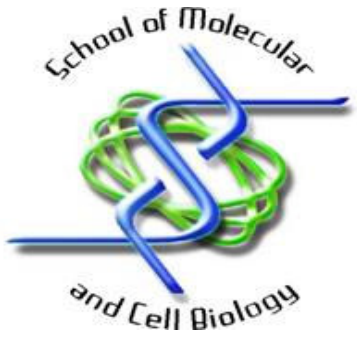

STUDIES OF HETEROLOGOUS EXPRESSION OF A MYCOBACTERIAL GLUTATHIONE S-TRANSFERASE GENE

OLUGBADE ADEBAYO ADETUNJI

A DISSERTATION SUBMITTED TO THE FACULTY OF SCIENCE, UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG, IN FULFILMENT OF THE REQUIREMENTS FOR THE MASTER'S DEGREE.

JULY, 2009

Declaration

I hereby declare that this dissertation is my own unaided work. It is being submitted to the University of the Witwatersrand, Johannesburg for Master's degree. It has not been previously submitted for any degree or examination at this or other University.

Adetunji, Olugbade Adebayo

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Dedication

This work is dedicated to the glory of the Lord Jesus Christ, the author and finisher of my faith.

Abstract

Resistance to antibiotics still poses a major challenge to human and animal health as a result of wide spread dissemination of resistance genes. Study of the evolutionary origin of these determinants is of importance in order to understand how the mechanisms involved arose in the first place. Two approaches were used to study antibiotic resistance from the evolutionary point of view; a model system of heterologous expression of Gram positive DNAs in a Gram negative background giving rise to macrolide resistance, and expression of the Gram positive glutathione-S-transferase (*gst*) gene from *Mycobacterium* in both Gram positive and Gram negative backgrounds.

Expression of the *gst* gene in *Escherichia coli* conferred a 3× increase in MIC in kanamycin and streptomycin and a ~ 2 fold increase for chloramphenicol. In the Gram positive *Rhodococcus erythropolis* ATCC 4277 it gave a ~ 5× increase in MIC for spectinomycin, streptomycin and nalidixic acid, while kasugamycin and chloramphenicol showed a 4× increase. Inactivation assay experiments also revealed the possibility of a novel chloramphenicol inactivation. Attempts made to create mutants with increased resistance by spontaneous selection, chemical mutagenesis or by use of an *E. coli* mutator strain were unsuccessful.

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Abbreviations

Amp: ampicillin

Amp-R: ampicillin resistant

amp: ampere

ATCC: American Type Culture Collection

ATP: adenosine triphosphate

APS: ammonium persulfate

Bp: base pairs

Cmp: chloramphenicol

Cmp-R: chloramphenicol resistant

$(CH_3COO)_2Mg$: magnesium acetate

CsCl: cesium chloride

dH_2O: distilled water

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

EDTA: ethylenediamenetetraacetic acid

EMS: ethyl methyl sulfonate

epPCR: error prone polymerase chain reaction

Ery: erythromycin

Ery-R: erythromycin resistant

EtBr: ethidium bromide

Fus: fusidic acid

fwd: forward

g: grams

GSH: glutathione

GST: glutathione s-transferase

HCl: hydrochloric acid

hr: hour(s)

Kan: kanamycin

Kan-R: kanamycin resistant

Kb: kilobase

kDa: kilodalton

kV: kilovolts

Ksg: kasugamycin

L: litre

LA: Luria-Bertani Agar

L(A-N): Luria-Bertani + phosphate buffer

L(A-N)Glu: Luria-Bertani + phosphate buffer and glucose

L(Glu): Luria-Bertani + glucose

LB: Luria-Bertani broth

LBCmp: Luria-Bertani + chloramphenicol

LBG: Luria-Bertani + glycine

LBSG: Luria-Bertani + sucrose and glycine

LDR: Lysozyme DNAse RNAse

M: molar

mg: milligram

MIC: minimal inhibitory concentration

min: minute(s)

ml: milliliter(s)

NaCl: sodium chloride

NAD: nicotinamide adenine dinucleotide

NADH: nicotinamide adenine dinucleotide, reduced

Nal: Nalidixic acid

Nal-R: nalidixic acid resistant

Rev: reverse

Rif: rifampicin

RNA: Ribonucleic acid

rpm: revolutions per minute

s: second(s)

sdH_2O: sterile distilled water

SDS: sodium dodecyl sulphate

Spc: spectinomycin

Spc-R: spectinomycin resistant

Str: streptomycin

TBE: Tris boric acid EDTA

Tet: tetracycline

Tris: Tris(hydroxymethyl)-aminomethane

Tween 80: polyoxyethylenesorbitan monooleate

UDPG: uridine diphosphate glucose

µF: micro Farad

µg: micro gram

µl: micro litre

v: volts

Chapter 1

Introduction

1.1 Actinomycetes

Actinobacteria comprise bacteria whose common feature is the formation of hyphae at some stage of development. They are Gram-positive with a high GC content. Members of this group of bacteria can be found in most ecological settings, soil having the greatest population density, especially Streptomycetes (Taber, 1960; Gottlieb, 1973). They occur in greatest numbers in the top inches of the soil and decrease with depth (Waksman and Purvis, 1932). Other members colonize plants and animals, including some pathogens, such as *Mycobacterium*, *Corynebacterium, Nocardia, Rhodococcus* and a few species of *Streptomyces*.

Most of the organisms in the order are aerobic, while some are facultatively anaerobic e.g. *Actinomyces israelii* and some strictly anaerobic species. Reproduction is usually asexual, though sexual processes have been shown to occur by genetic analyses. In the nonhyphal forms, asexual reproduction is by fragmentation or perhaps even by the usual fission of single cells. Where stable hyphae are produced, vegetative reproduction is by well formed spores resembling fungal arthrospores, borne either free or in sporangia.

Actinobacteria are well known as secondary metabolite producers and as such, they are of high pharmacological and commercial interest as the major antibiotics producers (Sykes and Skinner, 1973). Except for a few antibiotics produced by fungi e.g. penicillin and cephalosporin, and a few from other bacteria e.g. bacitracin and polymyxin, most antibiotics that are medically useful and have a wide application are produced by the Actinomycetes. Other important roles of this group of bacteria (e.g. *Streptomyces* and *Mycobacterium*) include their activities in the soil where they function in decomposing the organic matter of the soil (Waksman and Lomanitz, 1925; Reynold, 1954; Williams, 1966). They also exert their effect on the soil

structure in binding clay particles by their hyphal threads to impart a granular viable structure that is conducive to crop production. Actinomycetes are responsible for the earthy odour of soil (Gerber and Lechevalier, 1965). Other areas in which the production of the secondary metabolites by Actinomycetes has proved important include; their use as food preservatives, and incorporation into animal feeds with the primary effect of increasing the rate of growth in animals such as poultry, pigs and cattle. This is achieved by using the antibiotics to control low levels of infection in the animals and their effect on growth stimulation, specifically, in promoting the metabolism of the animals, thereby increasing hormone production, for example (Lucky, 1959; Wrenshall, 1959).

1.2 Mycobacteria

Mycobacteria usually occur as spherical or rod-like elements, though some species produce branched cells or even a branching mycelium (Besra et al., 1995). All strains contain mycolic acids, α-branched β-hydroxylated fatty acids (Sykes and Skinner, 1973). Their cell wall is thicker than in many other bacteria, which is hydrophobic and waxy. The presence of arabinogalactan bridges in their cell wall connects the peptidoglycan layer with the mycolic acids, in which the galactose is in the five-ring furanose, rather than the common six-ring pyranose form (Besra et al., 1995). These organisms are nonmotile, nonsporulating, Gram-positive, aerobic rods. Their DNA base composition ranges from 60 to 65 mole percent GC (Frobischer et al., 1974).

Many of these organisms grow on simple media with ammonia or amino acids as sources of nitrogen glycerol for the carbon source in the presence of mineral salts with varying optimum growth temperature between 25°C to over 50°C, but they are commonly cultivated on rich, solid organic media made with eggs, milk, and potatoes and containing selectively inhibitory dyes. When grown on simple media, the surface tension of the fluid must be lowered with a surfactant substance such as Tween 80 so that the fluid wets the waxy bacilli.

1.2.1 Pathogenicity of mycobacteria

Although most species of mycobacteria are harmless saprophytes living in the soil, several species of mycobacteria are pathogenic for man and result in chronic, destructive granulomas which may undergo further necrosis with ulceration or cavitation. The roles of the obligate pathogens *M. tuberculosis, M. bovis* and *M. africanum* (tuberculosis causative agents), and *M. leprae, M. microti* (leprosy causative agents) are well known but in some areas the commonest infections other than those caused by *M. tuberculosis* are caused by strains of the *M. avium-intracellulare* complex and the closely related species *M. scrofulaceum* (Runyon, 1971).

Another common pathogen, *M. ulcerans,* causes chronic skin lesions (Clancey, 1964; MacCallum et al., 1948; Perquis and Maret, 1965; Reid, 1967). Mycobacterial infections are difficult to treat since they are naturally resistant to a number of antibiotics that work by destroying cell walls because of their hardy cell wall which is neither truly Gram-positive nor Gram-negative and for this reason, they can survive long exposure to acids, alkalis, detergents, oxidative bursts, lysis by complement and antibiotics which naturally leads to antibiotic resistance. Most mycobacteria are susceptible to the antibiotics clarithromycin and rifamycin, but antibiotic-resistant strains are known to exist.

1.2.2 *Mycobacterium smegmatis*

Mycobacterium smegmatis was first described in 1884 by Lusgarten who found a bacillus with the staining appearance of tubercule bacilli in syphilitic chancres. Subsequently, Alvarez and Tavel found organisms similar to that in normal genital secretions (smegma), and it was later named *M. smegmatis.* It is generally considered a non-pathogenic microorganism; however in some cases it can cause disease, mainly in animals. In recent studies, it has been linked to the HIV virus and has been known to kill test animals (http://en.wikipedia.org/wiki/*Mycobacterium_smegmatis*).

The organism reduces nitrate, produces catalase, hydrolysis Tween, reduces tellurite, and grows in less than seven days at 37°C hence, classified as a rapid grower within the genus *Mycobacterium* (Frobischer, 1974). In molecular analyses, *M. smegmatis* has been an attractive model organism to work with because it can readily be grown in simple media and has been the preferred host for phage infections (Graham and William, 2000). It has been reported that strain mc^2155 is readily transformable with integration-proficient plasmids and pAL5000-based episomal plasmids, and it readily undergoes homologous recombination, allowing for the generation of targeted gene disruptions.

It has also been shown that genes from *M. tuberculosis* and *M. leprae* are readily expressed in *M. smegmatis*, allowing for the analysis of mycobacterial promoters and expression sequences. Numerous studies have also shown that mutations in the genes from M. smegmatis can be readily complemented with genes from *M. tuberculosis* or *M. leprae* thus proving mc^2155 to be a valuable surrogate host. It is of great importance that multicopy expression of *M. tuberculosis* genes in *M. smegmatis* has provided a means of identifying the previously unknown targets of isoniazid, ethionamide, ethambutol, and pyrazinamide (Banerjee et al., 1994; Belanger et al., 1996; Telenti et al., 1997).

1.3 Glutathione S-transferase

Glutathione (GSH) is a tripeptide of glutamic acid, cysteine, and glycine, the peptide link between glutamate and cysteine being on the γ-carboxyl. It is present in large amount in liver, muscle and other tissues. Its function is to establish a reducing situation in the cells and by virtue of its –SH group it is abbreviated to GSH. Glutathione is the most abundant low-molecular-weight cellular thiol in bacteria and eukaryotic cells, reaching levels of 1 to 10mM. Its major function include: the protection and detoxification of reactive chemical groups, by the high reactivity of its nucleophilic thiolate (Bernat et al., 1997; Elliot and Elliot, 2005).

The reaction of glutathione S-transferases (GSTs) prevents carcinogenic molecules from reacting with DNA and causing genetic damage. GSTs have been grouped into at least twelve distinct classes on the basis of substrate specificity and primary

structure. The nomenclature is in flux due to the continued identification of new GSTs, particularly in prokaryotes. In prokaryotes few bacterial representatives have been characterized in detail and the catalytic activities and substrate specificities observed have generally been modest. The few well studied bacterial GSTs have largely unknown physiological functions (www.pubmedcentral.nih.gov/article).

Prokaryotic GSTs are as diverse as their eukaryotic counterparts; they are broadly in prokaryotes as homodimers or heterodimers with ~25 kDa subunits. GSTs transform a wide variety of electrophilic compounds in a reaction typically involving GSH (γ-L-Glu-L-Cys-Gly) conjugation. Glutathione detoxifies by donating a reducing equivalent ($H^+ + e^-$) to other unstable molecules such as reactive oxygen species in its reduced state. It then becomes reactive after donating an electron and readily reacts with another glutathione to form glutathione disulfide (GSSG).

While most eukaryotic GSTs consume a single GSH that is conjugated to an electrophilic substrate during catalytic turnover, bacterial GSTs display considerable diversity with respect to the utilization of GSH in their detoxification and catabolic pathways functions. For example, enzymes such as maleylacetoacetate and maleypyruvate isomerases utilize but do not consume GSH. Some bacterial GSTs, such as tetrachloro-hydroquinone (TCHQ) dehalogenase from *Sphingomonas chlorophenolica*, consume two GSH equivalents. The first being used in the dehalogenation step leading to the formation of a mixed disulfide with the enzyme, and the second to regenerate the free enzyme, yielding a disulfide-bridged product, GSSG i.e. oxidized glutathione (Tocheva et. al., 2006).

Glu-Cys-Gly

|

SH

Fig. 1a Reduced glutathione (GSH)

Glu-Cys-Gly
|
S
|
S
Glu-Cys-Gly

Fig. 1b Oxidized glutathione (GSSG)

1.4 Mutagenesis

Mutagenesis is a process through which a change in the heritable characters is introduced into an organism. This change is not a result of transfer, segregation, or recombination of normal genes but of an alteration of the numbers, molecular structure or sequence of nucleotides that constitute the genes themselves. Mutations can arise spontaneously or be induced by mutagenic agents. Spontaneous mutations arise from errors in replication, spontaneous lesions, or transposition events. Replication errors can result in base pair substitutions. In transitions, a purine is replaced by a purine or a pyrimidine by a pyrimidine. In transversions, a purine is replaced by a pyrimidine and a pyrimidine by a purine. For the frameshift mutations, the codon frame is altered by deletion or addition of a single base pair to the sequence, and this can result in greatly altered protein products.

Induced mutations are those caused by the action of a specific mutagen. Each mutagen exhibits specificity, usually the result of the mechanism by which the mutagen alters the DNA. There are three basic mechanisms of induced mutagenesis; a base is replaced by another base, a base is altered so that it mispairs with another base, or a base is damaged so that it can no longer pair with any base (Graham and William, 2000). Mutagenic agents that cause induced mutations could be physical agents like a very high temperature, gamma radiation which has been used to induce mutations in *M. phlei*, an ultra violet light, or by chemical mutagens (Kolman and Herenberg, 1978). Konickova-Radochova et al., 1970 identified a wide range of effective agents as chemical mutagens and such include; acriflavin, ethyl methanesulfonate (EMS), hydroxylamine, nitrous acid, N-methyl-N′-nitro-N-nitrosoguanidine (NTG).

In vitro mutagenesis is a method for the generation of random point mutations in a given DNA segment. The DNA region to be mutagenized is amplified with a technique under such conditions that reduce the fidelity of DNA synthesis by *Thermus aquaticus* (Taq) DNA polymerase (Leung et al., 1989), and the technique is referred to as an error prone polymerase chain reaction (epPCR). This method of mutagenesis is a powerful tool for the study of structure and function of both proteins and regulatory DNA elements (Botstein and Shortle, 1985).

1.5 Antibiotics

Antibiotics are low molecular weight substances which have the ability in dilute solution to inhibit pathogenic bacteria (bacteriostatic effect) or to kill them (bactericidal effect). They are secondary metabolic products from microorganisms and they act by blocking some crucial processes in a prokaryotic cell without having a harmful effect on the infected host. Most antibiotics have a molecular weight of < 2 kDa. They may be natural products, semi-synthetic or synthetic chemicals. The existence and clinical development of both synthetic and natural product antibiotics reflect a dichotomy in antibiotic discovery programs in the 20th century. At one end of the spectrum was the medicinal chemistry view and at the other was the classical "magic bullet" approach that pure compounds could be made with therapeutic specificity and utility. The early introduction of the sulfa drugs as antibacterials, still in use six decades later, was an early success for this approach (Amyes, 2001).

Antibacterial agents can be classified on their target specificity: "narrow spectrum" antibiotics which target particular types of bacteria, such as Gram-negative or Gram-positive bacteria. Other factors to be considered while investigating these compounds include; the economic impacts, the bacterial disease they are prescribed for and their mode of action, which is the principal criterion for classifying them (Tipper and Strominger, 1965; Gale, 1981; Russell and Chopra, 1996).

1.5.1 Classes of antibiotics

With respect to their specific target sites in microorganisms, antibiotics are classified into four major groups; (i) cell wall biosynthesis e.g. penicillin, a member of β-lactam class, (ii) protein biosynthesis e.g. erythromycins and clindamycin, members of the macrolide class, (iii) DNA replication/repair e.g. ciprofloxacin, a member of the quinolone class, and (iv) RNA synthesis e.g. rifampicin, one of the rifamycins (Ali et al., 1989; Retch and Puglisi, 2001).

1.5.1.1 Antibiotics that act on cell wall biosynthesis

This class of antibiotics interdicts any of the several steps in bacterial cell wall assembly, from biogenesis of the specialized assembly, membrane translocation, and extracellular cross-linking and strengthening of the exoskeletal peptidogycan layers (Walsh, 2003). Many of the antibiotics that affect bacterial cell walls inhibit enzymes or sequester substrates involved in peptidoglycan assembly and cross-linking. The thick peptidoglycan layer of Gram-positive bacteria has been described as a surface organelle, for display of carbohydrates and proteins, while the outer membrane is the equivalent surface organelle in Gram-negative organisms (Lee and Schneewind, 2001; Navarre and Schneewind, 1999; Nikaido, 1994).

Gram-positive bacteria are susceptible to some antibiotics that do not work or work poorly against Gram-negative bacteria (e.g. pseudomonads), and this difference is related to the ability of antibiotics to be blocked by the limiting pore sizes of the porin proteins of the Gram-negative organisms' outer membranes (Koebnik et al., 2000). There is no such barrier to diffusion in Gram-positive bacteria. Penicillin, a member of the β-lactam antibiotics, cephalosporins and ampicillin are well known examples of this class of the antibiotics (Lund and Tybring, 1972; Tipper and Strominger, 1965).

1.5.1.2 Antibiotics that block bacterial protein biosynthesis

The antibiotics in this class exert their bacteriostatic or bactericidal action by blockage of one or more of the protein biosynthesis steps that occur on the 30S and 50S subunits of the bacterial ribosome. Bacterial ribosome is a two-subunit nucleoprotein

particle, about two-thirds RNA and one-third protein, of molecular weight 2.5 to 2.6 MDa (Yusupov et al., 2001). The small subunit, 30S, contains about 20 proteins and a 16S ribosomal rRNA of about 1,500 ribonucleotides. Streptomycin and neomycin, aminoglycosides, are examples of antibiotics that target this ribosomal unit (Retch and Puglisi, 2001). Those that bind the large subunit belong to the macrolides e.g. streptogramin B and lincosamides (Malbruny, et al., 2004; Motanari, et al., 2003). Chloramphenicol, the principal antibiotic in my work, also blocks bacterial protein synthesis as an amino acid antimetabolite. Binding occurs in the peptidyltransferase center of the 50S ribosome subunit (Walsh, 2003).

1.5.1.3 Antibiotics that block DNA replication/repair

Natural products from metabolic activities in bacteria have the ability to inhibit DNA replication and repair enzymes, resulting in the death of the other bacteria in the neighbourhood. The coumarins as represented by such streptomycetes metabolites as novobiocin and coumermycin target DNA type II topoisomerases, specifically DNA gyrase (Maxwell, 1997). DNA gyrase is important for controlling DNA topology in DNA replication, recombination, and transcription, while topoisomerases IV is also implicated in DNA replication and decatenation of linked daughter chromosomes at the end of bacterial DNA replication (Pan and Fisher, 1997). 15 quinolones have been evaluated against *S. aureus* MS 5935 and subdivided into three classes; (i) those that target topo IV preferentially e.g. norfloxacin, ciprofloxacin and levofloxacin, (ii) those that are more selective for DNA gyrase e.g. sparfloxacin and nadifloxacin, and (iii) those that target topo II and topo IV equally e.g. gatifloxacin and moxifloxacin (Takei et al., 2001)

Rifampicin which is a member of the rifamycins is an example of antibiotics that block RNA synthesis and they are very useful in the treatment of tuberculosis. Other antibiotics of clinical importance not mentioned in the above major classes include those that block precursor biosynthesis for nucleic acids as well as those that act to disrupt one or more aspects of bacterial membrane functions. Antibiotics that exert their activity by permeabilizing bacterial membranes include the antimicrobial peptides e.g. tigerinins (Sai, 2001).

Those that block folic acid metabolism include the synthetic sulfa drugs e.g. sulfamethoxazole, used in combination with trimethoprim for the treatment of patients with urinary tract infections and also for AIDS patients with *Pneumocystis carinii* infections (Scholar and Pratt, 2000). Sulfamethoxazole blocks the enzyme dihydropteorate synthesase in the biosynthetic pathway to folate, while trimethoprim inhibits dihydrofolate reductase, a key enzyme providing the pyrimidine thymidylate for DNA biosynthesis.

1.6 Mechanisms of antibiotic resistance

It has been widely reported that since the development of antibiotics, the drug-producing microbes have also developed intrinsic resistance mechanisms of protection to avoid self-destruction during antibiotic production. Such mechanisms include; modification of the susceptible molecular target, efflux of the antibiotic, and response to antibiotic challenge by inactivation of the drug. Protein modification have been found in penicillin-binding proteins which underlie the methicillin-resistant *Staphylococcus aureus* (MRSA) phenotype, also 23S rRNA methylation is prevalent in erythromycin resistance, and reprogramming of peptidoglycan intermediates in vancomycin resistance.

Antibiotic resistance genes such as *mef* A, *mef* E and *mre* A code for transport (efflux) proteins which pump the antibiotic out of the cell, keeping intracellular concentrations low and ribosomes free from antibiotic. The pumps act through proton- or ATP-driven membrane transporters, this mechanism is of major importance in the macrolide resistance (Giovanetti et al., 2002; Motaneri et al. 2003; Walsh, 2003).

Dabbs reported in 2004 that four mechanisms were identified in rifampicin inactivation enzymes and they include; decomposition, ribosylation, glucosylation and phosphorylation. Although the first report of rifampicin modification enzymatic activity was in *Rhodococcus rhodococcus* and in *M. smegmatis* (Dabbs, 1987), enzymatic inactivation of antibiotics occurs with several of the natural product antibiotic classes, but has not yet been observed as a major route of resistance development for the classes of synthetic antibacterials e.g. the fluoroquinolones and the oxazolidinones. The most wide spread of clinical development to β-lactam

antibiotics is the expression of β-lactamase that hydrolyze the antibiotic (Bush and Mobashery, 1998).

1.7 Evolution of antibiotic resistance

The growing health crisis of antibiotic resistance has been of concern over the years. During the five to six decades that antibiotics have been in ever-widening therapeutic use, the development of antibiotic resistance has followed. The intensive and essentially constant exposure of bacteria to antibiotics has led to selective pressure for antibiotic-resistant bacteria to maintain those determinants, survive, and even dominate the bacterial populations (Walsh, 2003). It has been reported that spontaneous mutations (although infrequent) in the bacterial chromosome may modify the target site of the antibiotic thus interfering with normal function of the target site and they are passed on to all of the bacterial progeny (Schnappinger et al., 1996).

Bacteria have been so evolutionary successful because of their fast generation time, large population sizes and unique method of gene acquisition. Bacteria are capable of exchanging resistance genes among nearby bacterial cells by horizontal gene transfer, referred to as conjugation. Such genes could also be disseminated among bacterial population through the uptake of extacellular DNA, the process known as transformation, and there could be genetic material transfer through infections of bacteria by virus (transduction).

Human behaviour in the excessive and wrong use of antibiotics has also contributed largely to the rapid increase in the resistance to antibiotics. Kunin, 1993 reported that excessive use of antibiotics in ambulatory practice has contributed to the emergence and spread of antibiotic resistance in the community. This ranges from unnecessary medical prescriptions, to the prophylactic use in livestock to promote growth in healthy animals.

It has been established that some livestocks were given animal feed containing avoparcin, and humans coming into contact with the animals have been shown to carry vancomycin-resistance enterococci. The public health concern about the

emergence and dissemination of this resistant strain in food animals and the food supply caused the European union to ban the use of avoparcin in animal feed in 1997 (Hammerum, et al., 2000; Manson, et al., 2003; Witte, 1998). It is now a major concern that infection from penicillin resistant pneumococci has increased at an alarming rate during the past two decades, particularly the last five years. It is more of a concern that many of these resistant pneumococci also exhibit resistance to chloramphenicol, co-trimoxazole, macrolides and tetracycline (Appelbaum, 1995).

Inappropriate use of antimicrobials by exposure to low and/or prolonged concentrations also has a role in the selection of resistance. This may occur by killing susceptible normal flora and allowing pre-existing resistant organisms to survive, or by increasing the likelihood for subsequent colonization of the host with resistant bacteria (Guillemont, et al., 1998; Morita et al., 2000).

1.7.1 Erm genes

Two examples will be given where the origin of resistant determinants is known. One of the earliest discoveries in the evolution of antibiotic resistance mechanisms is the alteration of the rRNA structure, by single base mutations. This mutation involves the A_{2058} adenine or one of the adjacent residues (A_{2057} or A_{2059}) in the peptidyltransferase loop in domain V of the 23S rRNA. An X-ray determination of the 50S subunit of the ribosome soaked with erythromycin visualized the antibiotic bound in the peptidyltransferase cavity, in the vicinity of both A loops and P loops, and near A_{2058}. This action is monomethylation or dimethylation of the N_6 exocyclic amino group of A_{2058} by a gene known as erythromycin ribosome methylation (*erm*) gene, the modification enzyme by the gene produces the Erm phenotype and reduced affinity of the RNA for the antibiotic, without affecting the role of A_{2058} in peptidyltransferase function (Walsh, 2003).

Over the last 30 years, a large number of different rRNA methylase genes have been isolated from a variety of bacteria that range from *E. coli* to *Haemophilus influenzae* in Gram negative species, and from *Streptococcus pneumoniae* to *Corynebacterium* spp. in Gram positive species (Roberts et al., 1999). There are many cellular N-methyltransferases known, including RNA N-methyltransferases, and evolution of the

Erm methyltransferases from such a precursor is likely. In particular *ermE* is a constitutively expressed enzyme of the erythromycin biosynthesizer *Saccharopolyspora erythraea*, where it provides protection to the antibiotic producer and may be a recent progenitor of the *erm*s in the resistant pathogens.

The methyl-A_{2058}-specific enzymatic modification in 23S rRNA not only decreases affinity for macrolide antibiotics of the erythromycin class, but also for those of the lincomycin/clindamycin class, as well as for a third group, the streptogramin B (MLS_B) phenotype of ribosomal drug resistance. Many of the *erm* genes occur on conjugative or non-conjugative transposons, on chromosomes or sometimes plasmids. They are often associated with other antibiotic resistance genes, especially those against tetracycline (Clewell et al., 1995; Salyer et al., 1995).

While the *ermE* enzyme in *S. erythraea* is produced constitutively, the MLS_B phenotype is usually inducible by erythromycin resistant pathogens. Studies have also revealed that one of the *erm* genes, the *ermC* gene transcription in *Staphylococcus aureus* indicate that a 141-bp leader sequence just upstream of the *ermC* start codon adopts a secondary structure that sequesters the ribosome binding site so *ermC* transcription is blocked. In the presence of low level erythromycin, the secondary structure of the leader is postulated to refold, exposing the ribosome binding site, permitting *ermC* transcription, and producing ErmC methytransferase which methylates A_{2058} and protects the ribosome before lethal concentrations of erythromycin build up in the cell (Walsh, 2003).

1.7.2 Aminoglycoside phosphotransferase

Bacterial resistance to aminoglycoside antibiotics is mediated primarily by covalent modification of these drugs by a variety of enzymes. One such modifying enzyme, the 3´-aminoglycoside phosphotransferase, produced by Gram positive cocci such as *Enterococcus* and *Streptococcus*, inactivates a broad range of aminoglycosides by ATP-dependent phosphorylation of specific hydroxyl residues on the antibiotics. (Mckay and Wright, 1995).

Aminoglycosides read specific regions of the 16S rRNA in the 30S ribosome subunit by a hydrogen bonding network through the various hydroxyl and amino substituent on the cyclitol rings to provide a high-affinity docking site for this class of antibiotics. The enzymatic destruction strategy for aminoglycoside-resistant bacteria is to covalently modify those specificity-conferring hydroxyl and amino groups in the aminoglycosides and thereby interfere with recognition by the 16S rRNA (Livermoore, 2000; Poole, 2001). This kind of enzymatic modifications of the hydroxyl and amino groups on aminoglycosides is of normal electrophilic group transfer enzyme that participates in primary metabolism (Kotra et al., 2000; Wright, 1999).

ATP is a reactant used in O-phosphoryl transfers, by attack of the γ-PO_3 group by a hydroxyl or amino nucleophile and the reaction is irreversible. Other kinds of enzymatic modifications of hydroxyl and amino groups on aminoglycosides include; adenylylation and acetylation. It is likely that the antibiotic-inactivating enzymes will have evolved from phosphotransferases, adenyltransferases and N-acetyltransferases that had been utilized for normal biosynthetic process in the bacterial cells.

The determination of X-ray structure of the APH (3')-IIIa phosphotransferase from enterococci revealed high similarity to eukaryotic serine/threonine protein kinases, consistent with an evolution from protein substrate –OH recognition for attack on the γ-PO_3 of ATP to recognition of the aminocyclitol framework –OH under selective pressure to survive (Hon et al., 1997). Given the multiplicity of hydroxyl and amino groups in the successive generations of aminoglycosides to see clinical use, it is not surprising that modifying enzymes of distinct regiospecificity for phosphorylation, acetylation and adenylylation would arise. The presence of the genes for modifying enzymes on transmissible plasmids helps spread the resistance determinants and perhaps speed the evolution of catalytic activities toward newly introduced aminoglycosides.

1.8 Chloramphenicol

The antibiotic chloramphenicol was isolated from *Streptomyces venezuelae* in 1948 (Malik, 1972; Vining and Stuttard, 1995). It is a bacteriostatic antimicrobial which was introduced into clinical practice in 1949. It was widely used for some decades as a broad spectrum antibacterial agent, active against both Gram-negative and Gram-positive bacterial infections by blockage of bacterial protein synthesis as an amino acid antimetabolite. It was the first antibiotic to be manufactured synthetically on a large scale. Though it has fallen out of favour in the West due to very rare but serious side effects, but is still very widely used in low income countries because it is very inexpensive. It has been recently discovered to be a life saving cure for chytridiomycosis in amphibians (http://en.wikipedia.org/wiki/chloramphenicol).

1.8.1 Mode of action of chloramphenicol

The antibiotic functions by inhibiting peptidyl transferase activity of the bacterial ribosome, binding to A2451 and A2452 residues in the 23S rRNA of the 50S ribosomal subunit. It directly interferes with substrate binding, and is a simple molecule with a nitrophenyl serinol skeleton in which the amino group has been acylated with a dichloroacetyl group. The backbone clearly comes via the chorismate pathway, via amination, to produce 4-amino-4-deoxychorismate, that on 3, 3-sigmatropic rearrangement and dehydrogenative aromatization gives para-aminophenylalanine.

The conversion of the β-CH_2 to the CHOH of aminophenylserine probably also occurs while the amino acid is installed on an A-PCP reductase three-domain subunit. The aminophenylseryl-S-PCP would then be reductively cleaved by the third domain in the protein to release the aminophenylserol. This is two steps away from the antibiotic. One is the dichloroacetylation, presumed to occur from dichloroacetyl-CoA (Vining and Stuttard, 1995) and the other is the N-oxidation of the *para*-amino to *para*-nitro substituent. Some of the logic and mechanism of nonribosomal peptide synthetase selection, activation, and modification of amino acid monomers is utilized in these amino acid-based antibiotics (Walsh, 2003).

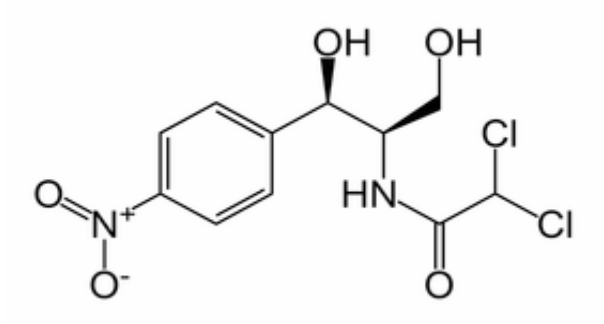


Fig. 2: Structure of chloramphenicol

Chemical formula: $C_{11}H_{12}C_{l2}N_2O_5$

Compound name: 2,2-dichloro-N-[(aR,bR)-b-hydroxy-a-hydroxymethyl-4-nitro phenethyl] acetamide

(http://www.freebase.com/view/wikipedia/images/commons_id924397)

1.8.2 Clinical uses of chloramphenicol

Because it functions by inhibiting bacterial protein synthesis, chloramphenicol has a very broad spectrum of activity: it is active against Gram-positive bacteria (including most strains of MRSA), Gram-negative bacteria and anaerobes. The original indication of chloramphenicol was in the treatment of typhoid, but the now almost universal presence of multi-drug resistant *Salmonella typhi* has meant that it is seldom used for this indication except when the organism is known to be sensitive. It may be used as a second-line agent in the treatment of tetracycline-resistant cholera. Due to its excellent CSF penetration (far superior to any of the cephalosporin), it remains the first choice treatment for staphylococcal brain abscesses.

It is also helpful in the treatment of brain abscesses due to mixed organisms or when the causative organism is not known. The antibiotic is active against the three main bacterial causes of meningitis: *Neisseria meningitides, Streptococcus pneumoniae and Haemophilus influenzae.* In the West, chloramphenicol remains the drug of choice in the treatment of meningitis in patients with severe penicillin or cephalosporin allergy. In low income countries, the World Health Organization recommends that oily chloramphenicol be used first-line to treat meningitis. In the U.S., it has been used in the initial empirical treatment of children with fever and a petechial rash, when the differential diagnosis includes *Neisseria meningitidis* and septicaemia, as well as

Rocky Mountain spotted fever, pending the results of diagnostic investigations. The antibiotic is also effective against *Enterococcus faecium*, which has led to it being considered for treatment of vancomycin-resistant *Enterococcus* (http://en.wikipedia.org/wiki/chloramphenicol).

1.8.3 Side effects of chloramphenicol

The most serious side effect of chloramphenicol treatment is aplastic anaemia, though the effect is rare, but it is generally fatal and has no treatment (Rich et al., 1950). There is no way of predicting who may have this side effect, it usually occurs weeks or months after the treatment has been stopped and there may be a genetic predisposition (Nagao and Maner, 1969). It is not known whether monitoring the blood counts of patients can prevent the development of this effect, but it is recommended that patients have a blood count checked twice weekly while on treatment. The highest risk is with oral chloramphenicol (affecting 1 in 24,000 - 40,000) and the lowest risk occurs with eye drops (affecting less than 1 in 224,176 prescriptions).

It is also common for chloramphenicol to cause bone marrow suppression during treatment. This is a direct toxic effect of the drug on the human mitochondria, but the effect is fully reversible once the drug is stopped and does not predict future development of aplastic anaemia. Intravenous chloramphenicol use has been associated with the so called gray baby syndrome, the phenomenon that occurs in newborn infants because they do not yet have fully functional liver enzymes, it therefore remains unmetabolized in the body and causes hypotension and cyanosis. The condition can be prevented by using chloramphenicol at the recommended doses and monitoring blood levels (Muhall et al., 1983).

1.8.4 Chloramphenicol resistance mechanisms

There are three mechanisms of resistance to chloramphenicol: reduced membrane permeability, mutation of the 50S ribosomal subunit and elaboration of chloramphenicol acetyl transferase. Resistance-conferring mutations at 50S are rare, and it is easy to select for reduced membrane permeability to the antibiotic in vitro by

serial passage of bacteria and this is the most common mechanism of low-level chloramphenicol resistance. The high level resistance is conferred by the *cat*-gene; this gene codes for an enzyme called chloramphenicol acetyl transferase which inactivates chloramphenicol by covalently linking one or two acetyl groups, derived from acetyl-S-coenzyme A, to the hydroxyl groups on the chloramphenicol molecule. The acetylation prevents the antibiotic from binding to the ribosome. Chloramphenicol resistance may also be carried on a plasmid that also codes for resistance to other drugs e.g. ACCoT plasmid, also called the R factors, and it mediates multi-drug resistance in typhoid.

1.9 Aims and objectives

Antibiotic resistance remains a major threat to human and animal health as a result of wide spread dissemination of resistance genes. These genes had existed long before the use of antibiotics: they have been detected in coliforms from glacial water and ice estimated at 2000 years old, evidence also suggests that R-genes long predate the use of antibiotic in medicine, evolving long ago in soil bacteria (Skinner et. al., 1983; Dancer et. al., 1997).

Study of the evolutionary origin of antibiotic resistant determinants is a key role in the determination of how the resistance evolved in the first place, and then to understand how the mechanisms have developed, hence the need for more work in this area of research.

In her work, Dorothy Nteo (2006) described a model system of heterologous expression of Gram positive DNA in a Gram negative background, thus providing another perspective of studying antibiotic resistance from an evolutionary perspective. The aim of this work therefore, was to express the DNA of a glutathione S-transferase gene from *Mycobacterium smegmatis* mc^2155 strain in different bacterial strains, and to obtain a mutant that would be used for molecular analysis with respect to antibiotic resistance. The DNA was from the clone pDN1 prepared by Duduzile Ndwandwe.

The specific objectives in this work include the following:

- expression of the *gst* gene in the Gram negative background;
- expression of the *gst* gene in the Gram positive background;
- phenotypic characterization of the clones from these bacterial strains;
- molecular analysis of the clones;
- carrying out mutagenesis on the clones; and
- characterization of additional clones from the libraries screened by Dorothy Nteo for macrolide resistance.

Chapter 2

Materials and Methods

2.1 Bacteria strains and plasmid vectors used in this work

Table 1: Bacteria strains used in this work

ORGANISM	STRAIN	CHARACTERISTICS	SOURCE
Escherichia coli	MM294-4	Nal-R, *end A1, hsdR17*	S. Quan
E. coli	GM2929-1	Rif-R, Dam^-, Dcm^-	E. Dabbs
E. coli	MM294-4 λ	Nal-R, *end A1,* , *hsdR17* lysogenised with phage λ	E. Dabbs
E. coli	CSH116	*mut D*, (*dna Q*), Δ(*gpt-lac*)5, *rpsL*, Tn10	E. Dabbs
E. coli	MM294-1	*rpoB*	E. Dabbs
Rhodococcus erythropolis	ATCC 4277		E. Dabbs
R. fascians	DSM20669		E. Dabbs
R. rhodochrous	HS1		S. Anderson
R. rhodochrous	HS6		S. Anderson
R. rhodochrous	HS13		S. Anderson
Gordonia rubropertincta	ATCC 25593		E. Dabbs
Bacillus subtilis	1 A3-1	Spc-R Antibiotic inactivation assay organism	E. Dabbs
Mycobacterium smegmatis	Mc^2155		E. Dabbs
Arthrobacter oxydans	C7		E. Dabbs

Table 2: Plasmid vectors used in this work

PLASMID	CHARACTERISTICS	SOURCE
pDA71	*E. coli-Rhodococcus* shuttle vector	E. Dabbs
pUC18/19	*E. coli* cloning vector *pBR322 ori,* MCS in *lacZ´,* Amp-R	Fermentas
pGem T-Easy	*E. coli* highly copy number vector*, lac Z'* gene, single 3´ thymidine, overhangs, *Sp6* and *T7* promoters	Promega
pNV18	*Nocardia-Rhodococcus* shuttle vector	Y. Mikami
pACYC184	Cmp-R, Tet-R	E. Dabbs
pDN1	Amp-R	D. Ndwandwe
pBA1	Kan-R	This work

2.2 Media and growth conditions

Luria-Bertani (LB) was used for the pre-culture of *E. coli* and other strains by inoculating the organism into 5 ml of the broth, *E. coli* MM294-4 was incubated at 37°C while the λ lysogen strain was incubated at 30°C. *Rhodococcus* and other Gram positive strains were incubated at 30^0C except *Mycobacterium smegmatis* which was incubated at 37°C.

Grown cultures of *E. coli* were kept on the wheel at room temperature while those of the Gram positive strains were kept at 4°C for short term storage. Grown strains on agar plates were also kept at 4°C for short term storage while freshly grown strains were kept in 33% glycerol at -70°C for long term storage.

2.3 Determination of MIC due to pDN1 and pBA1

Plates were prepared and dried overnight at 37°C. The plates contained 100 µg/ml Nal and 100 µg/ml Amp, plus different concentrations of the test antibiotics up to the concentration that would inhibit the growth of the clones in *E. coli*, while 100 µg/ml Kan was added to the plates for the Gram positive strains. The resistant clones to be used and the vector control were streaked on the LA Amp plates in order to obtain actively growing single colonies which were picked and re-suspended in 200 µl sterile distilled water, in the wells of the plate replicator. Preparation of different concentrations of antibiotics used is contained in appendix C on page 79.

2.4 DNA preparations

2.4.1 *E. coli* mini plasmid preparation

This procedure was carried out using alkaline lysis method; single colonies of bacteria or a group of clones was inoculated in 1 ml of LB supplemented with 100 µg/ml Amp and was grown on the shaker at 37°C overnight. The cultures were transferred into 1.5 ml Eppendorf tubes and harvested by spinning for 30s in micro-centrifuge to pellet the cells. The supernatant was discarded and the pellet re-suspended in 80 µl of solution I. 160 µl of solution II was then added and the contents mixed gently by the inversion of the tubes, they were then left to stand on the bench for 15 min or more at room temperature. 120 µl of solution III was added and the contents mixed vigorously by shaken, they were allowed to stand on ice for 5 min before centrifuging for 5 min at 10,000 rpm in the cold.

The supernatant was gently removed, put into a sterile Eppendorf tube and warmed at 42°C for 2 to 10 min. 220 µl of isopropanol was added and mixed gently by inversion; it was then allowed to stand at room temperature for 5 min. Precipitation of the DNA was carried out by centrifuging for 5 min at room temperature, the supernatant was discarded and the pellet washed with 150 µl of ethanol, mixed by gentle inversion and spun at room temperature for 1 min. The supernatant was discarded and carefully blotted on paper towel; the DNA was then dried in the speed-vacuum for 20 min. The dried pellet was re-suspended for 2 hr in 100 µl sterile distilled water. The

concentration of the DNA was checked by running 5 µl of the re-suspended DNA on 0.8% agarose gel with the ladder marker. Appendix B on page 75 contains the recipes for various solutions used in this work.

2.4.2 *Rhodococcus* mini plasmid preparation

Bacteria cultures were inoculated into 300 µl of LBG (LB + 2% glycine) containing the appropriate antibiotic and grown at room temperature for 2-3 days. 200 µl of the culture was transferred into a sterile Eppendorf tube and microfuged for 1 min at the room temperature. The pellet was resuspended in 200 µl TE to which a 5 mg/ml lysozyme had been added, the suspension was then incubated at 37^0C for 1 hr. A 40 µl volume of 5.0 M KAc (pH 6.0) was added, mixed vigorously, and left on ice for 5 min. The content was microfuged for 5 min at 4°C and the supernatant was transferred into a sterile Eppendorf tube. Purification of the DNA was carried out by the phenol-chloroform method, and the DNA was precipitated with 1.0 M NaCl and 96% ethanol. The plasmid DNA was then dried in the Speedvac for about 20 min and the pellet obtained was resuspended in 150 µl of sterile distilled water for 2 hr.

2.4.3 Large scale plasmid DNA isolation

The appropriate strain was inoculated into 100 ml LB supplemented with 100 µg/ml Amp and was grown overnight on the shaker at 37°C. The culture was harvested by pelleting in centrifuge in a JA 10 Beckman rotor at 6,000 rpm for 10 min. The cell pellet was re-suspended in 5 ml solution I in JA 20 tube. 10 ml of solution II was added and mixed gently by inverting the tube to lyse the cells; it was then incubated at room temperature for about 30 min. 7.5 ml of solution III was added to precipitate proteins, lipids and cell wall material, and the tube shaken vigorously for 15-20s. The tube was placed in ice/water slurry for 15 min and the precipitate removed by centrifuging in a pre-chilled JA 20 rotor for 10 min at 15,000 rpm.

The supernatant was decanted into a fresh JA 20 Beckman centrifuge tube as the white pellet was discarded. The supernatant was warmed in 37°C water bath, 12 ml of isopropanol was added, and the tube was covered with parafilm, mixed gently by inversion and placed at room temperature for 15 min. The DNA was precipitated by

centrifuging in JA 20 rotor at room temperature for 10 min, 15,000 rpm. The pellet was washed with 2 ml 96% ethanol and re-centrifuged for 1 min at room temperature. The pellet was dried in the speed-vacuum for 20 min and re-suspended in 4 ml TE buffer for 2 hr with gentle agitation in a 30^0C water bath. 4.1g of CsCl was added and dissolved by gentle agitation, 400 µl of a 1% solution of EtBr was also added and the refractive index was adjusted to 1.387-1.389 using a refractometer. A Pasteur pipette was used to load the solution into a quick seal tube and sealed with heat.

The solution was then placed in a vertical VTi 65.2 rotor and spun in an ultra-centrifuge at 10°C, 45,000 rpm for 16 hr. Plasmid DNA was extracted (the lower band in the tube) with a hypodermic syringe and needle; this was then transferred into a sterile Eppendorf tube.

2.4.4 Ethidium bromide removal

Ethidium bromide was removed from the plasmid DNA by adding 150 µl of butanol into the tube, it was inverted several times to mix the contents and the butanol layer which appeared on top was removed with a Gilson pipette. This process was repeated until the ethidium bromide was not visible in the tube, usually about three to four times. The DNA was stored in CsCl at -20°C until needed.

2.4.5 Precipitation of DNA

A 100 µl of the DNA from the large scale isolation was measured and transferred into a sterile Eppendorf tube, twice this volume of sterile distilled water was added, more than two times the combined volume of ethanol was added and the contents mixed by inversion. The DNA was recovered by spinning in a microfuge for 20 min at 4°C. The supernatant was gently decanted off and discarded while the tube was blotted on paper towel to remove excess liquid and then dried in the speed-vacuum for 20 min. The pellet was re-suspended in 200 µl of sterile distilled water.

2.4.6 Low gelling agarose electrophoresis

Plasmid DNA was digested with restriction enzyme(s) to release the band of interest; 5 µl of tracking dye was added to it in the tube and loaded on 0.6% low gelling agarose. The gel electrophoresis tank containing 0.5× TBE was placed at 4^0C overnight before use. The voltage was set at 100V, the current at 20 amp and the DNA was allowed to run on the gel until the tracking dye almost migrated to the edge of the gel. The bands of interest on the gel were cut out while viewing under UV light at 366 nm wavelength using a sterile scalpel blade.

2.4.7 DNA fragment purification

DNA extraction could be done by melting the excised bands on gel at 60°C in the water bath or by the freeze squeeze method. The latter was employed in this work as the excised band of interest was chopped into pieces in the Eppendorf tube by a sterile spatula, the tube closed, wrapped in a plastic and frozen at -70°C for more than 30 min. It was then thawed between 10-15 min and spun for 10 min at 4°C. The supernatant was extracted and transferred into a new sterile Eppendorf tube.

2.4.8 Phenol-chloroform extraction

For phenol-chloroform extraction to be carried out, the DNA recovered from $CsCl_2$ density gradient in the tube must be up to 300 µl. Phenol at a volume of 1/3 of the content was added to the DNA, gently mixed and spun in the microfuge for 5 min at 4°C. The top layer was extracted and transferred into a sterile Eppendorf tube for the process to be repeated three times. Chloroform at a volume of 1/3 was then added to the DNA, mixed gently, and spun for 30s at room temperature. The top layer was then extracted and transferred into a sterile Eppendorf tube.

2.4.9 Salt-ethanol precipitation

One tenth volume of 1M sodium chloride was added to the DNA gently mixed and ethanol at two times the total volume of the content was added and mixed by inverting the tube. The precipitated DNA was recovered by spinning in the microfuge for 20

min at 4°C. The supernatant was decanted off and the DNA was dried in the speed-vacuum for 20 min. The dried pellet was re-suspended in 50 µl of sterile distilled water and the DNA concentration was checked by running about 2 µl on a 0.8% agarose gel to determine the volume needed for ligation.

2.5 DNA manipulations

2.5.1 Restriction enzyme digestions

Restriction enzymes were obtained as commercial products from New England Biolabs, Amersham, Promega or Boehringer Mannheim and the total volume of the mixture for digestions were kept at 15 µl. 13.5 µl of the DNA was transferred into a sterile Eppendorf tube to which 1.5 µl of 10× appropriate buffer was added and mixed gently, briefly spun and 0.5 µl of the enzyme then added to the tube, tapped briefly and microfuged for 1-2s followed by incubation at the required optimum temperature from the manufacturer's guide. In order to carry out a double digestion, the guide from the manufacturer was followed to know the buffer best suited for both enzymes as well as the ratio of the enzymes and the required optimum activity temperature for incubation. Where no common buffer could be found, double digestions were done sequentially starting with the enzyme that required the lower salt. All restriction digestions were carried out for a minimum of 3 hr. A "no DNA" control was included to allow for comparison.

2.5.1.1 Extraction of DNA from the AVS clone

The resistant clone was inoculated into a 100 ml volume of LB supplemented with Amp at 100 µg/ml for a large scale DNA isolation. It was then grown on a shaker overnight at 37°C, the plasmid DNA was extracted, ethidium bromide was removed, and CsCl gradient centrifugation was performed to purify the DNA.

2.5.1.2 Extraction of erythromycin resistance insert

The DNA was digested overnight with *Hind* III in order to separate the insert from the vector DNA. This was followed by 0.6% low gelling agarose electrophoresis. The

band on the agarose carrying the insert was excised with a sterile scapel on a trans-illuminator, phenol-chloroform extraction was carried out and the DNA was precipitated using the salt-ethanol method.

2.5.2 Ligation

T4 DNA ligase from Boehringer Mannheim was used in this work to join the DNA with the vector at the required site(s) for the required library or in preparation for sequencing. The total ligation volume was kept at 20 µl and the ligation was performed at 22°C for 16-22 hr.

2.5.3 Dephosphorylation

This procedure was carried out after the vector to be used for ligation was treated with the restriction enzyme and incubated. This is to prevent it from re-ligating itself and denying the introduction of the insert. It acts by removing the 5´-phosphates that are required for ligation by DNA ligase however, not affecting its ligation to the insert that retains its own 5´-phosphates. Calf intestinal alkaline phosphatase from Boehringer Mannheim was used in this work. It was inactivated by phenol-chloroform extraction after an overnight incubation and the DNA was recovered by salt-ethanol precipitation method.

2.6 Transformations

2.6.1 *E. coli* calcium chloride transformation

E. coli strain was inoculated into 5 ml LB supplemented with 100 µg/ml Nal and was grown on the wheel at 37°C overnight. 200 µl of this culture was added to pre-warmed 20 ml LB supplemented with 0.5% glucose and grown with vigorous aeration on the shaker at 37°C until an $O.D_{590}$ of 0.2-0.4 had been reached (within 1 hr 45 min to 2 hr). The cells were then placed in ice-water slurry for 5 min and then centrifuged for 5 min at 10,000 rpm in a pre-chilled JA 20 Beckman rotor. The supernatant was decanted off, the cells re-suspended in 10 ml transformation buffer and left on ice for 15 min or more. The cells were centrifuged again for 5 min at 10,000 rpm, the

supernatant was discarded, the cells were gently re-suspended in 1.33 ml transformation buffer and left to stand on ice for 2-24 hr.

The DNA to be used was placed on ice for more than 10 min and to each tube of 10 µl, 50 µl of competent *E. coli* cells were added, tapped gently to mix and left on ice for 10 min to allow diffusion of the DNA into the host/recipient cells. The competent cells were heat shocked for 90s at 44°C to transform the DNA and 0.5 ml of LB was added to the tube(s). Incubation was carried out at 37°C for 1 hr to allow for phenotypic expression of antibiotic resistance, the cap(s) of the tube(s) were left opened during incubation.

The transformed cells were then spread on plates that had been dried at 37°C for 24-48 hr which contained 100 µg/ml Amp; chloramphenicol was also added when necessary. The plates were incubated overnight at 37°C. Competent cells only were also spread on the plate to serve as the control and vector only DNA also included during ligation.

2.6.2 Electroporation

Cultures of *Rhodococcus* and other Gram positive strains were inoculated into 5 ml LBSG with 2% glycine for 2 days at 30°C, the culture was then transferred into a sterile Eppendorf tube and spun for 1 min in a slow microfuge. The supernatant was discarded and 1 ml of sterile distilled water was added to wash the pellet, this process was repeated three times, a 100 µl volume of the cells was transferred into a sterile Eppendorf tube and about 1-10 µl of DNA was added. It was mixed by bubbling air through the mixture and allowed to stand for 5 min on the bench.

Cells were transferred into a cuvette and tapped gently to make sure that the cells were evenly distributed. The outside of the cuvette was wiped to dry and placed in the white chamber of the Gene Pulser to electroporate the cells. The parameters under which the electroporation was carried out include; 25µF capacitance, 2.5kV voltage and 400Ω resistance. The time constant at which electroporation took place was recorded and LB was added to the cells immediately, it was then allowed to stand on

the bench for 10 min, followed by incubation on the shaker at 30 rpm (30°C) for 3 hr. Aliquot of the cells were then added onto the relevant antibiotic plates.

2.7 Mutagenesis

2.7.1 Spontaneous mutation

The *Mycobacterium smegmatis gst* gene carried in plasmid pDN1 was transformed into *E. coli* MM294-4 strain using the calcium chloride method and into *Rhodococcus* by electroporation. The transformants containing the insert were inoculated into 5 ml LB + 100 µg/ml Nal to select for the growth of *E. coli*, 100 µg/ml Amp was added to maintain the plasmid in the Gram negative strain, while 100 µg/ml Kan was added to maintain the plasmid in the Gram positive strains. A 100 µl volume of the pre-culture was spread on LA Amp plates as well as LA Kan plates at high concentrations of the test antibiotic(s). The plates were incubated at 37°C and 30°C for *E. coli* and the *Rhodococcus* strains respectively. Colonies were found after 2 days in *E. coli* and after 4 days in *Rhodococcus*.

2.7.2 Chemical mutagenesis (EMS)

Strains carrying the insert were grown in 5 ml LB with 100 µg/ml Nal + 100 µg/ml Amp, and 5 ml LB + 100 µg/ml Kan up to O. D. 0.2-0.4 (590nm) in *E. coli* and *Rhodococcus* strains respectively. A 100 fold dilution was made from the overnight culture in LB and grown in the flasks on the shaker at 37°C and 30°C for about 4 hr and overnight. 1 ml of the 100 fold diluted culture was transferred into a sterile Eppendorf tube and microfuged for 30s, the pellet obtained was washed with 1 ml buffer E (pH 5.0). 2% v/v of the mutagen was dissolved in the buffer and 0.5 ml volume of this was added to the cells that had been resuspended in 0.5 ml of the buffer to a 1 ml volume.

The content was incubated at 37°C in a pre-set water bath for 1 hr. The mutagen was removed by spinning down in the microfuge for 30s and the cells washed with buffer E twice to stop the reaction. The cells were dissolved with LB and added to the tube containing 5 ml LB + 100 µg/ml Nal and 100 µg/ml Amp, and 5 ml LB + 100 µg/ml

Kan. They were then incubated at 37°C and 30°C overnight and a 100 µl of the mutagenized cells were then spread on selective plates.

2.7.3 Marker Rescue

It was necessary to verify that the mutations obtained from both the spontaneous selection and after chemical mutagenesis were plasmid borne, and to ascertain this the pools of colonies found on different selective plates were washed off with 2-3 ml LB into a McCartney bottle and the content briefly vortexed to have a homogenous mixture. 100 µg/ml Amp was added into each bottle and grown for 4 hr on the shaker at 37°C. Plasmid DNA extraction was carried out on the grown cultures according to alkaline lysis method. The DNA obtained from the mini prep was used to transform *E. coli* MM294-4 strain via calcium chloride transformation method and cells were spread on selective plates in which the same concentration of the test antibiotic(s) that was used for the mutagenesis had been added, ampicillin was also added to maintain the plasmid.

2.7.4 Plasmid curing

This procedure was carried out in order to remove the plasmid from the *E. coli* strain used for transformation to verify whether the mutation was carried on the plasmid in the clone obtained from mutation, or as a result of changes in the chromosomal DNA of *E. coli.*

A colony was inoculated into 5 ml LB and was grown to stationary phase at 37°C overnight. A 1000 fold dilution was carried out on the culture and grown on the wheel at 37°C overnight again. This procedure was repeated for 5 days to obtain a ~50 generation from the dilutions at which stage the strain should have lost the plasmid. Serial dilutions were made from the culture obtained after the fifth day up to 10^{-9} and 100 µl of the dilutions plated on LA Nal. Single colonies were picked from these plates and were patched unto LA + Cmp, LA and LA Amp plates. Pre-cultures of the strain from the different plates were made by inoculating into 5 ml LB and grown on the wheel at 37°C overnight; these were used for transformation with the pool of DNA

obtained from spontaneous mutation, while the parental DNA was also included as the control.

2.7.5 Error prone PCR (epPCR)

The sequenced oligonucleotide from Fermentas was used as the template and epPCR was used to introduce random point mutations into the *rgt* ORF. This was achieved by adding a 0.5mM concentration of $MnCl_2$ and increasing the concentration of the $MgCl_2$ (10mM) in the reaction mixture. The increased concentration is usually of insignificant impact on the level of amplification obtained from the PCR.

M13/pUC sequencing primer (-20), 17-mer from Fernentas was used with 5´-d (GTAAAACGACGGCCAGT)-3´ and 5´-d (CAGGAAACAGCTATGAC)-3´ as the forward and reverse primers respectively. The reaction mixture was carried out in a thin-walled PCR tube and it was amplified by using a MJ MINI ™ personal thermocycler supplied by Biorad. The following tables show the composition of the components used and the amplification parameters under which cycling was carried out;

Table 3a: Table showing the reaction mixture for error prone PCR

Component	**Final concentration**	**Control vol (µl)**	**Component vol for 50µl mixture (µl)**
Water, Nuclease free	-	30.75	28.5
10× Taq buffer	1×	5	5
2 mM dNTP mix	0.2 mM each	5	5
Forward primer	0.5 µM	2.5	2.5
Reverse primer	0.5 µM	2.5	2.5
Taq DNA polymerase	1.25 units	0.25	0.25
25 mM $MgCl_2$	2.0 mM	4	4
Template DNA	0.1 µg	-	2.25
		50	50

Table 3b: Table showing the cycling conditions for the error prone PCR

Cycle	Temperature (°C)	Time (minute)
First: Initial Denaturation (DNA Template)	95	10
Second: Denaturation Annealing Extension	95 45 72	2 2 4
Third: Final extension	72	10

The second cycle was repeated 25 times to generate the amplification of ~1.8 Kb *rgt* gene region. The PCR product was then analyzed on a 1.2% agarose gel electrophoresis to verify the amplification of the fragment of interest. Thereafter, the product was digested and run on a 0.6% low gelling agarose gel electrophoresis to separate the fragment. The DNA was excised from the gel with a sterilized scalpel while viewing under UV light at 366 nm and the DNA was extracted by phenol-chloroform method, followed by salt-ethanol precipitation, re-suspended in sterile distilled water and ligated into pDA71 for transformation in *E. coli* MM294-4 for post amplification analyses.

2.8 Plate assays of antibiotic inactivation

2.8.1 Antibiotics concentration calibration

Prior to the inactivation assay experiments, antibiotics to be tested for inactivation assay were calibrated on the plates in order to determine the concentration of the antibiotic that will give the required zone of inhibition. Different concentrations of antibiotics were added to 5 ml LB in the test tube and were incubated on the wheel at 37°C overnight. A 100 µl of assay organism was spread on the plate and agar plugs were removed by the sterile end of a Pasteur pipette. A 70 µl volume of the different

concentrations of antibiotics were added to the holes, incubated at 4°C for 4 hr to allow diffusion and was later incubated at 37°C overnight.

Zones of inhibition were measured, calibration curve was drawn and the best concentration at which the assay was to be carried out was determined, this concentration was made use of in all the inactivation assay experiments carried out.

2.8.2 *In vivo* inactivation

E. coli cultures containing plasmid pDN1 *gst* gene was grown in 5 ml LB overnight at 37°C on the wheel. The overnight culture was diluted 100 fold and grown for 4 hr in LB on the shaker at 37°C, the cells were then challenged with the test antibiotic at a concentration of 20 µg/ml and grown overnight at 37°C. *Bacillus subtilis* 1 A3-1 and *E. coli* MM294-4 strains were used as the assay organisms. They were diluted 1:100 and spread on LA ½ agar plates (the plates contain half of the normal agar) + 200 µg/ml Spc/Str. Agar plugs were removed by the sterile end of a Pasteur pipette and 70 µl of the challenged culture(s) poured into each well. It was incubated for 4 hr in the cold room to allow diffusion, followed by an overnight incubation at 37°C. Diameters of zones of inhibition found on the plate(s) were measured and recorded.

2.8.3 *In vitro* inactivation

Clones were grown in 10 ml LB with 100 µg/ml Nal and 100µg/ml Amp + the test antibiotic(s) in a flask on the shaker at 37°C overnight. The cultures were centrifuged at 15,000 rpm in a JA 20 Beckman rotor for 5 min to pellet the cells. The cells were washed with sonication buffer twice to remove the antibiotic. This was followed by the extraction of crude cells by sonication for 10s in ice about 3 or 4 times and spun down in the microfuge at 4°C. The clarified supernatant was transferred into a sterile Eppendorf tube where antibiotic with the appropriate co-factor were added, this was then followed by incubation at 37°C overnight.

The reaction mixture with the following constituents was used:

	Vol (µl)
Sonication buffer	31.5
Antibiotic (Cmp) 4 mg/ml	1.5
Acetyl-CoA 40mM	15
Magnesium acetate 2mM	15
Glutathione 80mM	7.5
LDR 1 µg/ml each	6
Cell homogenate	58.5
Total	135

The reaction was stopped after the incubation period with the enzyme proteinase K (300 µg/ml) and boiled at 56°C for 15 min. The assay organism was diluted 1:100 and spread on the LA half agar plate + 200 µg/ml Spc/Str. Agar plugs were removed by the sterile end of a Pasteur pipette and 70 µl of the reaction mixture was added to the wells. The plate was incubated for 4 hr at 4°C to allow diffusion; this was then followed by an overnight incubation at 37°C. Diameters of the zones of inhibition were measured and compared with the control.

2.9 Automated DNA sequencing

2.9.1 Preparation of DNA for sequencing

GFX micro plasmid prep kit from Amersham Biosciences was used to prepare the DNA for sequencing. The protocol is an improved alkaline lysis procedure for the purification of DNA as outlined in the instruction manual (2004). A 2 ml volume of an overnight culture was prepared using the clone from the transformation plate.

Cell lysis was carried out by transferring 1 ml of the culture into a 1.5 ml microfuge tube. It was then spun for 30s to harvest the cells. Supernatant was removed by aspiration without disturbing the cell pellet and was re-suspended in 150 µl of solution I with vigorous vortexing. 150 µl of solution II was added and mixed by inverting the tube 15 times. A 300 µl volume of solution III was added at the soonest

time possible and mixed by inverting the tube until a flocculent precipitate appeared, this was continued until the precipitate was evenly dispersed. It was important for the precipitate to be broken up effectively to ensure consistent yield, the mixing was however done gently in order not to share genomic DNA with the plasmid DNA and co-purify them. The content was centrifuged for 5 min at room temperature to pellet debris and one GFX column for each prep was prepared while centrifuging by placing the column in a collection tube.

The supernatant was transferred to the prepared GFX column and it was incubated for 1 min at room temperature, followed by spinning for 30s. The flow through was discarded by emptying the collection tube and 400 µl of wash buffer was added to the column. The buffer was removed by centrifuging for 1 min at room temperature and the matrix dried prior to elution. The GFX column was transferred to a fresh Eppendorf tube and a 100 µl volume of TE buffer was added directly to the top of the glass fiber matrix in the GFX column. The sample was incubated for 1 min at room temperature, and then centrifuged for 1 min to recover the purified DNA.

2.9.2 Spectrophotometric Analysis

Concentration of the plasmid DNA extracted from the mini prep was checked on the Nano drop Spectrometer, supplied by Inqaba Biotec in preparation for sequencing. A concentration of 10 ng/µl was needed for 100-500 bp, and 20 ng/µl for 500 - 1000 bp, an additional 100 ng/µl was needed for every additional 500 bp of plasmid DNA up to 5 Kb. The DNA was diluted by sterile distilled water until the required concentration was achieved. It was then sent to Inqaba Biotec for automated sequencing.

2.9.3 Sequencing Analysis

Bioinformatics programmes were used to analyze the results obtained from the sequencing analysis by converting the result from Inqaba into nucleotides with the fasta format. The data were then analyzed using the following web addresses:

Table 4: Website addresses of the sequence analysis programs

Program	Web Site
FASTA	http://www.ebi.ac.uk
BLAST	Http://www.ncbi.nlm.nih.gov/BLAST
WEBCUTTER	http://www.ccsi.com/cgi-bin/firstmarket/cutup
ExPASy	http://www.expasy.org
GENEMARK	http://genemark.biology.gatech.edu/GENEMARK/webgenemark.html

Chapter 3

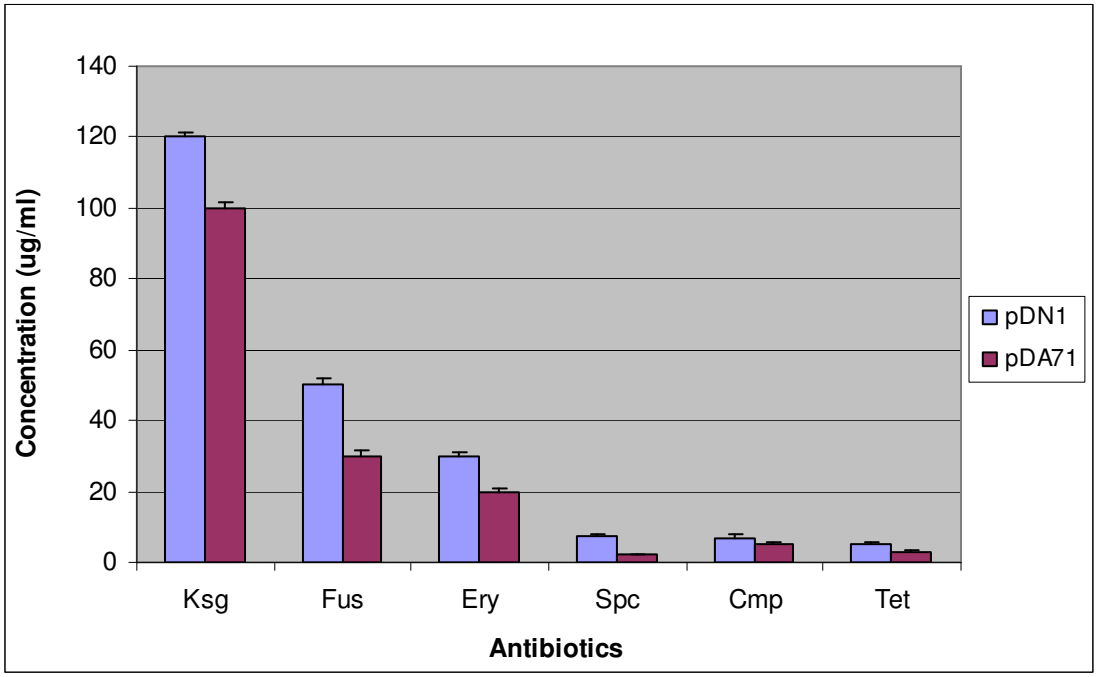
RESULTS AND DISCUSSION

3.1 Expression of GST gene in *E. coli*

Plasmid pDN1 was prepared by Duduzile Ndwandwe. She isolated the *gst* gene from *Mycobacterium smegmatis* mc^2155 strain and amplified it by the polymerase chain reaction method. The primers she constructed contain: 5´ GTGATGGCTCAGGGCACCTACGTC 3´ for the forward reaction, and 5´ TCAGCCCGCGCCGTGACCGGCG 3´ for the reverse reaction. The 1000 bp size DNA fragment was excised, purified and cloned into pUC18 vector. It was sent for sequencing, and the sequence analysis confirmed glutathione S-transferase gene (D. Ndwandwe, personal communication).

3.2.1 Determination of MIC with respect to pDN1 in *E. coli*

To determine the minimum inhibitory concentrations (MICs) conferred by pDN1, actively growing cells were used for spot tests. This was carried out at an increasing concentration of chloramphenicol, kanamycin, kasugamycin, erythromycin, tetracycline, spectinomycin, streptomycin, and fusidic acid in *E. coli* MM294-4 strain on LA plates, and *E. coli* MM294-1 strain was used for Nalidixic acid because of its resistance to MM294-4 strain, shuttle vector pDA71 was used for the cloning of pDN1 and therefore was necessary to be included in this experiment as the vector control. The plates were supplemented with 100 µg/ml Amp + 100 µg/ml Nal (except the plate where Nal was tested), and were incubated at 37°C for 24 hr and 48 hr respectively. The MIC increase was < 2 fold for most of the antibiotics tested but for Kan which showed a 3 fold increase, and no significant difference was detected between the plates incubated for 24 hr and those of 48 hr. The results obtained after carrying out the experiment five times are shown in figures 3a and 3b.



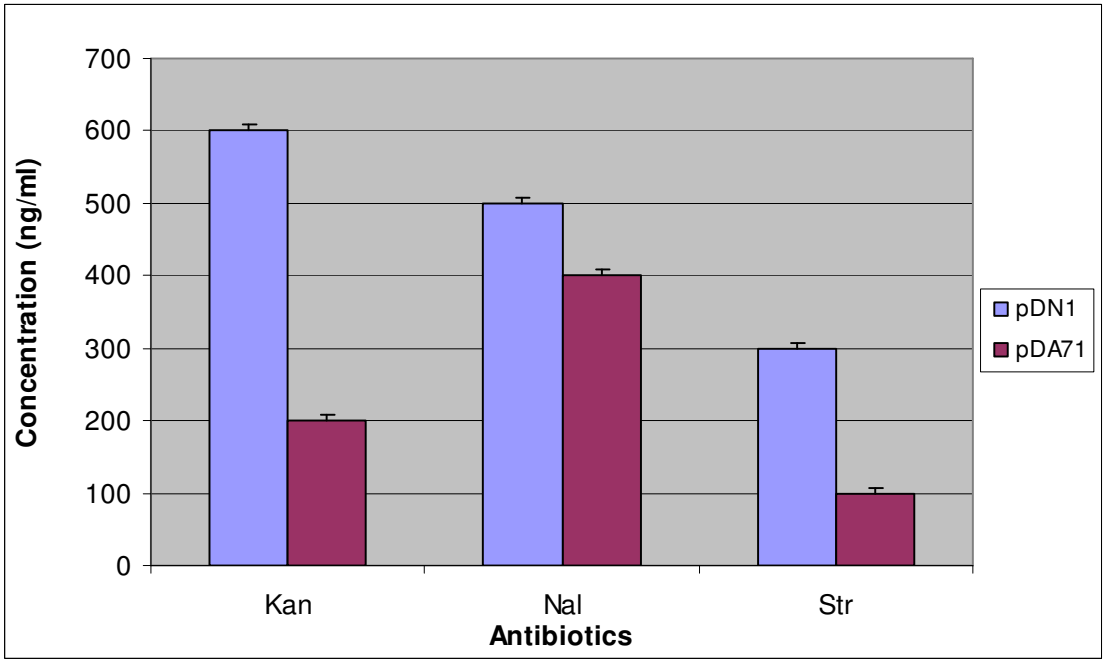


Fig. 3a and b: MICs due to pDN1 in *E. coli* MM294-4 and 294-1 strains at 37°C. Control without *gst* gene: pDA71

3.2.2 MIC of pDN1 with respect to tetracycline and its derivatives

Spot tests were further carried out on the strain *E. coli* MM294-4 transformed with pDN1 for expression of resistance to tetracycline and its derivatives: chlortetracycline (chlortet) and oxytetracycline (oxytet). The plates were again incubated for 24 hr and 48 hr. The results showed that the resistance observed to tetracycline was not due to its derivatives with the highest MIC at 2µg/ml in chlortetracycline after 24 hr, and no growth was found in oxytetracycline even after a 48 hr incubation period.

The MIC of tetracycline was slightly higher than the vector control and the result after 48 hr of incubation showed that the best MIC in tetracycline is at 5 µg/ml concentration as manifested by secondary mutant at 6 µg/ml after a 48 hr incubation period.

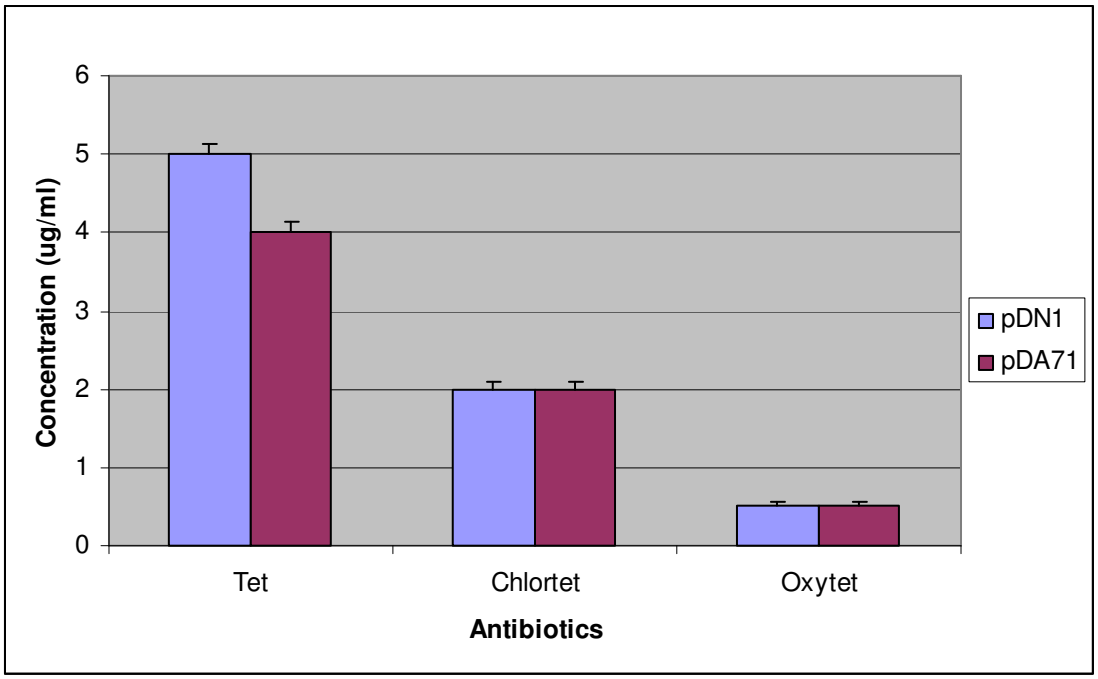


Fig. 4: MIC of pDN1 with respect to tetracycline and its derivatives

3.2.3. Phenotypic characterization of *M. smegmatis* chloramphenicol resistant clone

Spot tests for chloramphenicol resistance were carried out on LA plate and the plates enriched with 0.5% glucose (LA(Glu), 80% phosphate buffer (L(A-N), and 0.5% glucose + 80% phosphate buffer (L(A-N)Glu). Effect of temperature on the growth of the clone in those media was also tested by incubating the plates at varying degrees of temperature i.e. 24°C, 37°C and 42°C. The result showed a < 2 fold MIC increase in all the media used and there was no noticeable difference on the growth at 24°C and 37°C. The best MIC differences were observed on L(A-N) plates at 42°C where the clone conferred the highest resistance to the test antibiotic. A slight increase in MIC was also noticeable on the L(A-N)Glu plate at this temperature compared with the other plates, hence it could be concluded that LA supplemented with phosphate buffer at 42°C gave the best phenotypic expression of the clone in *E. coli*.

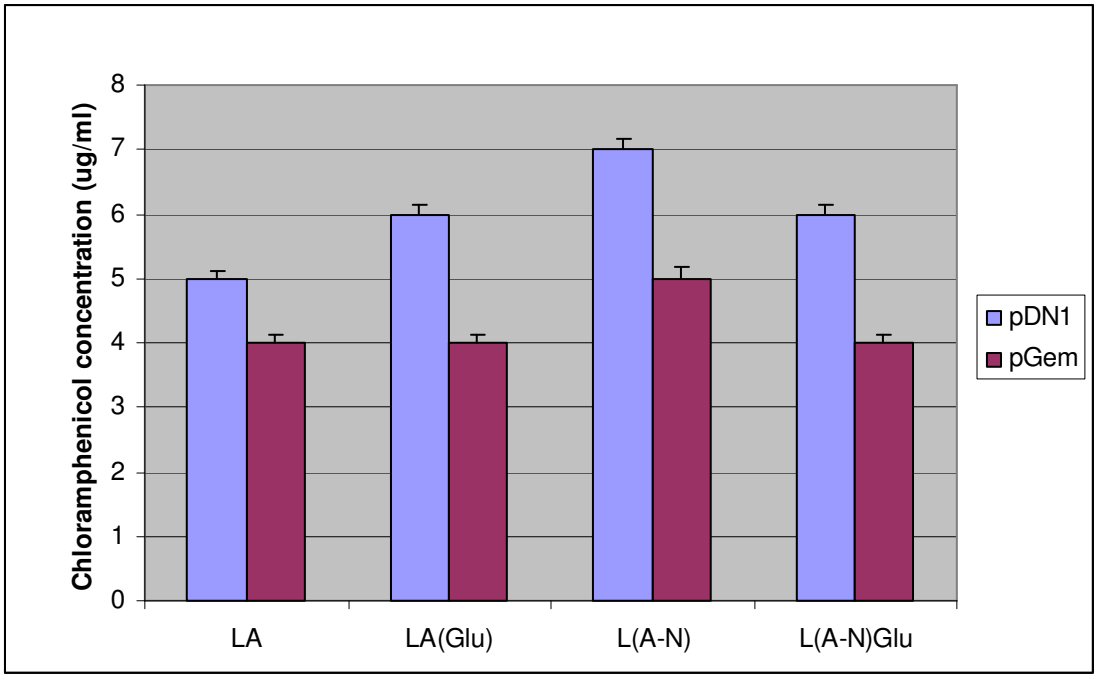


Fig. 5: Effect of different rich media compositions on chloramphenicol resistance phenotype in *E. coli* MM294-4 at 24°C

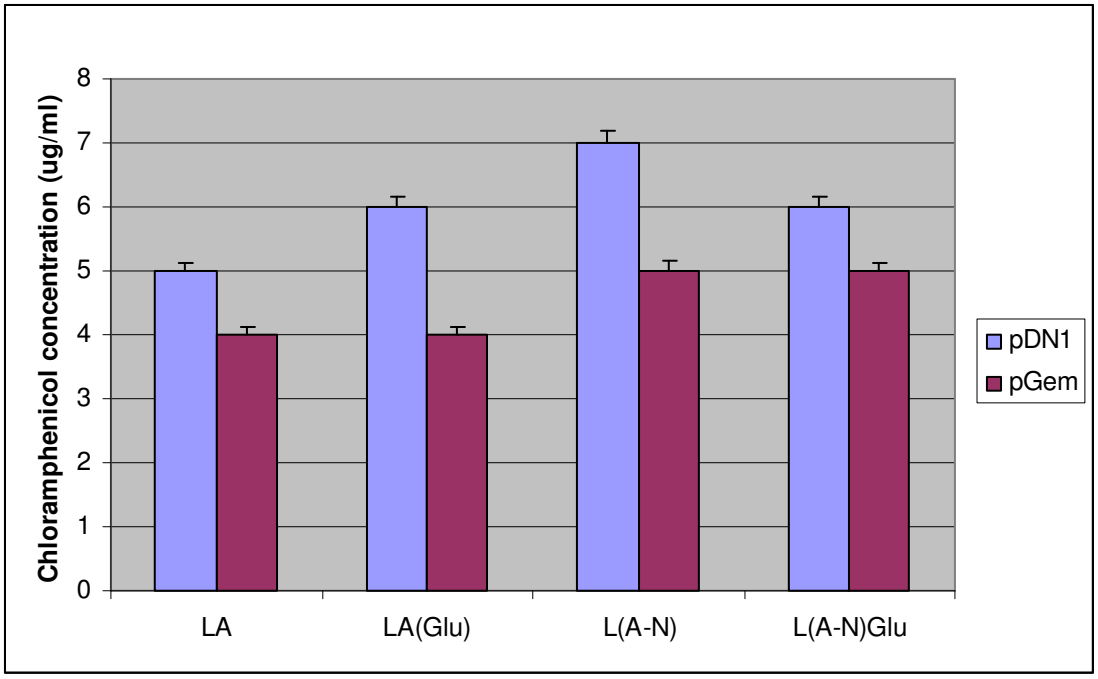


Fig. 6: Effect of different rich media compositions on chloramphenicol resistance phenotype in *E. coli* MM294-4 at 37°C

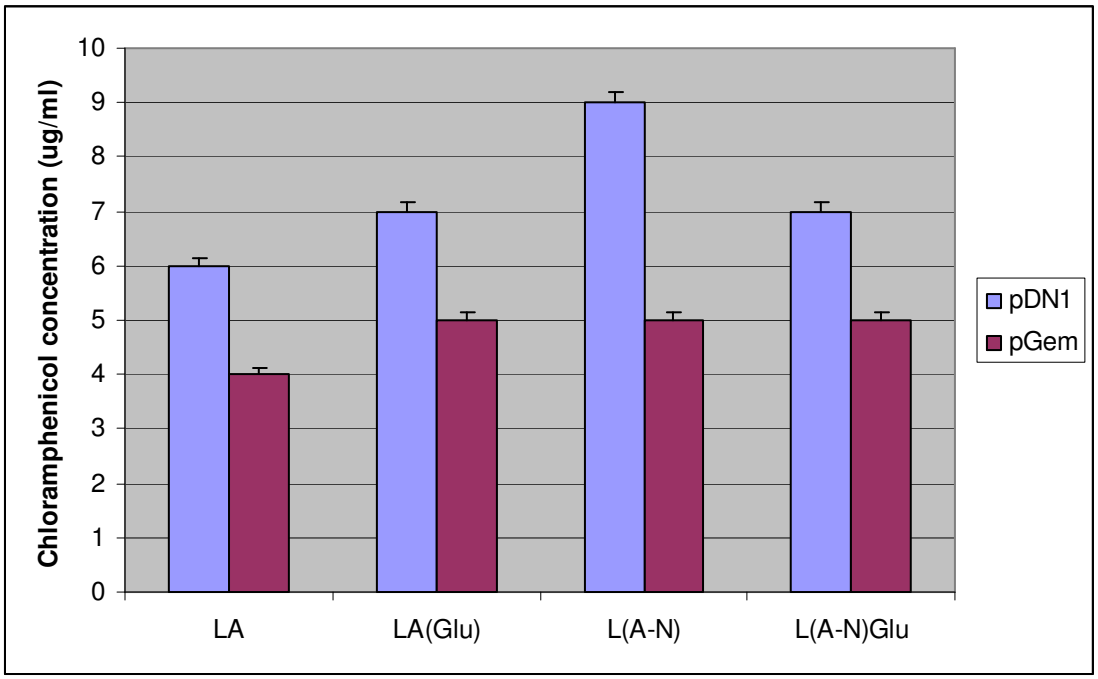


Fig. 7: Effect of different rich media compositions on chloramphenicol resistance phenotype in *E. coli* MM294-4 at 42°C

3.3 Expression of *gst* in the Gram positive background

To investigate the expression of the *M. smegmatis* mc^{2}155 *gst* gene in the Gram positive background, this gene was ligated into pNV18 shuttle vector via the *Pst*I and *Sph*I sites. The construct, termed pBA1, was electroporated into Gram positive bacteria: *Rhodococcus sp*., *Mycobacterium sp*., *Gordonia sp.* and *Arthrobacter sp*. Time constants were recorded, cells were spread on LA supplemented with 100 µg/ml Kan and incubated at 30°C. Growth was observed on the plates after 3 days, some plates gave a very small number of colonies while there was no growth on others. The experiment was reapeated three times to ensure its accuracy. The incubation period was increased for up to 10 days in order to obtain growth on the plates where there were no growth since all the conditions necessary for the uptake of DNA in the srains have been observed, yet no colonies were found.

The results obtained from the electroporation showed that only pBA1 transformants in strains *Rhodococcus erythropolis* ATCC 4277 and *Rhodococcus rhodochrous* HS1, HS6 and HS13 could be used for further experiments, this is because there were too little number of colonies in *Mycobacterium smegmatis* and *Rhodococcus fascians* strains and no growth at all in *Arthrobacter oxydans* and *Gordonia rubropertincta* strains. Results are presented in table 5.

Table 5: Electroporation of pBA1 into the Gram positive strains

Organism	Strain	No. of colonies
Mycobacterium smegmatis	mc^{2}155	7
Rhodococcus fascians	DSM20669	14
Rhodococcus erythropolis	ATCC 4277	Confluent growth
Rhodococcus rhodochrous	HS1	Confluent growth
Rhodococcus rhodochrous	HS6	Confluent growth
Rhodococcus rhodochrous	HS13	Confluent growth
Arthrobacter oxydans	C7	0
Gordonia rubropertincta	25593	0

3.4 Antibiotic MICs conferred by pBA1 in the Gram positive background

Spot tests were carried out on LA + Kan 100 µg/ml plates with increasing concentrations of the test antibiotics using the clones obtained from the electroporation of *Rhodococcus rhodochrous* strains HS1, HS6, HS13 and *R. erythropolis* ATCC 4277.

R. rhodochrous HS strains were considered for this experiments because of their susceptiblity to a wide range of antibiotics and as such, any noticeable increase in MIC could be deemed to have occurred as a result of the presence of the *gst* gene in the clone. Several classes of antibiotics were tested on the clone which include: macrolides, aminoglycosides, β-lactam, quinolone, tetracycline and chloramphenicol.

3.4.1 MIC of pBA1 in *Rhodococcus rhodochrous* HS1

Resistance to chloramphenicol and the aminoglycosides kasugamycin, spectinomycin and streptomycin was investigated in *R. rhodochrous* HS1. Other antibiotics tested include, nalidixic acid, rifampicin, ampicillin and erythromycin. Changes of resistance in this strain were found to be very low as the highest increase in MIC obtained was 3× with respect to rifampicin. There was a 2 fold increase in resistance to chloramphenicol and a 3 fold increase in ampicillin resistance. MIC increases to the other antibiotics tested were < 2 fold in this strain, and it could therefore be concluded that the expression of the gene was generally poor in the strain HS1.

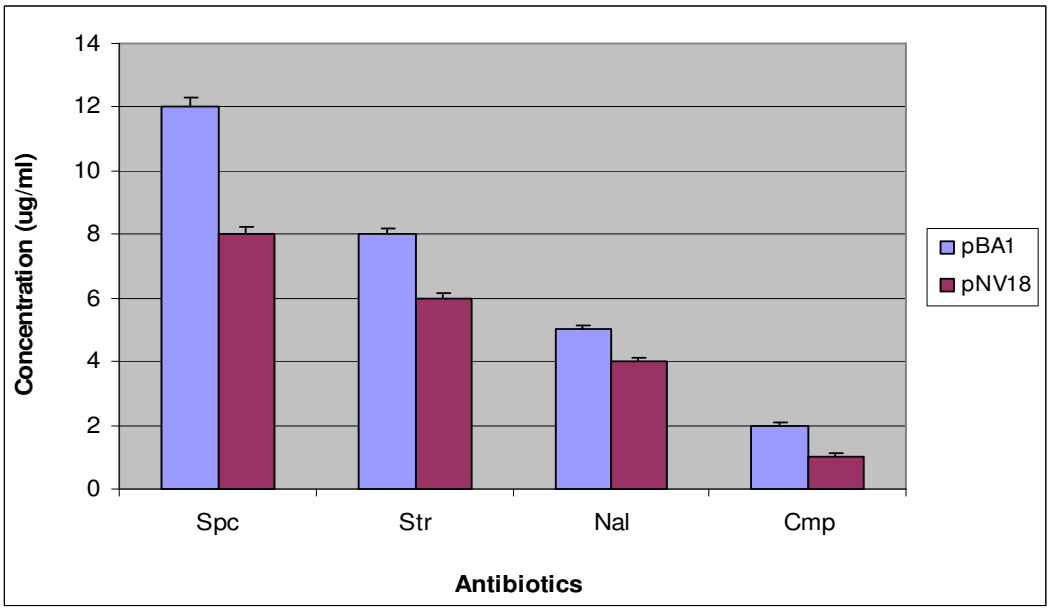


Fig. 8a: Antibiotic MICs conferred by pBA1 in *R. rhodochrous* HS1:pNV18 Vector control (Micrograms concentrations)

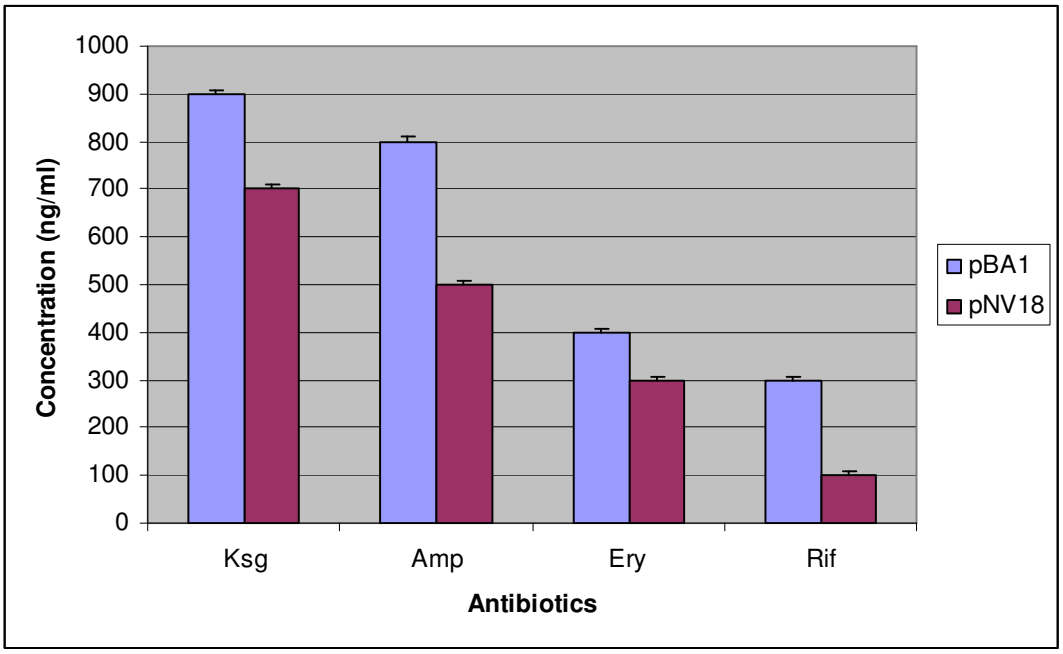


Fig. 8b: Antibiotic MICs conferred by pBA1 in *R. rhodochrous* HS1:pNV18 vector control (Nanograms concentrations)

3.4.2 MIC of pBA1 in *Rhodococcus rhodochrous* HS6

Following tranformation of pBA1 into this *Rhodococcus* strain, spot tests were carried out to determine the MIC of different classes of antibiotics in this strain. There was a 2 fold increase in MIC in chloramphenicol at 4 µg/ml concentration. The class aminogycosides as represented by streptomycin and spectinomycin also gave the same level of increase in resistance, except kasugamycin where a very slight increase in MIC was observed at 1600 ng/ml concentration. A slight increase in MIC of < 2 fold was found in ampicillin and erythromycin but increase in resistance was not found in nalidixic acid. The very low MIC for kasugamycin was not surprising because it has always shown a weaker activity in the class aminoglycoside antibiotics used in this work. The folowing histograms (Fig.9a and 9b) show the results of MICs obtained in the strain HS6.

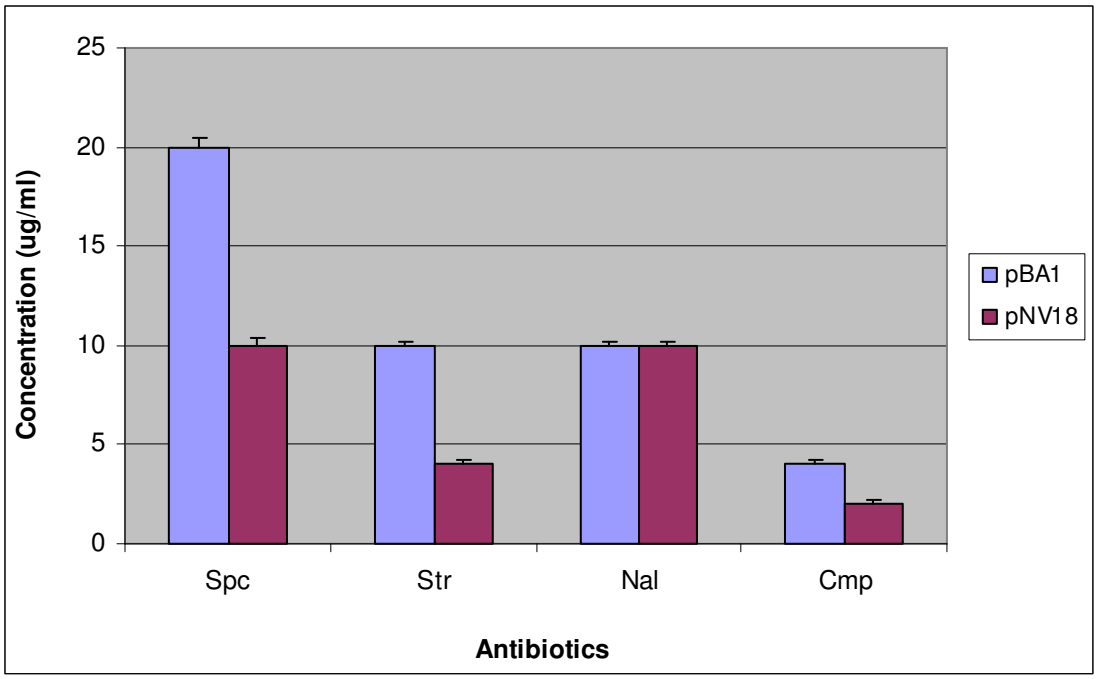


Fig. 9a: MICs conferred by pBA1 in *R. rhodochrous* HS6 (Micrograms concentrations)

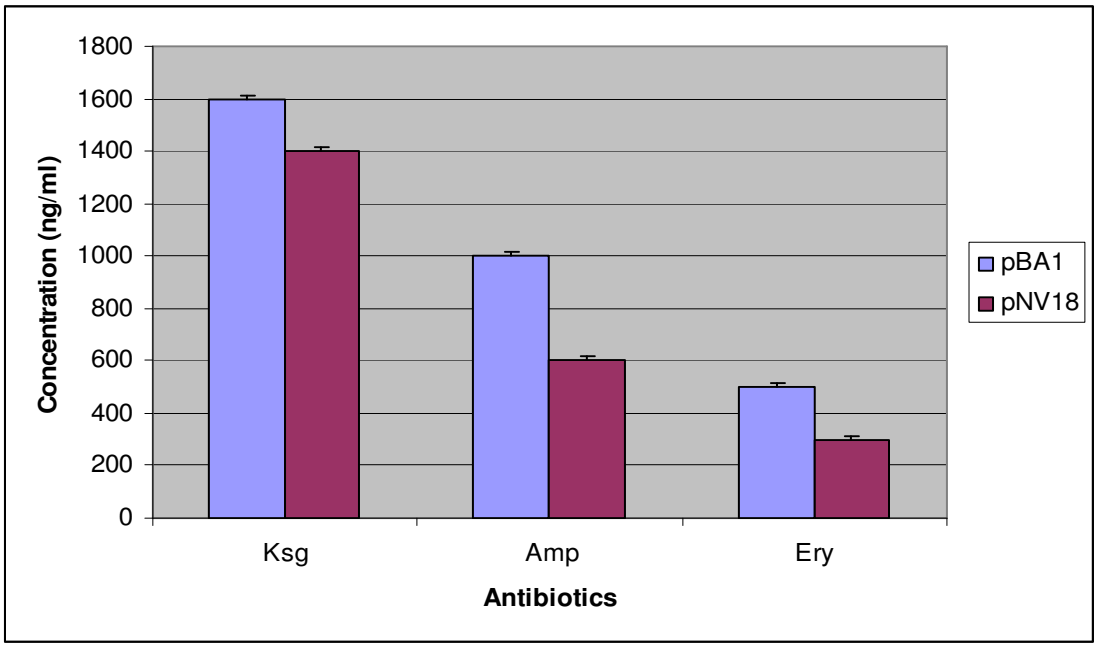


Fig. 9b: Antibiotic MICs conferred by pBA1 in *R. rhodochrous* HS6 (Nanograms concentrations)

3.4.3 MIC with respect to pBA1 in *Rhodococcus rhodochrous* HS13

Investigation of the level of resistance conferred on the antibiotics used for MIC in HS1 and HS6 strains was also carried out in HS13 strain. Spot tests were carried out using actively growing colonies obtained from the transformation in to this strain of Rho*dococcus*. The level of resistance obtained was similar to the one obtained for HS6, except for the fact that resistance to the antibiotics was higher in this strain. For instance, the resistance in streptomycin was up to 100 µg/ml and it was also higher in kasugamycin at 1,800 ng/ml although these were < 2× increase.

A major difference was found in the MIC of nalidixic acid which had the same level of increase in MIC with the vector in HS6, but showed a 2 fold increase in MIC in HS13 at 20 µg/ml concentration. Ampicillin also gave a 2× increase in resistance in HS13 but at a much higher concentration compared with what was obtained in HS6, while chloramphenicol showed a less increase in resistance in this strain, but at a higher concentration of 8 µg/ml. Results of the MICs of HS13 are presented in the histograms (Fig. 10a and 10b) that follow.

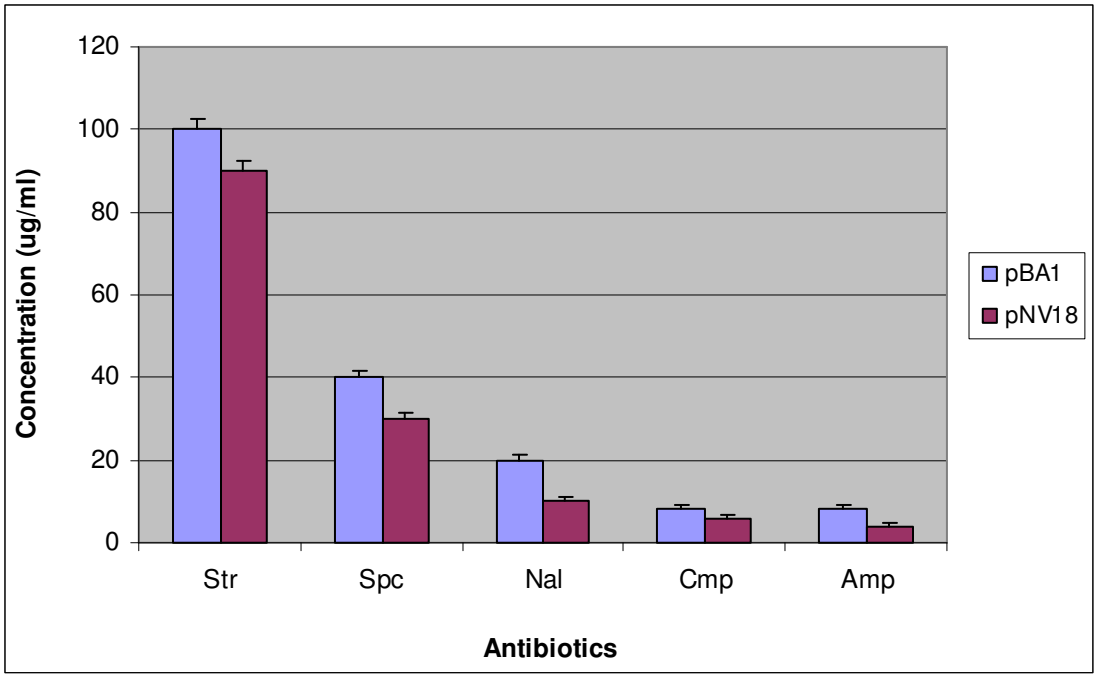


Fig. 10a: MICs conferred by pBA1 in *R. rhodochrous* HS13 (Micrograms concentrations)

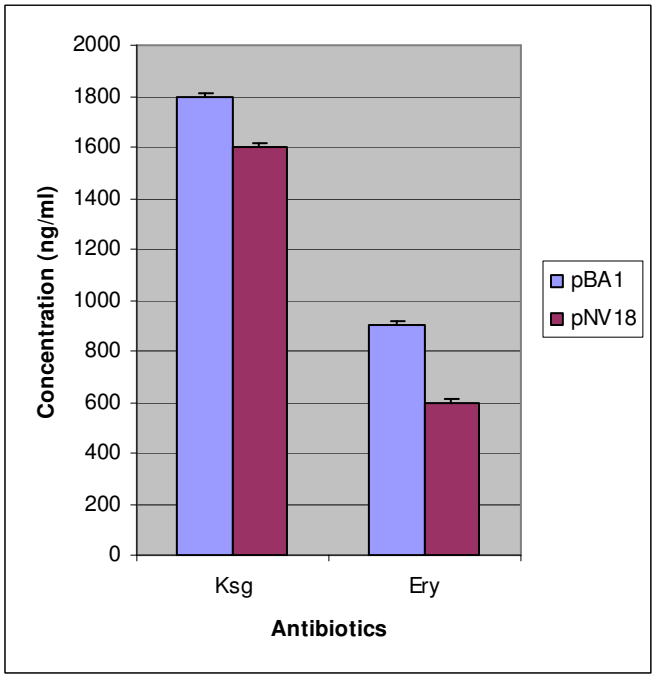


Fig. 10b: MICs conferred by pBA1 in *R. rhodochrous* HS13 (Nanograms concentrations)

3.4.4 MIC of pBA1 in *Rhodococcus erythropolis* ATCC 4277

Transformants of pBA1 into *R. erythropolis* ATCC 4277 were also tested. Results obtained showed that of all the strains in which the *gst* gene from *M. smegmatis* mc^2155 strain was expressed, the best phenotypic differences were obtained in this organism. Levels of resistance conferred on different classes of antibiotics were up to 5× increased MIC for spectinomycin at 20 µg/ml pBA1 and 4 µg/ml vector control, approximately 5× increased MIC for streptomycin at 14 µg/ml pBA1 and 3 µg/ml vector control and 5× increase for nalidixic acid at 10 µg/ml pBA1 and 2 µg/ml vector control. A 4 fold increase to 8 µg/ml was obtained for chloramphenicol, the principal antibiotic in this work, it was also a 4× increase for kasugamycin while < 3× increase was found for erythromycin.

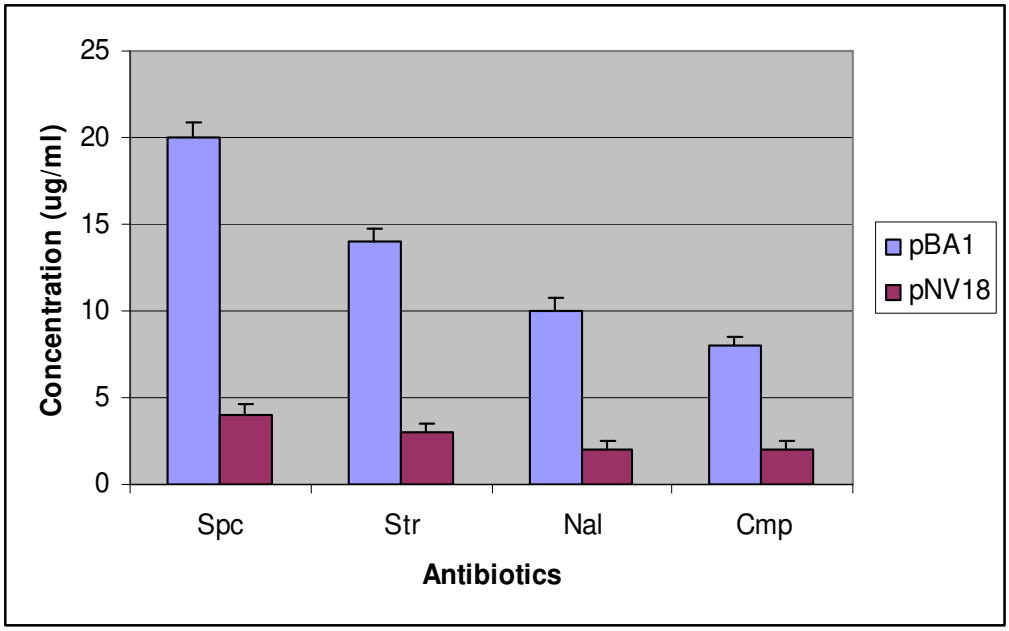


Fig. 11a: MICs conferred by pBA1 in *R. erythropolis* ATCC 4277 (Micrograms concentrations)

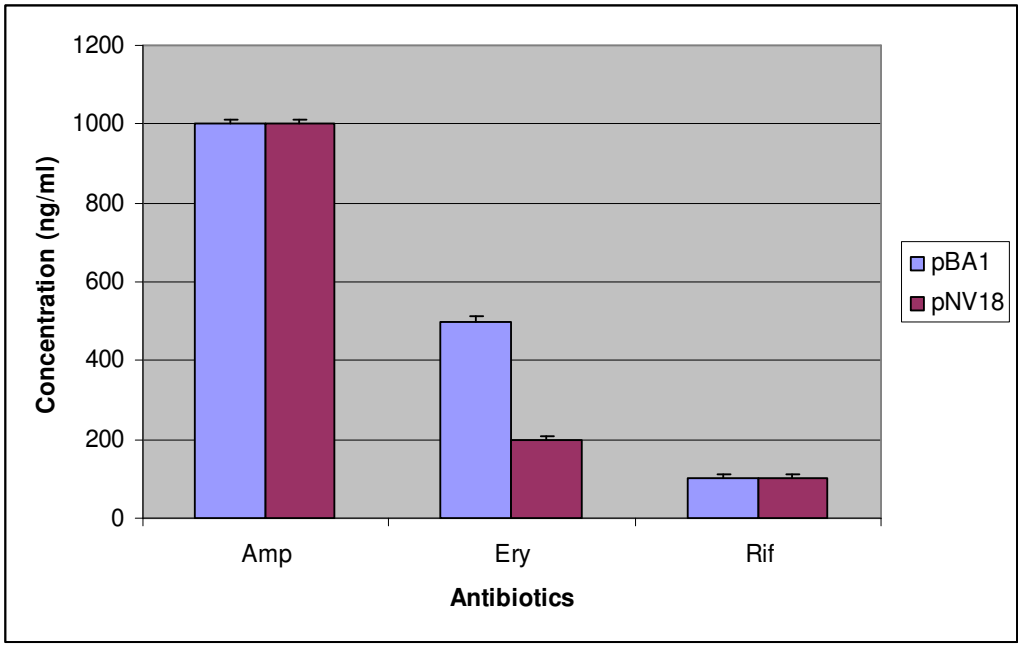


Fig. 11b: Antibiotic MICs conferred by pBA1 in *R. erythropolis* ATCC 4277 (Nanograms concentrations)

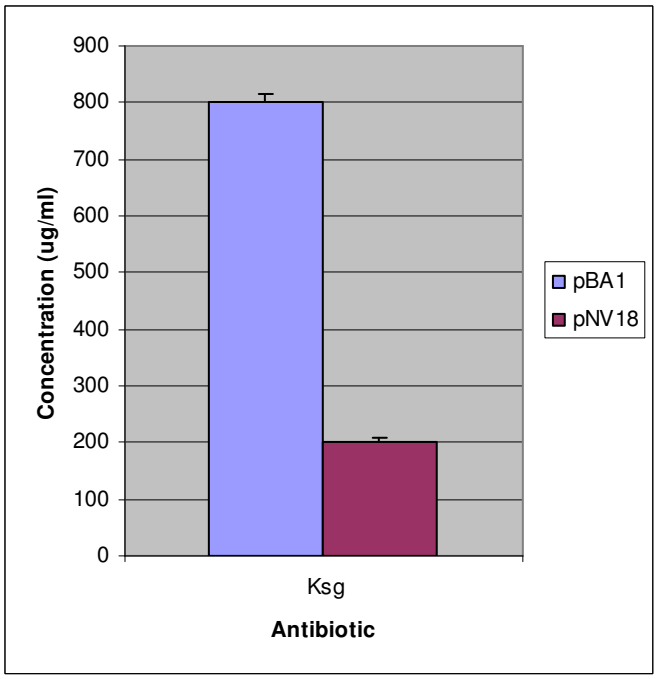


Fig. 11c: Kasugamycin MIC of *R. erythropolis* ATCC 4277 conferred by pBA1

From the results of the the MICs of the *gst* gene, it could be deduced that the presence of the gene confers increased resistance to a wide range of antibiotics compared to the MICs obtained in the control without *gst* gene in figures 3a and 3b above. Also it was

found that the highest increase in MIC of chloramphenicol in the *R. rhodochrous* HS strains was at 2 fold in HS1 and HS6 while it was < 2× increase in HS13.

The highest MIC increase in HS strains was at 3× increase in the antibiotic rifampicin while increase in MIC of the *gst* gene was very distinct in *R. erythropolis* ATCC 4277 where up to 5× increase was obtained in the antibiotics spectinomycin and nalidixic acid, 4× increase in chloramphenicol and kasugamycin, 3× increase in streptomycin, and 2× increase in erythromycin. Therefore, the best increase in MIC of the *gst* gene was obtained in *R. erythropolis* ATCC 4277.

3.5 Analysis of the Ery-R Gram positive DNA expressed in *E. coli*

Analysis of the libraries containing the erythromycin resistant clones is the other major component of my Master's work. Erythromycin is produced by the actinomycete bacterium *Saccharopolyspora erythraea*. It is a macrocyclic compound that contains a 14-membered lactone ring with two deoxy sugars attached. It inhibits replication by binding to the 50S subunit of the bacterial 70S rRNA complex and interferes with protein synthesis.

The set of experiments involved in this part of my research work was to expand on heterologous expression of Gram positive DNA in the Gram negative background. Gram positive libraries carried by shuttle vector pDA71 which were maintained in *E. coli* were screened (Nteo M. D., MTech, 2006), and clones in many libraries were identified. Out of the 23 screened, ery-R clones were found in 9. I carried out further analysis on 6 of these. These six clones from the ery-R libraries include: RX, K24, MP15, IP6, MCX and AVS. Three of the clones, MP15, MCX and AVS gave the highest MIC to erythromycin and two of these (AVS and MCX) were further tested on azithromycin.

3.6.1 MIC of ery-R clones in *E. coli*

Since it was reported that the clones were resistant to erythromycin after their screening, I first confirmed this. The DNA from the afore-mentioned clones were transformed into *E. coli* MM294-4 and the transformants obtained were used to carry

out spot tests in the antibiotic erythromycin. Colonies picked from the transformation plate were spotted on to LA plates supplemented with Amp at 100 µg/ml with the increasing concentrations of erythromycin up to 300 µg/ml. Plates were incubated at 37°C and the plates were allowed to grow for 24 hr as well as 48 hr together with the pDA71 vector control.

The MICs were obtained at 100 µg/ml concentration of the antibiotic which gave a 2× increase in 3 of the 6 clones; MP15, MCX and AVS. There was no significant difference between the growth of the clones after 24 hr compared to the growth after 48 hr, it was therefore concluded that the MIC was best at 100 µg/ml. Figure 12 shows the levels of resistance of 6 clones to erythromycin.

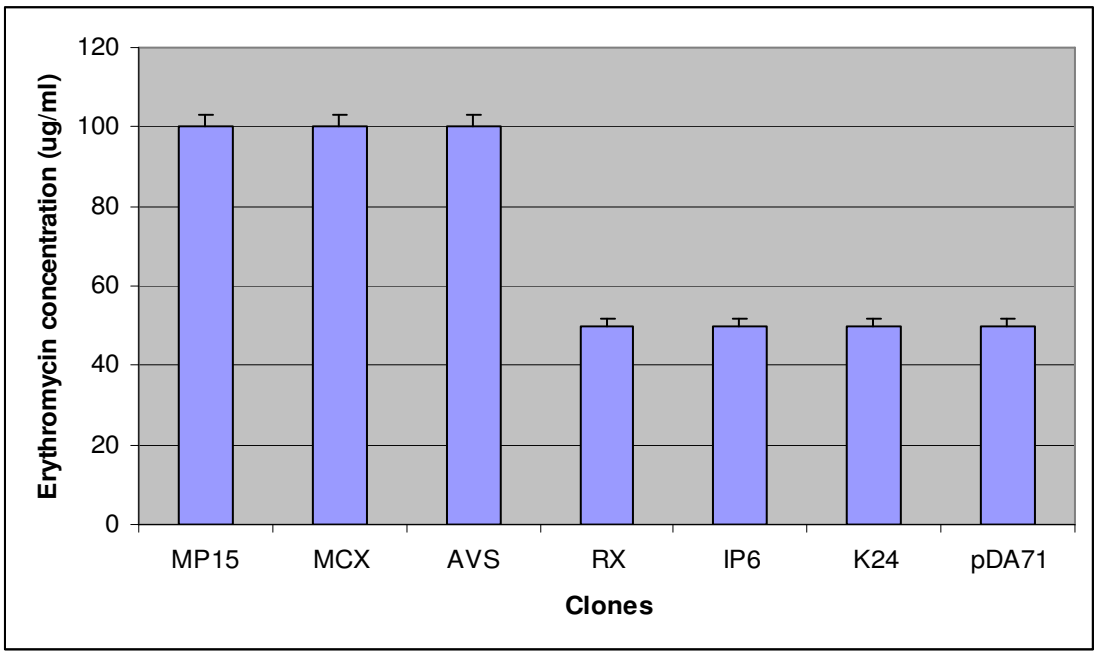


Fig. 12: MIC of selected Ery-R clones and pDA71 in *E. coli*

3.6.2 MIC of Ery-R clones in pUC 18

Two of the three clones that conferred the highest resistance on erythromycin were investigated further by ligating their DNA into pUC18, a high copy number vector because the MIC increase in vector pDA71 was modest. Transformants were again obtained in the *E. coli* MM294-4 strain and these were used to carry out spot tests on LA plates supplemented with Amp at 100 µg/ml with an increasing concentration of

erythromycin. Almost the same result was found in the pUC18 vector as obtained in pDA71 vector with the highest resistance to erythromycin at 100 µg/ml but slightly > 2 fold increase resistance as shown in Fig 13.

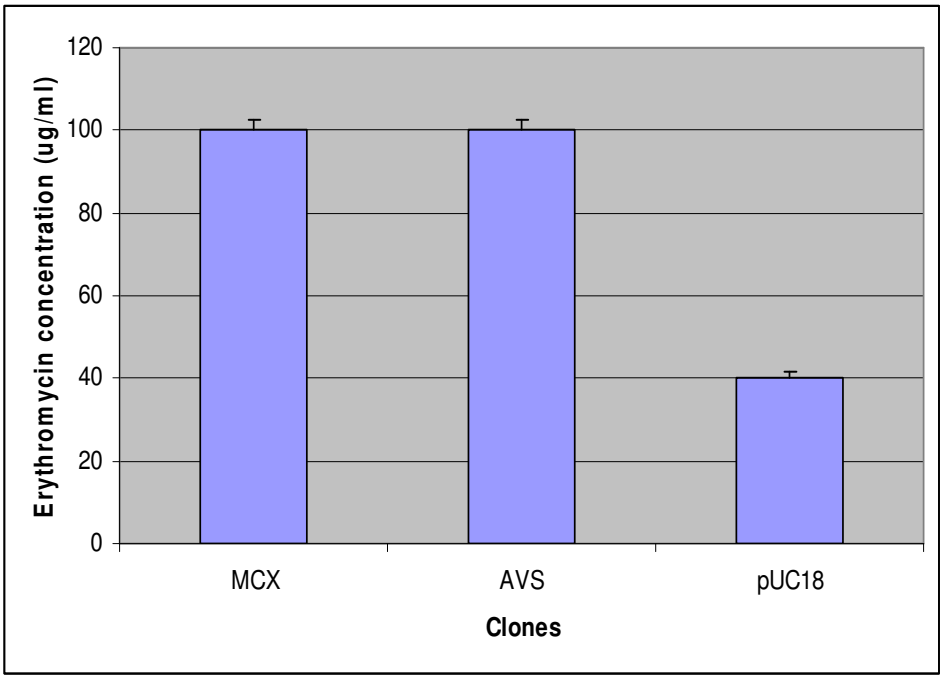


Fig. 13: MIC of Ery-R clones in *E. coli* using pUC18 Vector

3.6.3 Azithromycin MIC of Ery-R clones

Further experiment was carried out on clones MCX and AVS to investigate their levels of resistance in another member of the macrolide class of antibiotic, azithromycin. This macrolide was developed for better stability and a broad spectrum action. It differs chemically from erythromycin by incorporating a methyl-sustituted nitrogen atom into the macrocyclic compound to make it a 15-membered lactone ring. It binds to the 50S subunit of the bacterial ribosome to inhibit translation of the mRNA thus interfering with the protein synthesis.

This experiment was carried out to determine which of the clones had resistance to more than one member of the macrolide class. Spot tests were carried out on LA plates supplemented with Amp at 100 µg/ml at an increasing concentration of azithromycin up to 10 µg/ml. AVS showed a higher resistance in this antibiotic with a 5 fold increase resistance while the level of resistance in MCX was 3 fold. This result

was an indication that AVS has a higher level of resistance to the class of macrolide, it was therefore chosen for further experiments. Figure 14 shows the results obtained.

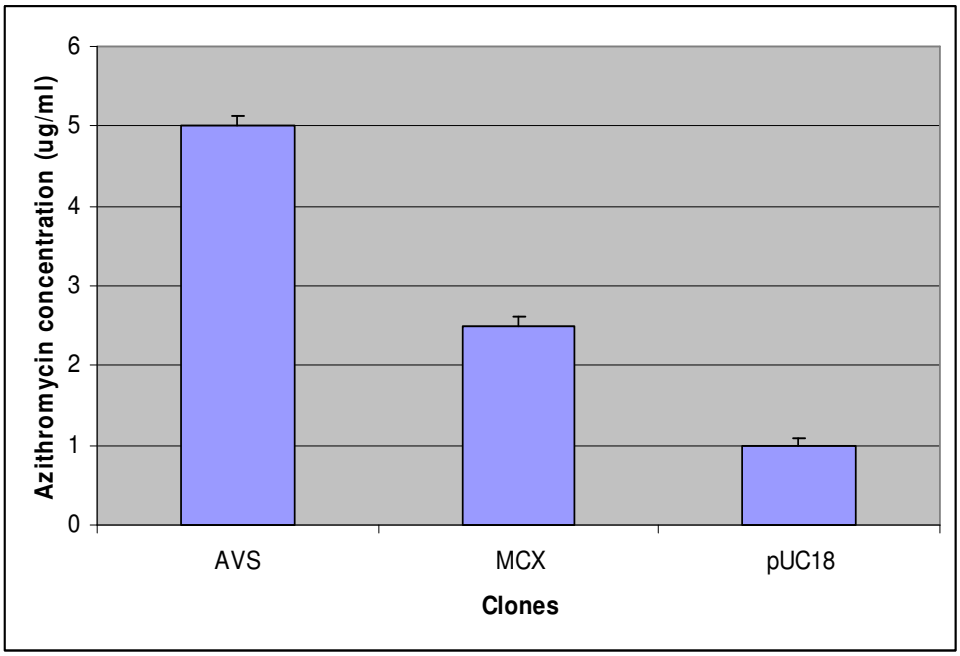


Fig. 14: Azithromycin MIC conferred by Ery-R clones in *E. coli*

3.7 Restriction digestions of the DNA from AVS

Several restriction enzymes were used to carry out single and double digestions on the DNA. This was to construct a restriction map by finding the number of sites and their sizes in the insert. Sizes were calculated by using the standard curves generated from the distances migrated by the known molecular weight size markers run on the same gel, Gene Ruler Ladder Plus from Fermentas was used. The digestion was carried out overnight and run on 0.8% agarose. The number of sites obtained from the enzymes used are presented in table 6 as follows;

Table 6: Restriction digestions of DNA from AVS

ENZYME	NUMBER OF SITES
*Nsi*I	1
Bam HI	0
*Nar*I	2
*Acc*I	2
*Taq*I	Numerous
*Sau*3A	Numerous
Eco RI	0
*Stu*I	0
*Nae*I	1
*Nru*I	1
*Sma*I	0
*Xho*I	1
*Sal*I	1

3.7.4: Restriction map of AVS

From the series of digestions carried out, it was found that *Nsi*I and *Nru*I were cutting the insert once and these were used to construct the restriction map. The other enzymes; *Sal*I, *Xho*I and *Nae*I that cut once were found cutting very close to the *Hind*III ends of the insert, and from these results the restriction map was constructed as shown in figure 15 below.

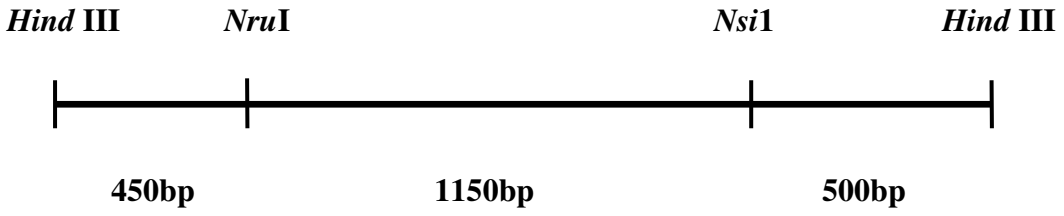


Fig. 15: Preliminary restriction map of AVS

3.7.5 Sequencing of the ery-R DNA

The approximately 1.2Kb insert (*Nsi*I - *Nru*I fragment) from AVS was ligated into pUC18 and transformed into the *E. coli* strain MM294-4. DNA from the transformants was purified using the plasmid prep kit from Amersham and the concentration of the DNA checked on the Nanodrop. The DNA obtained from this preparation was sent to Inqaba Biotec for sequencing, and the following nucleotide sequence was obtained.

gccatacgggtgtacgcaaaatggtggataaaaacgcattaagtctg
tggatgcsagaacgtagcgatctttgggtgcagccaaaagttgatggcgttgcggtaacc
ctggtttatcgggacgggaaactgaacaaagcaatcagtcgcggtaatggcctgaaaggc
gaggactggacgcagaaagttagcttaatttccgctgtgccgcaaaccgttagcgggcct
ttagccaacagtacgcttcagggggaaatatttctccagcgcgaggggcatatccaacaa
caaatgggggaataaatgcccgcgcaaaagttgctggcttgatgatgcgccaggacgat
agcgacacgctgaattctctgggcgtttttgtctgggcatggccggatggaccgcagtta
atgtctgatcgtttaaaagagctggctaccgcaggttttactctaacgcagacgtatacc
cgtgcggtgaaaaatgctgatgaagttgcgcgcgtacgcaatgagtggtggaaagcggaa
ttacccttcgtcaccgatggcgtagttgtacgagcggcgaaagagccagaatcccgccat
tggctaccgggccaggcagagtggctggtggcctggaaatatcaacctgtagctcaggtt
gccgaagtgaaggcaattcagtttgcggtgggtaagagcggtaaaatatcggtggttgcg
tcactcgcacctgtcatgctggatgataaaaaagtccagcgggtgaatattggttccgtc
aggcgctggcaggagtgggatattgcgcctggtgatcagattctcgtcagccttgccggt
caggtatt

Fig. 16: DNA sequence of AVS insert

3.7.6 Nucleotide alignment of the AVS sequence

Nucleotide alignment was carried out using BLASTN search for the 1150bp *Nsi*I – *Nru*I fragment. In this search, the zero e-value indicates the most significant alignment. It was most surprising that the nucleotide blast result showed it was an *E. coli* K12 DNA ligase, NAD (+) –dependent suggesting that contaminating *E. coli* DNA could have been cloned into the vector during library construction. Further investigation on the clone AVS was therefore discontinued but the results are summarized below.

Table 7: BLASTN nucleotide alignment search result

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP000948.1	*Escherichia coli* str. K12 substr. DH10B, complete genome	1496	1496	99%	0.0	99%
CP000946.1	*Escherichia coli* ATCC 8739, complete genome	1496	1496	99%	0.0	99%
CP000802.1	*Escherichia coli* HS, complete genome	1496	1496	99%	0.0	99%
AP009048.1	*Escherichia coli* str. K12 substr. W3110 DNA, complete genome	1496	1496	99%	0.0	99%
U00096.2	*Escherichia coli str.* K-12 substr. MG1655, complete genome	1496	1496	99%	0.0	99%
L10328.1	*E. coli*; the region from 81.5 to 84.5 minutes	1496	1496	99%	0.0	99%

```
gb|CP000948.1| Escherichia coli str. K12 substr. DH10B,
complete genome
Length=4686137
Features in this part of subject sequence:
   DNA ligase, NAD(+)-dependent
 Score = 1496 bits (1658), Expect = 0.0
 Identities = 833/835 (99%), Gaps = 1/835 (0%)
 Strand=Plus/Minus
```

```
Query2
CCATACGGGTGTACGCAAAATGGTGGATAAAAACGCATTAAGTCTGTGGATGCSAGAACG  61
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CCATACGGGTGTACGCAAAATGGTGGATAAAAACGCATTAAGTCTGTGGATGCGAGAACG  3916425
Sbjct3916484
Query62
TAGCGATCTTTGGGTGCAGCCAAAAGTTGATGGCGTTGCGGTAACCCTGGTTTATCGGGA       12
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TAGCGATCTTTGGGTGCAGCCAAAAGTTGATGGCGTTGCGGTAACCCTGGTTTATCGGGA  3916365
Sbjct3916424
Query122
CGGGAAACTGAACAAAGCAATCAGTCGCGGTAATGGCCTGAAAGGCGAGGACTGGACGCA      181
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CGGGAAACTGAACAAAGCAATCAGTCGCGGTAATGGCCTGAAAGGCGAGGACTGGACGCA  3916305
Sbjct3916364
Query182
GAAAGTTAGCTTAATTTCCGCTGTGCCGCAAACCGTTAGCGGGCCTTTAGCCAACAGTAC      241
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GAAAGTTAGCTTAATTTCCGCTGTGCCGCAAACCGTTAGCGGGCCTTTAGCCAACAGTAC  3916245
Sbjct3916304
Query242
GCTTCAGGGGGAAATATTTCTCCAGCGCGAGGGGCATATCCAACAACAAATGGGGGGAAT      301
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GCTTCAGGGGGAAATATTTCTCCAGCGCGAGGGGCATATCCAACAACAAATGGGGGGAAT  3916185
Sbjct3916244
Query302
AAATGCCCGCGCAAAAGTTGCTGGCTTGATGATGCGCCAGGACGATAGCGACACGCTGAA  361
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AAATGCCCGCGCAAAAGTTGCTGGCTTGATGATGCGCCAGGACGATAGCGACACGCTGAA  3916125
Sbjct3916184

Query  362
TTCTCTGGGCGTTTTTGTCTGGGCATGGCCGGATGGACCGCAGTTAATGTCTGATCGTTT      421
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TTCTCTGGGCGTTTTTGTCTGGGCATGGCCGGATGGACCGCAGTTAATGTCTGATCGTTT  3916065
Sbjct3916124
Query422
AAAAGAGCTGGCTACCGCAGGTTTTACTCTAACGCAGACGTATACCCGTGCGGTGAAAAA  481
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AAAAGAGCTGGCTACCGCAGGTTTTACTCTAACGCAGACGTATACCCGTGCGGTGAAAAA  3916005
Sbjct3916064
```

```
Query482
TGCTGATGAAGTTGCGCGCGTACGCAATGAGTGGTGGAAAGCGGAATTACCCTTCGTCAC  541
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TGCTGATGAAGTTGCGCGCGTACGCAATGAGTGGTGGAAAGCGGAATTACCCTTCGTCAC  3915945
Sbjct3916004
Query542
CGATGGCGTAGTTGTACGAGCGGCGAAAGAGCCAGAATCCCGCCATTGGCTACCGGGCCA  601
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CGATGGCGTAGTTGTACGAGCGGCGAAAGAGCCAGAATCCCGCCATTGGCTACCGGGCCA  3915885
Sbjct3915944
Query602
GGCAGAGTGGCTGGTGGCCTGGAAATATCAACCTGTAGCTCAGGTTGCCGAAGTGAAGGC  661
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGCAGAGTGGCTGGTGGCCTGGAAATATCAACCTGTAGCTCAGGTTGCCGAAGTGAAGGC  3915825
Sbjct3915884
Query662
AATTCAGTTTGCGGTGGGTAAGAGCGGTAAAATATCGGTGGTTGCGTCACTCGCACCTGT  721
  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AATTCAGTTTGCGGTGGGTAAGAGCGGTAAAATATCGGTGGTTGCGTCACTCGCACCTGT  3915765
Sbjct3915824
Query722
CATGCTGGATGATAAAAAAGTCCAGCGGGTGAATATTGGTTCCGTCAGGCGCTGGCAGGA  781
  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CATGCTGGATGATAAAAAAGTCCAGCGGGTGAATATTGGTTCCGTCAGGCGCTGGCAGGA  3915705
Sbjct3915764
Query  782
GTGGGATATTGCGCCTGGTGATCAGATTCTCGTCAGCCTTGCCGGTCA-GGTATT  835
|||||||||||||||||||||||||||||||||||||||||||||||| ||||||
GTGGGATATTGCGCCTGGTGATCAGATTCTCGTCAGCCTTGCCGGTCAGGGTATT  3915650
Sbjct  3915704
```

Fig. 17: Mega BLAST discontinuous search

```
Localization Scores:
    Cytoplasmic            0.01
    CytoplasmicMembrane    0.00
    Cellwall               0.02
    Extracellular          9.97
  Final Prediction:
    Extracellular          9.97
```

Fig. 18: Prediction of protein localization

I did not investigate how it might be that an *E. coli* DNA ligase conferred resistance to the macrolide azithromycin since these substrates bear no apparent similarity.

3.8 Mutagenesis

Mutagenesis was carried out on the *gst* gene clone, this was done to create mutant libraries from the clone, and to carry out the molecular analysis of such libraries whether the mutant(s) of the clone would confer an increase resistance to different classes of antibiotics.

3.8.1 Spontanous mutation of the *gst* clone in *E. coli*

The plates used for this experiment were incubated in a dark box at 37°C since tetracycline is photolabile. Growth was monitored after every 24 hr. Colonies appeared on plates with no Cmp after 4 days and growth was found on the 24 µg/ml Cmp concentration plates after 6 days. It was therefore possible that mutation took place in the clone since the plates without Cmp gave growth 2 days before any growth could be found on the plates containing the test antibiotic, also at the higher concentrations of 18 µg/ml and 21 µg/ml Cmp there were 400 and 500 colonies of pDN1 respectively while there were no growth of pGem-T-easy (the vector control) indicating that the growth was inhibited by the presence of the antibiotic.

3.8.2 Spontanous mutation of the *gst* clone in *Rhodococcus erythropolis*

The vector pNV18 vector was used used as the vector control in the Gram positive organism. The plates containing chloramphenicol had colonies appearing at 20 µg/ml after 4 days but there was no growth on the 30 µg/ml Cmp plates even when it was incubated further for 9 days. It was observed that mutagenesis could have occurred in the clone pBA1 since 43 colnies were found on its plate at 20 µg/ml compared to the vector control (pNV18) which gave 17 colonies at the same concentration of the antibiotic.

3.8.3 Chemical mutagenesis of the clones pDN1 in *E. coli* and pBA1 in *R. erythropolis*

The cells were treated with 2% EMS solution in buffer E, incubated for 1 hr, and washed with the phosphate buffer before being added to LB + Amp/Kan for overnight incubation with chloramphenicol at an increasing concentration. The plates were incubated at 37°C and 30°C respectively. There were confluent growth on the control plates while pDN1 and pGem-T-Easy (vector control) showed growth only at 18 µg/ml with 250 colonies on their plates, the same no of colnies found on these plates could be due to the fact that mutagenesis did not take place. pBA1and pNV18 (the vector control) in the Gram positive organism gave growth at 20 µg/ml concentrations of chloramphenicol with 192 colonies and 73 colonies in *R. erythropolis* ATCC 4277 respectively. Tables 9 and 10 show the results obtained from these experiments.

3.8.4 Mutagenesis by the use of a mutator strain

This method of mutagenesis involves transformation of the clone into the mutator strain which serves as the host cell to introduce mutation into the clone. A pre-culture of *E. coli* CSH116 (*mutD*) strain was inoculated into 5 ml LB and incubated overnight at 37°C. DNA from pDN1 was transformed into this strain via $CaCl_2$ method and mutants were selected for on LA Amp100 µg/ml plate supplemented with 15 µg/ml and 20 µg/ml Cmp, with pGemT-Easy as control.

Mini prep was carried out using the transformants from the plate, and the extracted DNA was ligated with pNV18 by utilizing the *Pst*I and *Sph*I sites in the vector, transformations into the Gram positive strains of *R. erythropolis* ATCC 4277, *R. rhodochrous* HS6 and HS13 were carried out by the electroporation method. Cells were spread on LA Kan 100 µg/ml plates, including the vector and cells only plates. The plates were incubated at 30°C and colonies were found after 2 days. Serial dilutions up to 10^{-6} were made to determine the number of colony forming units and for the subsequent calculation of the mutation rate.

Table 8: Spontanous mutation of pDN1 in *E. coli*

Cmp conc. (µg/ml)	No of colonies (pDN1)	No of colonies (pGem)
0	Confluent growth	Confluent growth
12	Confluent growth	Confluent growth
15	Confluent growth	40
18	400	0
21	250	0
24	1	0
27	0	0
Tet conc. (µg/ml)		
0	Confluent growth	Confluent growth
10	200	400
12	100	250

Table 9: Chemical mutagenesis of pDN1 in *E. coli*

Chloramphenicol conc. (µg/ml)	No of colonies (pDN1)	No of colonies (pGem)
0	Confluent growth	Confluent growth
18	250	250
21	0	0
24	0	0
27	0	0

Table 10: Spontanous and chemical mutagenesis of pBA1 in *Rhodococcus erythropolis* ATCC 4277

Clone	No of colonies (spontanous)	No of colonies (EMS)	No of colonies (10^{-6} dilution)
4277 (pBA1)	43	192	388
4277 (pNV18)	17	73	187
HS6 (pBA1)	1	1	168
HS6 (pNV18)	3	1	127
HS13 (pBA1)	1	1	104
HS13 (pNV18)	2	1	96

Table 11: Mutagenesis in *E. coli* CSH116

Clone	No of colonies	No of colonies (10-6 dilution)
4277 (pBA1)	1	76
4277 (pNV18)	3	78
HS6 (pBA1)	2	56
HS6 (pNV18)	3	61
HS13 (pBA1)	1	63
HS13 (pNV18)	4	69

3.8.5 Mutation rates of the clone pBA1

Following the results obtained from the mutagenesis, it was found that the rate of mutation of the clone pBA1 was low by calculating the mutation rates in each of the strains used for the experiment. Thus from the chemical mutagenesis, the mutation rate of the clone in *R. erythropolis* ATCC 4277 strain was $< 5 \times 10^{-7}$ mutations/cell division, and those of the *R. rhodochrous* HS6 and HS13 strains were both $< 1 \times 10^{-9}$ mutations/cell division, while the mutation rates for all the three strains in the mutator strain *E. coli* CSH 116 were $< 1 \times 10^{-8}$. With respect to the results obtained from the mutagenesis experiments, I concluded that the methods of mutagenesis employed

were not effective in introducing the desired mutations into this *gst* gene, hence a more powerful approach will be required to achieve this objective, and that would be error-prone PCR.

3.9 Screening for plasmid borne mutants via marker rescue technique

This technique was carried out to identify the plasmid borne mutations by pooling of chloramphenicol resistant colonies. Mini prep was then performed on the culture using alkaline-lysis method. The DNA extracted from the clones pDN1 and pNV18 were transformed into the *E. coli* MM294-4 strain and cells were spread on LA Amp 100 µg/ml plates supplemented with concentrations of chloramphenicol used for the original selection, pGem-T-Easy and pNV18 controls were included. After an overnight incubation, no growth was found on all the plates containing chloramphenicol. Plates were left for further incubation period of up to 10 days, but there was still no growth. Therefore the mutants obtained were not due to *gst* but were in the *E.coli* chromosome.

3.10.1 Mutagenesis of the glycosyltransferase gene

A further minor project of mine was based on previous work done in our laboratory, C. Lephoto (2002) had cloned and characterized a glycosyltransferase gene – *rgt* – from *Nocardia brasiliensis* IFM 0236. referred to as pCL1, the plasmid conferred resistance to rifampicin. Chemical mutagenesis was also carried out in this clone in her work, the result of which was not reported, this could probably be due to the fact that mutants were not found after the mutagenesis experiment.

The aim of carrying out this experiment was to utilize an *in vitro* method of mutagenesis to create mutants by generating a random point mutation in a given segment of the DNA from the clone pCL1, and to investigate the effect of this mutation on its resistance to rifampicin as well as other classes of antibiotics.

3.10.2 MIC of the glycosyltranferase gene in *E. coli*

Prior to the mutagenesis, spot tests were carried out to determine the level of resistance to rifampicin. Clones obtained from the transformation carried out in *E. coli* MM294-4 were used for the spot test on LA Amp 100 µg/ml plates with an increasing concentration of rifampicin up to 400 µg/ml and were incubated at 37°C. The vector pDA71 in which it was cloned into was used as the vector control. The result obtained from the MIC revealed that the clone was resistant to rifampicin at a concentration of 400 µg/ml which is about 80 fold increase as was reported before. The following chart shows the result obtained from the spot test.

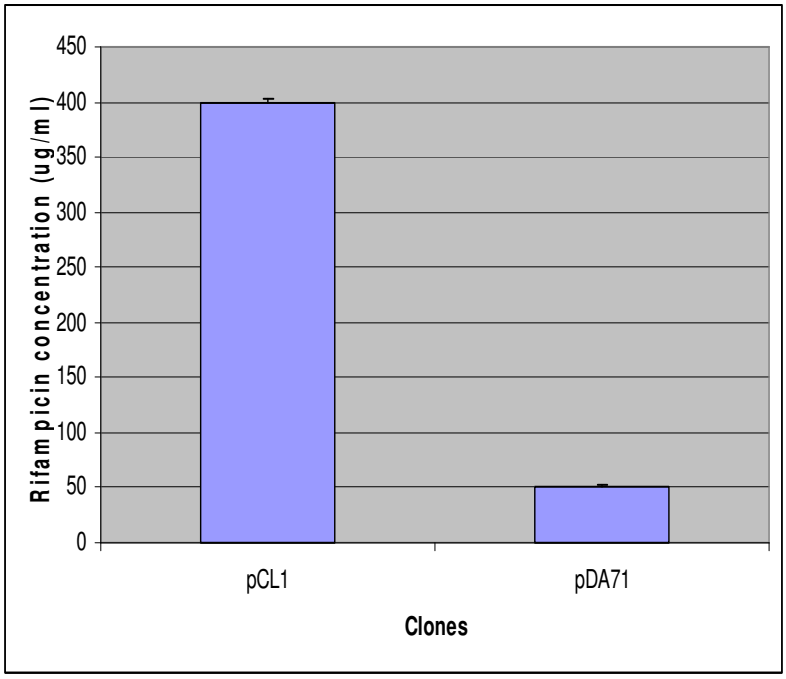


Fig. 19: Rifampicin MIC of *E. coli* due to pCL1 in pDA71

3.10.3 MIC of glycosyltransferase gene in *E. coli* using pUC18

Following the result found in the vector pDA71, it was imperative to determine the MIC of the clone in the vector pUC18 that was used for the epPCR. The insert and the

clone were digested with the restriction enzyme *Pst*I and were ligated, followed by transformation in to *E. coli* MM294-4 strain.

The transformants obtained were used to carry out spot tests on LA Amp 100 µg/ml + rifampicin at an increasing concentration. Incubation was carried out at 37°C, and growth were found after an overnight incubation on the LA Amp plate where rifampicin was not present, but no growth was found on the plates containing rifampicin, even when incubated further for up to 6 days.

3.10.4 Orientation of the clone pCL1 in pUC18

The reason for the clone not conferring resistance to rifampicin in pUC18 might be due to the fact that pUC18 has the *lacZ* gene instead of the λ promoter found in pDA71 which could have favoured the growth of the clone, or the orientation of the clone in the vector was in the opposite direction. Extraction of the DNA was carried out for double digestion, and the restriction enzymes *Sfu*I and *Xba*I that are closest to the *Pst*I site of the insert from where it was sitting on both sides of the vector were used for the digestion. The digestion was carried out overnight, and run on 0.8% agarose gel.

An approximately 1.6Kb size of the insert band was obtained on the gel which gave the possibility that, the enzymes must have cut the vector just before the *Pst*I site and also cut the insert just before the *Pst*I site on the opposite side giving the approximately 1.6 Kb size, and not the second possiblity where both could have cut very close to just one site of *Pst*I whereby the total size of the area cut would be approximately 210 bp. From this result, it was concluded that the insert was cloned in the opposite direction to the *lacZ* gene in pUC18.

3.10.5 Error prone PCR

pCL1 DNA was resuspended in 100 µl of sd$H_2$0, and 20 µl of this was diluted with 100 µl sd$H_2$0 to give a concentration of 45.2 ng/µl on the nanodrop. 2.25 µl of this concentration was used for the reaction to give a final concentration of 0.1µg in the reaction mixture. Error prone PCR was carried out as stated in the materials and

method section and several controls were utilized including; standard PCR, fwd primer control, rev primer control and no template DNA control. The amplified products were checked on 1.2% agarose gel electrophoresis, digestion of the epPCR product was carried out with the vector pDA71 for ligation with *Pst*I.

The amplified product of epPCR ligated into pDA71 was transformed into *E. coli* MM294-4 hereafter referred to as pVOA1, mini prep was carried out to check for the amplified band on the agarose gel. The band of interest was excised by a sterile scapel and purified. The concentration of pVOA1 DNA was checked on the spectrometer and the DNA was sent to Inqaba Biotec for sequencing.

3.10.6 Sequencing result

The nucleotide sequence result obtained from Inqaba was aligned with the existing sequence of pCL1 in the data base using the web tools, and it matched the sequence by aligning with the sequence of the *N. brasiliensis* IFM 0236 from which the mutagenesis was carried out. However, multiple mutations occurred in the DNA including base pair substitution, insertion, and deletion of the nucleotides. It was therefore not suitable for further analysis.

3.11 Antibiotic inactivation assayed on plates

Plate inactivation assays were carried out in the Gram negative background using the DNA from the clone pDN1, and in the Gram positive background using the DNA from the clone pBA1. These were done in order to investigate the concentration of chloramphenicol that could be utilized by the DNA from these clones which could lead to a novel chloramphenicol inactivation mechanism.

3.11.1 Calibration for inactivation assay

A70 µl volume of chloramphenicol in LB at 2, 4, 8, 16, 32 and 64 µg/ml were added to the wells made on LA half agar plates covered with a *B. subtilis* 1A3-1 lawn. These plates were incubated in the cold for 4 hr to allow for antibiotic diffusion before an overnight incubation at 37°C. Diameters of the zones of inhibition were measured on

the plates to plot a calibration curve where the zones of inhibition obtained from the inactivation assays performed in *E. coli* and *Rhodococcus* strains were compared. The results obtained from the calibration experiment is presented in fig 20 below.

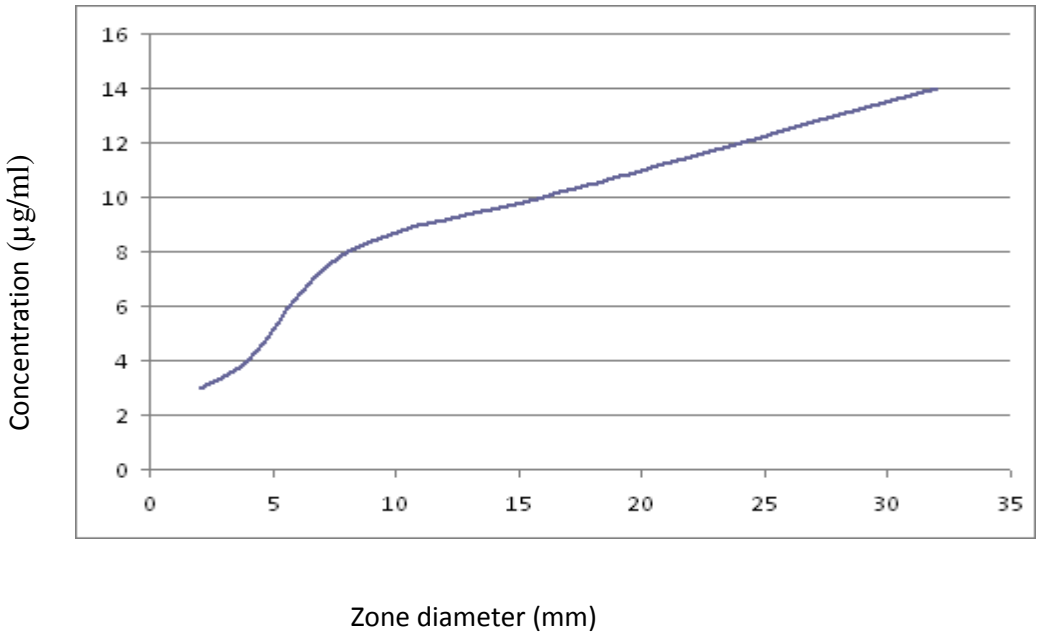


Fig. 20: Calibration curve for chloramphenicol concentration

3.11.2 *In vivo* inactivation in *E. coli* and *Rhodococcus*

E. coli clones were grown overnight at 37°C while *Rhodococcus* clones were grown for 2-3 days for *in vivo* inactivation assays. A 100 fold dilution of these cultures were made and chloramphenicol at 10 µg/ml concentration was added to each culture for overnight incubation.

A 1:100 fold dilution of *B. subtilis* 1A3-1, the assay organism was made and spread on LA half agar plates to which 200 µg/ml streptomycin had been added. Wells were made on the plates and a 70 µl volume of the cultures were added to the wells. Plasmid pACYC184 (which inactivates chloramphenicol due to the acetyl-transferase) and pGemT-Easy were used as controls in *E. coli,* while transformant Rhodococcus pNV18 (vector only) was a the negative control. After 4 hr in the cold to allow diffusion, incubations were carried out at 37°C for the *E. coli* strain, and 30°C for the *Rhodococcus* strain, diameters of the zones of inhibition were measured. Partial

inactivation were obtained with the *gst* clones, while pACYC184 showed complete inactivation, as expected (table 12).

Table 12: Results of *in vivo* plate inactivation assay

Cell cultures and antibiotic	Diameter of the zones of inhibition(mm)
pDN1	20
pGem-T Easy	25
pACYC184	0
LBCmp	30
pBA1	14
pNV18	23
LBCmp	30

3.11.3 *In vitro* inactivation in *E. coli* and *Rhodococcus*

E. coli and *Rhodococcus* cultures were centrifuged to pellet the cells at 15,000 rpm for 5 min and the residual antibiotic were removed from the cells by washing with the sonication buffer. Crude cell extracts were obtained by sonication and clarified supernatants were obtained by spinning down in the cold. Reaction mixtures were set up using GSH as the cofactor and acetyl CoA as the co-enzyme to transfer the acetyl group in to the reaction. After an incubation period of 16 hours, the reaction was stopped by boiling for 10 min and 70 µl of the reaction mixture was added to the wells on the half agar plates spread with assay organism. The plates were incubated in the cold for 4 hr before an overnight incubation at 37°C.

Cell homogenates from pGemT-Easy and pACYC184 were included for the reaction in *E.coli* and pNV18 for *Rhodococcus*, this is to ensure that the chloramphenicol phenotype obtained was due to the *gst* in pDN1 and pBA1 respectively, while LB + Cmp was used as the negative control. Diameters of the zones of inhibition were measured to compare the level of inactivation shown by the clones.

From the results obtained, the clone pBA1 in *Rhodococcus* showed partial inactivation compared to the clone pDN1 in *E. coli.* This suggests that this could be a novel chloramphenicol inactivation mechanism since there are no reports in the literature of GST-mediated inactivation of this drug. Figure 21 shows the results obtained from the inactivation plates, while the diameters of the zones of inhibition measured are presented in the table that follows.

Table 13: Results of *in vitro* plate inactivation assay

Crude cell homogenates	Diameter of zone of inhibition (mm)
pDN1	23
pGem-T Easy	27
pACYC184	0
LBCmp	30
pBA1	1.5
pNV18	24
LBCmp	30

A **B**

Fig. 21: *In vitro* inactivation assays in *E. coli* (plate A) and *Rhodococcus* (plate B)

3.12 Discussion

3.12.1 Expression of the *gst* gene in *E. coli* and *Rhodococcus* strains

It has been reported that majority of mycobacterial promoters function poorly in *E. coli*, and this is mainly due to the differences in their sigma factor sub regions which reflect different hexamer specificities. There has also been a suggestion that low GC content may impose a selective pressure for RNA polymerase with higher specificity for more conserved AT-rich promoters, therefore organisms like mycobacteria with high GC content in the chromosome could be characterized by having less specific RNA polymerase than that of organisms with lower GC content, and this can further explain why mycobacterial genes are, in general, better expressed in the Gram positive strains (Bashyam et., al. 1996; Fernandes et. al.,1999; Manganelli et. al., 1999).

In this work, the expression of *M. smegmatis* mc^2155 *gst* gene was poor in *E. coli*. Rich media were used to characterize the antibiotic resistance phenotype in *E. coli* at varying degrees of temperature, and the slightly increase in MIC observed could have been triggered by a sigma factor. This is because no noticeable increase was found at lower temperatures, and the effect of media differences was not of noticeable impact on the phenotype. In the Gram positive background changes in MIC were greater. Therefore future works should to be carried out in *R. erythropolis* ATCC 4277 where the gene expressed the best phenotypes.

3.12.2 Chloramphenicol resistance phenotype

Known resistance to chloramphenicol may be due to the *cat* gene (chloramphenicol acetyl-transferase). The product of the gene catalyzes the transfer of acetyl group from acetyl coenzymeA to chloramphenicol to form monoacetylated (1-acetyl and 3-acetyl chloramphenicol) and diacetylated (1, 3 diacetyl chloramphenicol) derivatives. Studies carried out in the Gram positive background revealed that spontaneous resistance to chloramphenicol occurs in mycobacteria at a frequency of 10^{-4} and can give rise to false positive clones (Bashyam et. al., 1996). From my experiments, it was

observed that the *gst* gene from *M. smegmatis* mc^2155 gave a well defined increase in MIC in the Gram positive.

3.12.3 Resistance to other structurally unrelated antibiotics

Resistance to the aminoglycosides is mainly due to genes exchanged on plasmids and transposons. With respect to the antibiotics tested in this work, this class of antibiotics gave the best increase in MIC. Streptomycin, Spectinomycin and Kanamycin all gave a 3 fold increase in *E. coli*, it was only kasugamycin that gave a < 2× MIC increase in this strain and this is not surprising because it is considered the weakest of them all. In the Gram positive strains these antibiotics still gave the best increase in MIC especially in *R. erythropolis* where streptomycin and spectinomycin both gave a 5× increase in MIC, kasugamycin gave a 4 fold increase while Kanamycin was not tested in this strain as it was used as the selectable marker.

Resistance to the antibiotic erythromycin was very poor in all the strains used in this work. The increase in MIC was < 2 fold in all the strains and this could be due to the fact that the antibiotic was able to bind to the region of the ribosome where some degree of inhibition could have occurred, and that the *gst* gene did not express the enzyme methylase that could have prevented the binding of the antibiotic to the region of the ribosome, the plasmid that would have encoded this characteristic might not be present in the gene.

Resistance phenotype to Nal was poorly expressed in *E. coli* with a < 2× increase in MIC, but it gave one of the best results in *R. erythropolis* with a 5 fold increase. It could therefore be possible that the enzyme responsible for resistance in this antibiotic could not be activated in *E. coli*.

3.12.4 Mutagenesis

Since spontaneous mutation and chemical mutagenesis of the *gst* gene did not give rise to any mutant after marker rescue, mutagenesis by the use of a mutator strain *E. coli* CSH116 was employed. Its plasmid encodes a non-functional mutant of the *mutD* gene, and it was expected that this would produce mutants in thousands fold. The

result from this experiment was quite disappointing as only 1 colony was obtained from *R. erythropolis* while HS6 and HS13 gave 2 colonies and 1 colony respectively. It was therefore concluded that none of the methods of mutagenesis employed could give the expected plasmid borne mutant.

3.12.5 Inactivation assays

Preliminary work explored the possibility of obtaining a novel chloramphenicol inactivation phenotype from the cloned *gst* gene fragment. Partial inactivation was found on the *in vitro* assay plate of the clone pBA1, the diameter of the zone of inhibition was measured at 1.5 mm compared with the cell homogenate from the vector control which gave the zone of inhibition of 24 mm, and 30 mm for the antibiotic control. This is an indication that further work could be done to achieve the objective of cloning and characterizing the chloramphenicol inactivation phenotype from the *gst* gene DNA.

3.12.6 Future work

Different methods of mutagenesis techniques were employed to create a plasmid borne mutant from the *gst* gene DNA which did not yield the desired result. A more powerful *in vitro* mutagenesis method by the error prone PCR is therefore suggested to create point mutation in the DNA. A mutant library is to be created for the molecular analysis by carrying out the sequencing, cloning and characterizing the DNA to obtain the inactivation phenotype as well as carrying out the biochemical analysis for the determination of the protein(s) that the mutant encodes. An explanation needs to be sought for the apparent conferral of azithromycin resistance by *E. coli* DNA ligase.

CHAPTER 4

APPENDICES

APPENDIX A

GROWTH MEDIA:

LB (100 ml)

Tryptone	1g
Yeast extract	0.5g
NaCl	0.5g

LA (100 ml)

Tryptone	1g
Yeast extract	0.5g
NaCl	0.5g
Agar	1.5g

LA ½ Agar (100 ml)

Tryptone	1g
Yeast extract	0.5g
NaCl	0.5g
Agar	0.75g

LBSG (100 ml)

Tryptone	1g
Yeast extract	0.5g
NaCl	0.5g
Sucrose	10g
Glycine:	1g for 1%
	2g for 2%
	3g for 3%

LBG (100 ml)

Tryptone	1g
Yeast extract	0.5g
NaCl	0.5g
Glycine	2g

Autoclave for 20 min at 121°C. For LBSG, sucrose was dissolved in water by microwave before adding other components; it was then made up to 100ml. LB was stored at 4°C, while LBSG and LBG were stored at -20°C and melted in the microwave before use.

APPENDIX B

SOLUTIONS

Solutions for *E. coli* mini plasmid preparation

Solution 1

50 mM glucose

25 mM Tris-HCl

10 mM EDTA

pH 8.0

Solution 2

0.2 M NaOH

1.0% SDS

Solution 3

5 M potassium acetate pH 4.8

88.5 ml sdH_2O

11.5 ml glacial acetic acid

Ribonuclease

10 mg/ml RNase dissolved in sdH_2O

Boil at 100°C for 10 min before use

Deoxyribonuclease

10 mg/ml solution in sdH_2O

Boil at 95°C immediately before use

Solutions for *Rhodococcus* mini plasmid preparation

TE buffer

0.5M EDTA

1M Tris-HCl

pH 8.0

TE lysozyme

TE + 5mg/ml lysozyme

TE SDS

TE + 10% SDS

5.0 M KAc (pH6.0)

Solutions for *E. coli* calcium chloride-mediated transformation

$CaCl_2$ transformation buffer

20 mM Tris-HCl

100 mM $CaCl_2$

pH 7.6-8.0

20% glucose

4g glucose

20 ml dH_2O

Autoclave for 20 min at 121°C.

Solution for agarose gel electrophoresis

5× TBE

54.0 g Tris

27.5 g boric acid

20 ml 0.5 M EDTA pH 8.0

Add distilled water to 1 litre, mix and autoclave at 121°C for 20 min.

Running buffer (0.5× TBE)

25 ml 5× TBE

225 ml sdH_2O

25 µl EtBr

Agarose gels (200 ml):

1.6g agarose = 0.8%

2.4g agarose = 1.2%

20 ml 5× TBE

180 ml sdH_2O

Dissolve the agarose in the microwave. 25 ml was used for each gel, and 25µl of ethidium bromide (10 mg/ml) was added.

Tracking dye

30% glycerol (w/v) in TE

0.025% bromophenol blue

Solutions for inactivation assay

Sonication Buffer

2.0 ml 1 M Tris-HCl pH 7.2

100 µl 0.5 M EDTA

100 µl 1 M DTT

97.8 ml dH_2O

Autoclave before use (121°C, 20 min) and store at 4°C.

LDR 1 µg/ml each

Solutions for chemical mutagenesis

Buffer E

10× A-N buffers diluted 1:10

10× A-N buffer

70g K_2HPO_4

26.8g KH_2PO_4

5.0g $Na_3C_6H_5O_7$ (tri-sodium citrate)

1.0g $MgSO_4$

Add distilled water to 1 litre

APPENDIX C

Antibiotics used in this work

ANTIBIOTICS	STOCK (mg/ml)	SOLVENT	SUPPLIER
Ampicillin	100	Ethanol 70%, water 30%	Boehringer Mannheim
Azithromycin	10	Ethanol 70%, water 30%	Unmarked
Chloramphenicol	4	Ethanol	Boehringer Mannheim
Chlortetracycline	10	Methanol	Sigma
Erythromycin	20	Ethanol 70%, Water 30%	Boehringer Mannheim
Fusidic acid	10	Ethanol	Leo pharmaceutical
Kanamycin	100	Water	Boehringer Mannheim
Kasugamycin	10	Water	Boehringer Mannheim
Nalidixic acid	100	Ethanol 70%, Water 30%	Sigma
Rifampicin	10	Methanol	Sigma
Spectinomycin	20	Water	Sigma
Streptomycin	20	Water	Boehringer Mannheim
Oxytetracycline	10	Methanol	Sigma
Tetracycline	20	Methanol	Sigma

APPENDIX D

Calculation of mutation rate

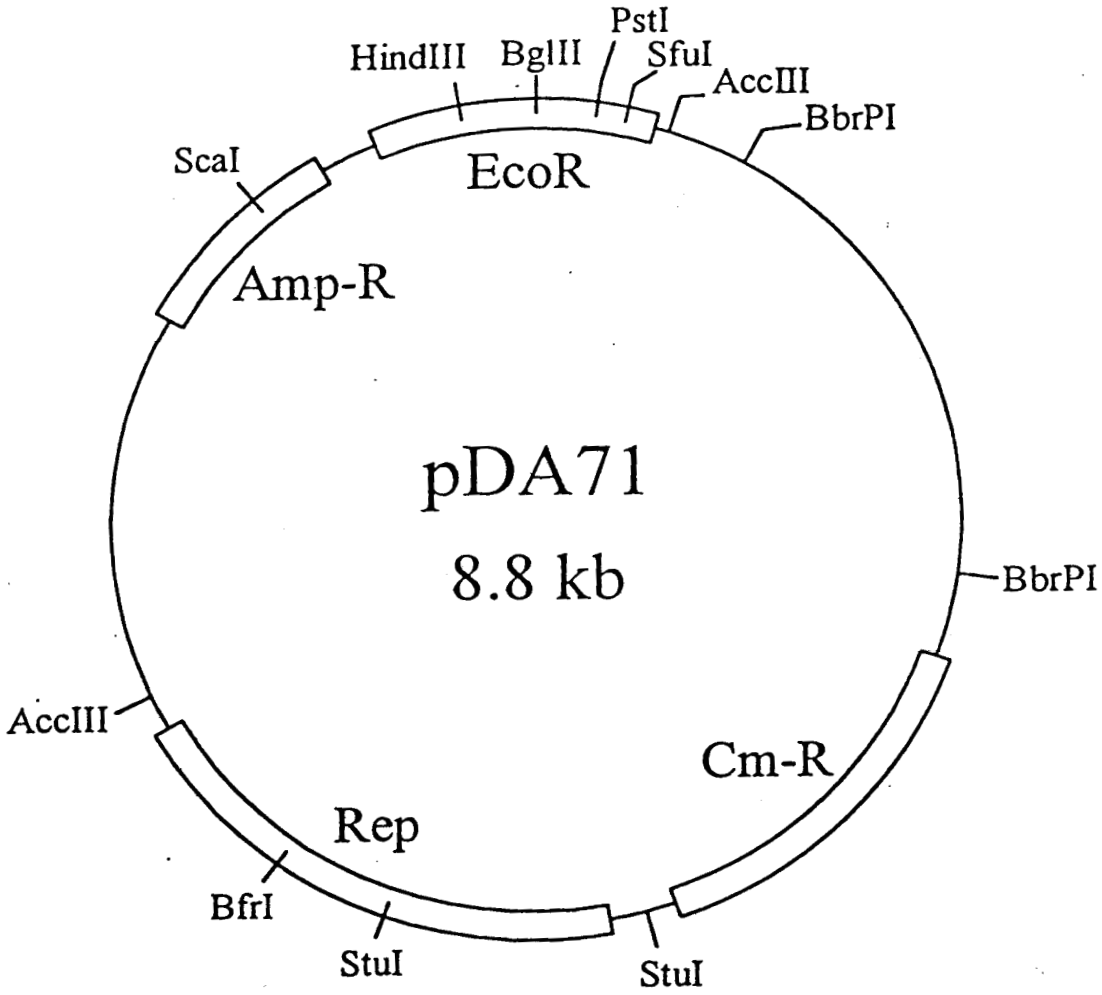
$$\text{Mutation Rate} = \frac{\text{No. of resistant cells/ml}}{\text{Total no. of cfu/ml}}$$

$$\text{Cfu/ml} = \frac{\text{No. of colonies on the LA plate} \times \text{dilution factor}}{\text{Vol. of the culture spread on the plate}}$$

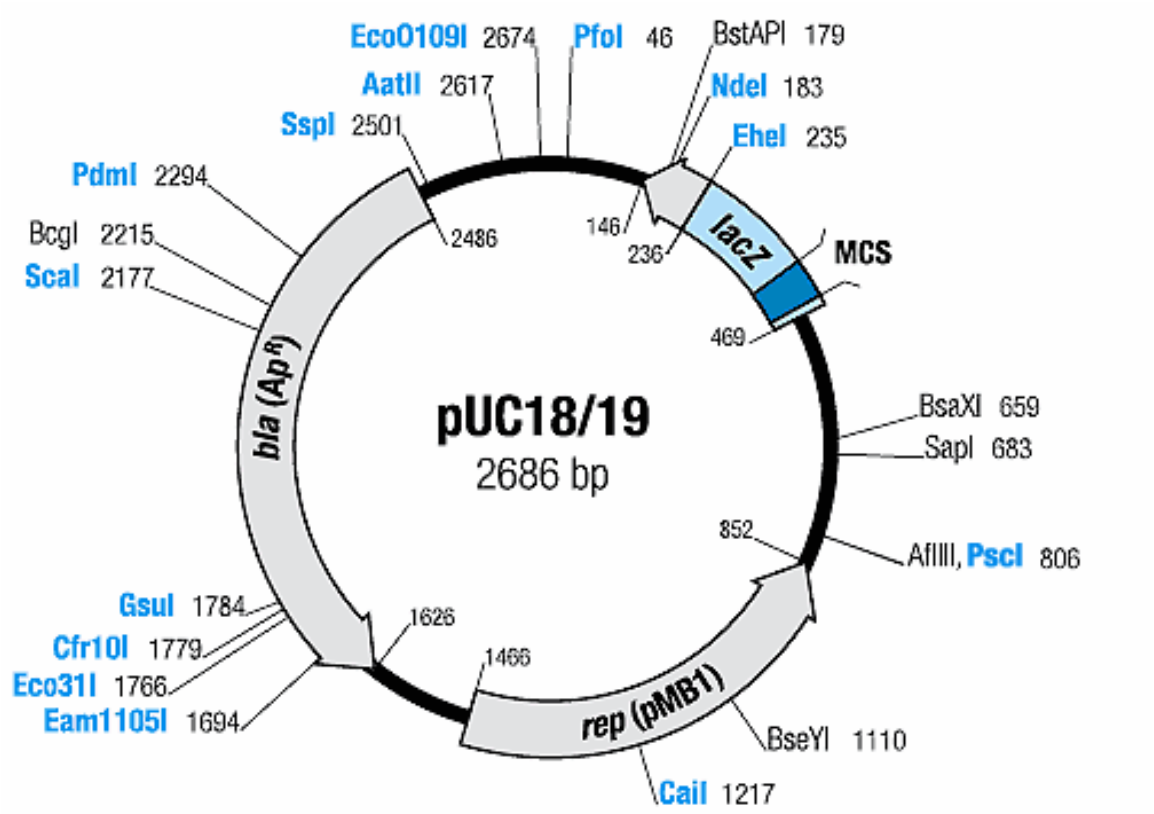
$$\text{No. of resistant cells/ml} = \frac{\text{No. of antibiotic resistant colonies}}{\text{Vol. of the culture spread on the plate}}$$

APPENDIX E

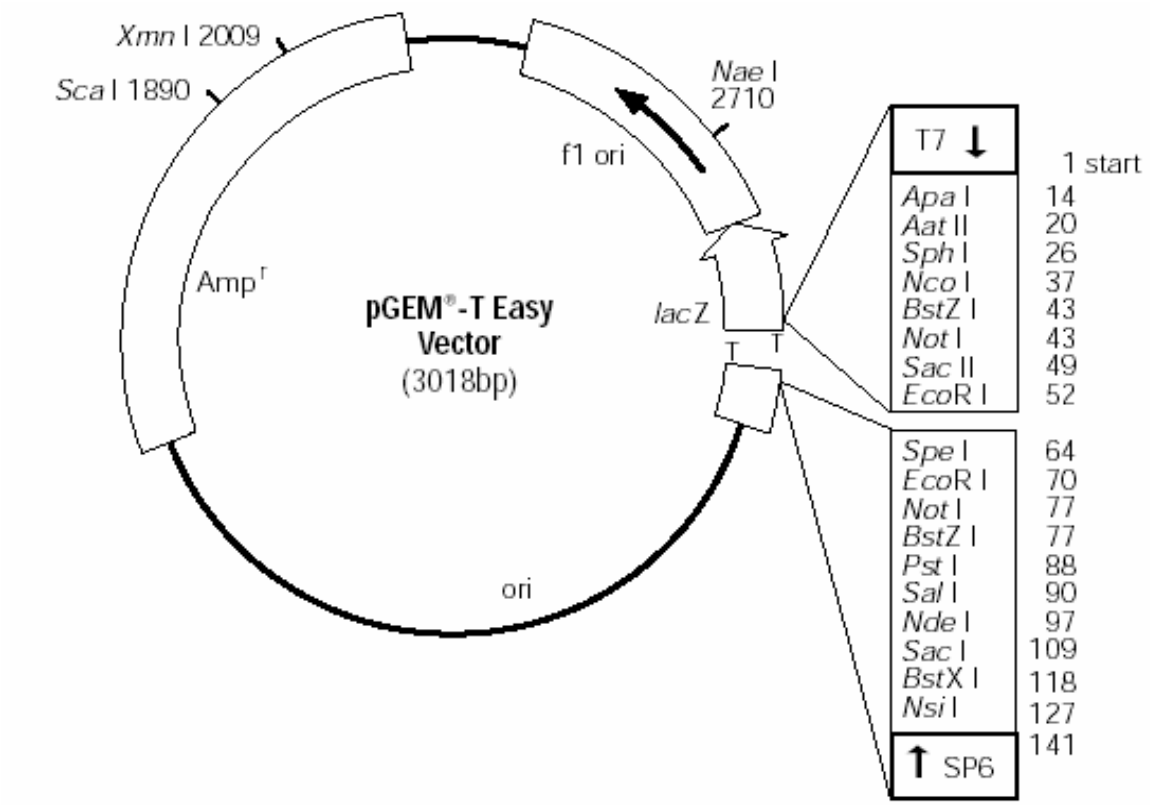
Restriction maps of plasmids



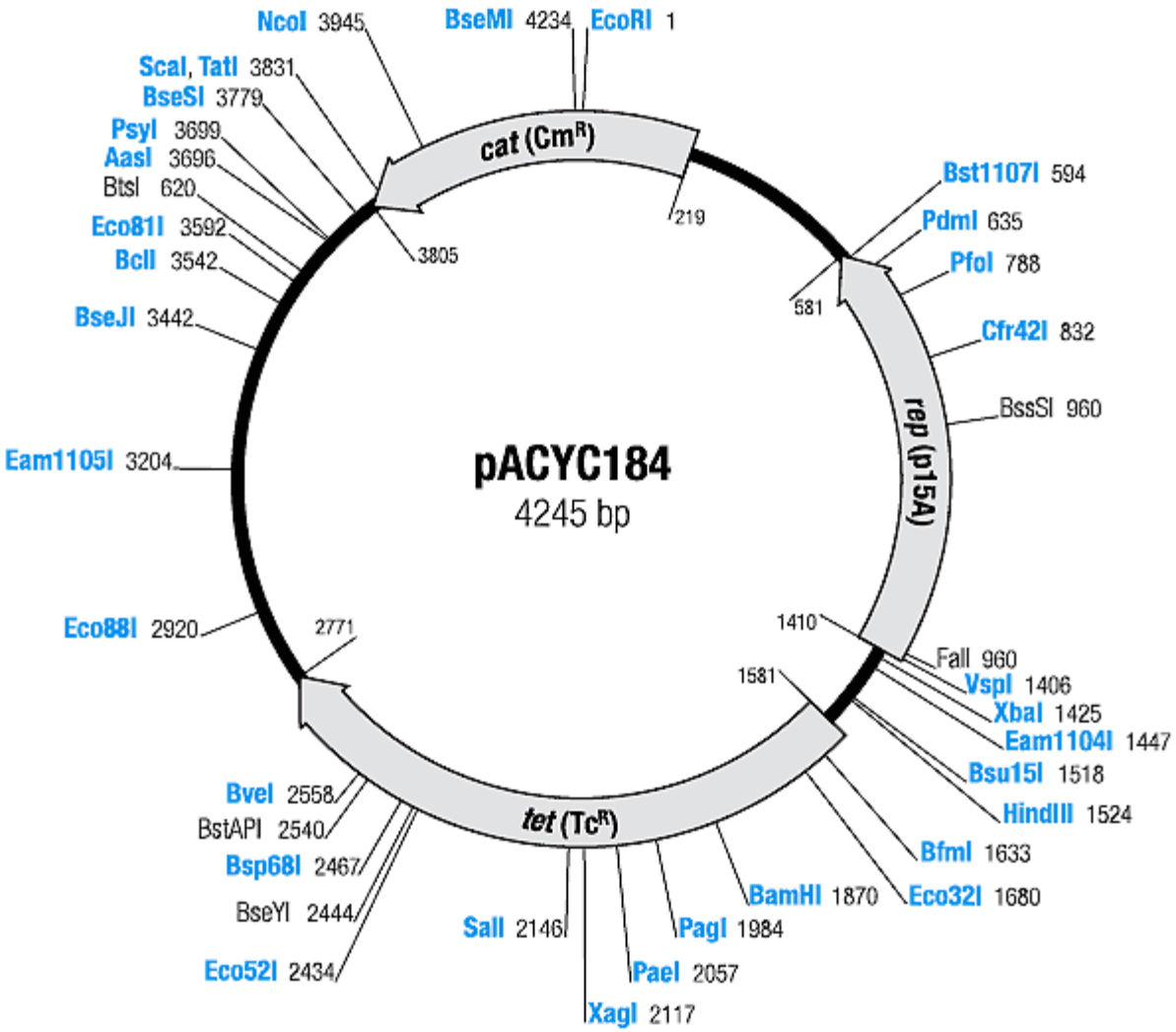
http://seq.yeastgenome.org/vectordb/vector.html



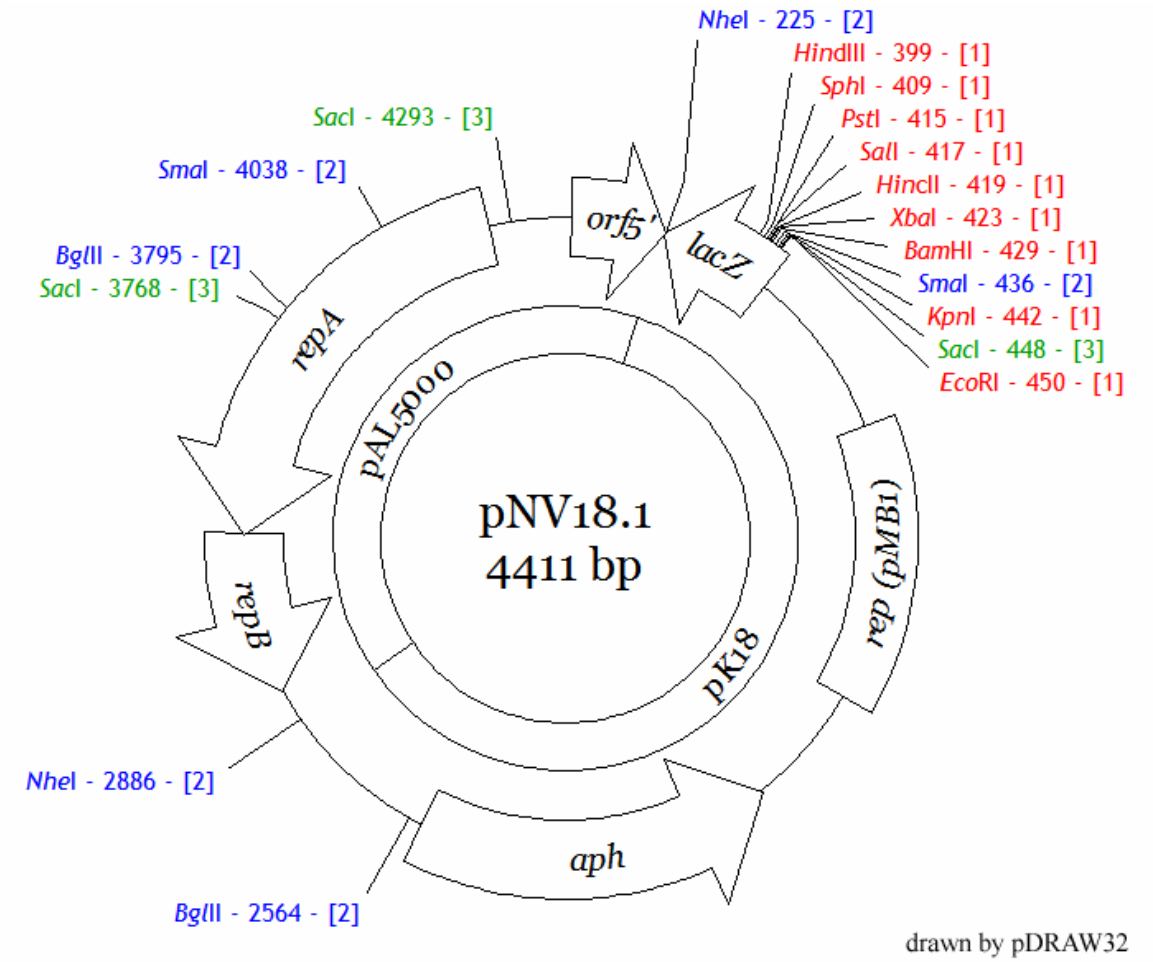
http://www.fermentas.com/techinfo/nucleicacids/mappuc1819.hth



Adapted from www.promega.com, technical manual No. 042)



www.fermentas.com/techinfo/nucleicacids/**mappacyc184**.htm



http://nocardia.nih.go.jp/vectors

CHAPTER 5

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