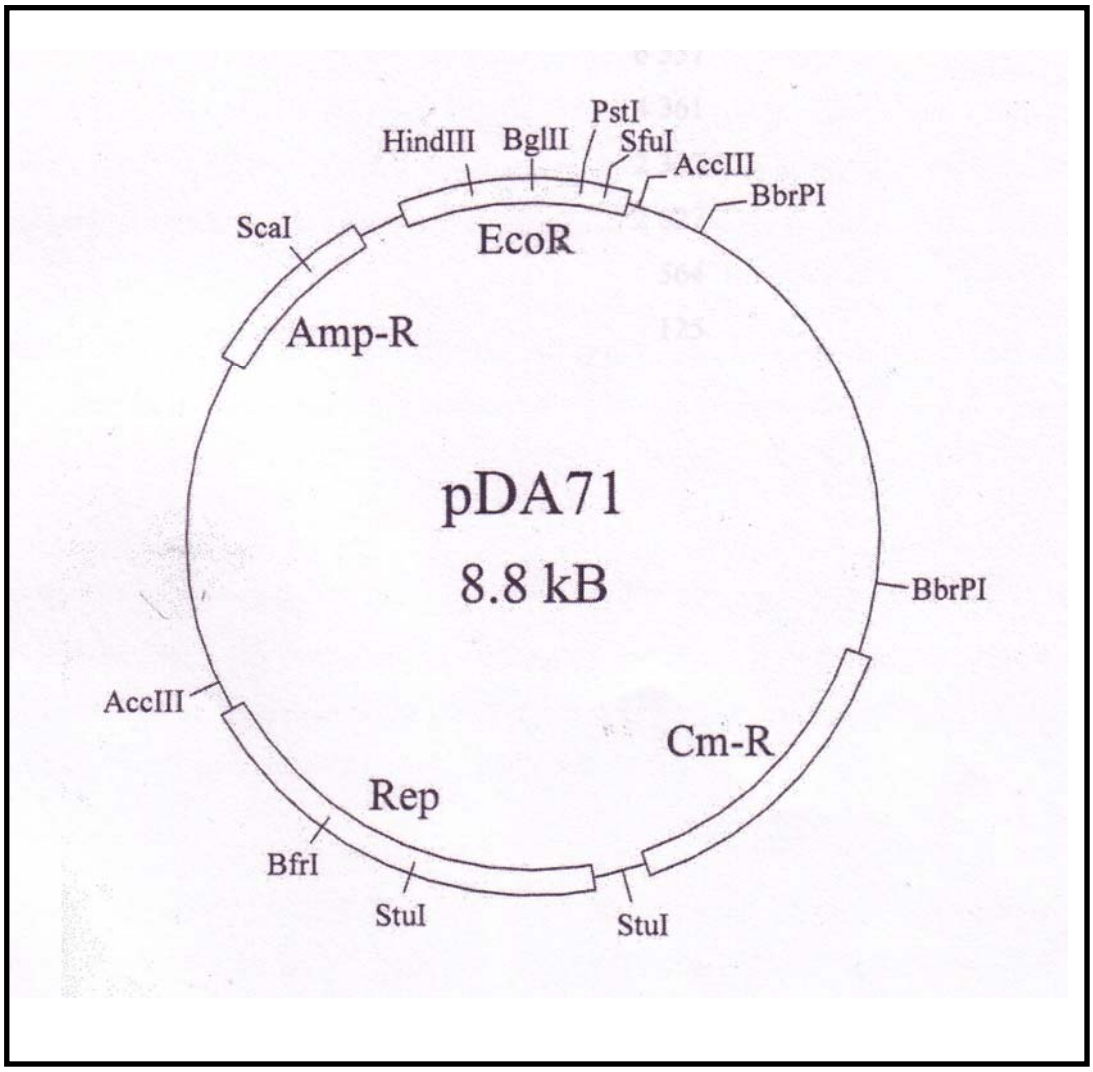
5.5.2 pACYC184



Restriction map of pACYC184, a low copy number cloning vector comprised of 4245 bp's. It does not contain a *Pst* I site or an *Nsi* I site.

(http://www.fermentas.com/techinfo/nucleicacids/mappacyc184.htm)

5.5.3 pDA71



Restriciton map of pDA71

(adapted from Dabbs et al., 1995)

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