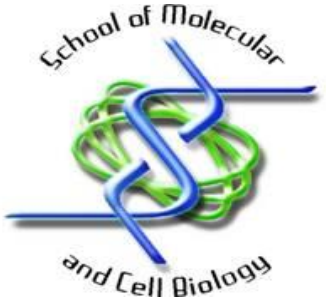
A NEW MYCOBACTERIAL RIFAMPICIN RESISTANCE

Tatenda Shungu Motsi



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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

As an adolescent I aspired to lasting fame, I craved factual certainty, and I thirsted for a meaningful vision of human life - so I became a scientist. This is like becoming an archbishop so you can meet girls.

~M. Cartmill

Research is what I'm doing when I don't know what I'm doing. *~Wernher Von Braun*

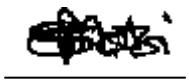
No one should approach the temple of science with the soul of a money changer. *~Thomas Browne*

ABSTRACT

Rifampicin is a major chemotherapeutic agent used against mycobacterial and nocardial infections. However, drug resistance in many of these strains has emerged as a one of the major challenges being faced by researchers. High level resistance to rifampicin is primarily due to mutational alterations in the *rpoB* gene encoding the β subunit of RNA polymerase. A number of cases have been reported in which there is no mutation in this gene leading to the discovery of the inactivation methods: decomposition, ADP-ribosylation, glucosylation and phosphorylation. Decomposition occurs mostly in *Nocardia*, *Rhodococcus* and *Mycobacterium* species. I isolated a new rifampicin resistance gene isolated from the *M. smegmatis* 43756Km1. Its sequence showed that it coded for the enzyme 2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase. This enzyme takes part in the hydrolysis of C-C bonds of aromatic compounds especially in the metabolic pathway of phenylalanine. Rifampicin is bright red but is completely decolourised by *iri*-mediated decomposition but 2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase showed only 75% decolourisation. This may be because this enzyme does not completely breakdown the rifampicin molecule.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.



Tatenda Shungu Motsi

11th day of April 2011,

ACKNOWLEDGEMENTS

First of all I would like to dedicate this work to my family: Abia, Lanna, Rebecca and Tafadzwa Motsi. Thank you for being there for me in every possible way you could. More so to my mother, I hope she will be pleased. I owe her a lot. I would also like to express my sincere gratitude and appreciation to my supervisor, Prof. Eric Dabbs. He has given me encouragement, guidance and advice throughout my project. His contribution was invaluable and I thank him for sharing his knowledge and experience during this very challenging process.

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And to my surrogate family, (Shasha, Zviemurwi, Tariro, Tsungi, Vaidah, Lenny and Julie), who have had to put up with my emotional ups and downs over the past years, and have been lovely all the time. Lastly, I want to acknowledge and thank God for enabling me and encouraging me through it all.

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ABBREVIATIONS

aa	Amino acids
ADP	Adenosine diphosphate
AIDS	Acquired Immunodeficiency Syndrome
AMP	Adenosine monophosphate
Amp	Ampicillin
arr	ADP-ribosyltransferase
ATP	Adenosine triphosphate
bp	Base pair
C	Carbon
$CaCl_2$	Calcium chloride
cm	Centimetre
Cam	Chloroamphenicol
CMN	*Corynebacteriaceae*, *Mycobacteriaceae* and *Nocardiaceae*
CsCl	Caesium chloride
Da	Daltons
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DNAP	DNA-dependent-RNA-polymerase
DNAse	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethylmethanesulfonate
EtBr	Ethidium bromide
EtOH	Ethanol
Ery	Erythromycin
g	Gram
HIV	Human Immunodeficiency Virus
HCl	Hydrogen chloride
hr	Hour
IPTG	Isopropyl-β-D-thiogalactoside
iri	Inactivation of rifampicin

Kan	Kanamycin
kb	Kilobase
kbp	Kilobase pairs
kDa	KiloDaltons
LA	Luria-Bertani Agar
LB	Luria-Bertani Broth
µg	Microgram
µl	Microlitre
M	Molar
mA	Milliampere
MAC	*Mycobacterium avium-intracellulare* complex
MCS	Multiple cloning site
MDR	Multi-drug resistant
mg	Milligram
MIC	Minimal inhibitory concentration
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
Mw	Molecular weight
NA	Not applicable
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
Nal	Nalidixic acid
NaOH	Sodium hydroxide
Ni-NTA	Nickel-nitrilotriacetic acid
nt	Nucleotide
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
Ω	Ohm
OD	Optical density
OH	Hydroxyl group
ORF	Open reading frame

Pers. Comm.	Personal communication
rdn	rifampicin decomposition gene from *M. smegmatis*
rif	rifampicin (rifampin)
RNA	Ribonucleic acid
RNAP	RNA-dependent-RNA polymerase
RNAP/rif	RNA-dependent-RNA polymerase and rifampicin complex
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal RNA
sd	sterile distilled
SDS	Sodium dodecyl sulphate
sec	Second
Spc	Spectinomycin
Str	Streptomycin
TB	Tuberculosis
TE	Tris-EDTA
TES	N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid
Tet	Tetracycline
Tris	Tris(hydroxymethyl)-aminomethane
V	Volt
XDR	Extremely-drug resistant

CHAPTER 1

INTRODUCTION

1.1 Mycobacteria and nocardioforms

Actinomycetes are a large group of Gram-positive filamentous aerobic or facultative anaerobes with G+C rich DNA. Mycobacteria and nocardioforms are part of the actinomycetes family. They are ubiquitous in nature and are wide spread in soil and water. Mycobacteria as well as the nocardioforms produce characteristic lipids known as mycolic acids which are high molecular weight, long chain 3-hydroxyl fatty acids. They also have many phenotypic characteristics in common and form a distinct phyletic line. In evolutionary terms nocardioforms are also closely related to *Mycobacterium*. Nocardioforms have been assigned to five genera: *Corynebacterium*, *Gordona*, *Nocardia*, *Rhodococcus* and *Tsukamurella*, based on 16s rRNA (Chun and Goodfellow, 1995). Actinomycetes have considerable practical and economical significance as they produce the majority of medically useful natural antibiotics.

Mycobacterium smegmatis is a saprophytic species that has been used to explore mycobacterial genetics and molecular biology and as a surrogate host to study the virulence and the regulatory pathways of *Mycobacterium tuberculosis.* Because mycobacterial genes are poorly expressed from their own promoters in *Escherichia coli*, *M. smegmatis* is used as a host strain for gene cloning of other mycobacterial pathogenic species. *M. smegmatis* besides sharing more than 2000 homologues with *M. tuberculosis*, also shares the same unusual cell wall structure of *M. tuberculosis* and other mycobacterial species, making it a perfect candidate for such studies (Hopwood, *et al.,* 1988; Snapper *et al.,* 1988, 1990 and Jacobs *et al.,* 1989). Other reasons why *M. smegmatis* is used so much for research as an important model for the genetic studies of mycobacterial species is because it rarely causes disease, it grows in days instead of weeks and it has a 90% homology to *M. tuberculosis.* The very slow growth rates and pathogenicity issues in working with pathogenic mycobacteria prompted the use of *M. smegmatis* as a model for mycobacterial studies

1.1.1 Pathogenicity of mycobacteria and nocardioforms

Most nocardioforms are non-pathogenic and since they are primarily soil inhabitants there are widely distributed. Usually nocardioforms are free-living micro-organisms; however, many are pathogens of plants, animals and humans. From a medical perspective, mycobacteria cause more harm than nocardioforms. The mycobacterial genus comprises of over a 100 species many of which are pathogenic to humans and animals. This genus contains some major human pathogens, such as *Mycobacterium tuberculosis, M. leprae* and *M. ulcerans*. There are also a number of important opportunistic pathogens within this group, for example the *Mycobacterium avium-intracellulare* complex, commonly referred to as MAC, *M. bovis*, *M. kansasii*, *M. smegmatis* and *N. asteroids* (**Table 1**). The previously regarded non-pathogenic strains including *Rhodococcus equi, R. erythropolis, R. rhodochrous, Gordona bronchialis* and *Tsukamurella paurometabolum* are presently described as opportunistic pathogens (Finnerty, 1992).

Table 1: Several diseases caused by pathogenic actinomycetes.

Disease	**Causative Agent**	**Body sites chiefly involved**
Diphtheria	*Corynebacterium diphtheria*	Throat, occasionally wounds
Equine pneumonia	*Rhodococcus equi*	Lung
Leprosy	*Mycobacterium leprae*	Skin
Tuberculosis	*Mycobacterium tuberculosis*	Lung, spine

Mycobacteria other than the tubercle bacillus sometimes infect humans but they are much less virulent in humans than is *M. tuberculosis*. Infections with these organisms have been called nontuberculous mycobacterial infections. *Mycobacterium bovis* is pathogenic in animals and causes a tuberculosis-like disease in cattle whilst *M. tuberculosis* is mostly pathogenic in humans. Mycobacteria can be classified into several major groups for purpose of diagnosis and treatment of disease in the case of pathogenic strains:

- *M. tuberculosis* complex, which can cause tuberculosis: *M. tuberculosis*, *M. bovis*, *M. canetti*, *M. pinnipedii*, *M. africanum*, and *M. microtii*.
- Nontuberculous mycobacteria (NTM) are all the other mycobacteria, which can cause pulmonary disease resembling tuberculosis, lymphadenitis, skin disease, or any other disseminated disease.

Most exposures and infections by these organisms do not cause disease, infection is usually due to a compromised immune system of the hosts; the frail elderly and the HIV- infected are highly targeted. *M. avium* complex (MAC) which is a close relative of *M. avium* and *M. intracellulare,* accounts for most of these diseases. Other pathogenic species are *M. kansasii*, *M. xenopi, M. marinum*, *M. ulcerans,* and the *M. fortuitum* complex (*M. fortuitum, M. abscessus,* and *M. chelonae*).

Mycobacteria are among the most notable human pathogens responsible for much morbidity and mortality in Southern Africa as well as elsewhere in the developing world (Lucas *et al.,* 1994). They are accountable for more fatalities worldwide than any other single pathogen as it includes the causative agents of tuberculosis, leprosy and other opportunistic organisms. Most mycobacterial infections are difficult to treat since they are capable of withstanding adverse conditions such as long periods of exposure to acids, alkalis, detergents, oxidative bursts and many antibiotics due to their mycolipid rich cell wall, which is neither truly Gram negative nor positive. Furthermore, they are naturally resistant to a number of antibiotics that disrupt cell-wall biosynthesis, such as penicillin. Tuberculosis (TB) is the most notable disease caused by mycobacteria and statistics for incidence, deaths distribution are discussed below.

It is estimated that one third of the world's population, that is, about two billion people are infected with TB-causing bacteria (World Health Organization (2009): Fact sheet on Tuberculosis) and every 20 sec a person dies from TB. According to the World Health Organization (WHO) 98% of TB deaths occur in developing countries and the incidence of TB is decreasing or stable in all regions of the world except for Africa. Most of the estimated number of cases in 2008 occurred in Asia (55%) and Africa (30%), with small proportions of cases in the Eastern Mediterranean Region (7%), the European Region (5%) and the Region of the Americas (3%). The five countries that rank first to fifth in terms of total numbers of incident cases in 2008 were India (1.6–2.4 million), China (1.0–1.6 million), South Africa

(0.38–0.57 million), Nigeria (0.37–0.55 million) and Indonesia (0.34–0.52 million). India and China alone account for an estimated 35% of TB cases worldwide (TB WHO 2009 Report). Tuberculosis is a chronic, progressive infection which has a period of latency following initial infection; therefore some people only get sick possibly years after infection. TB occurs most commonly in the lungs but in other active cases, the infection moves from the lungs, causing extrapulmonary tuberculosis (Golden and Vikram, 2005). Extrapulmonary infection sites include the pleura in tuberculosis pleurisy, the central nervous system in meningitis, the lymphatic system and bones and joints in diseases of the spine. Extrapulmonary TB may co-exist with pulmonary TB as well. TB is also a primary cause of death among people who are HIV positive and is responsible for more than 13% of AIDS-related deaths worldwide. HIV fuels XDR–TB (Extensively drug-resistant tuberculosis) and once someone is infected with TB there is a 5–10% lifetime risk of developing this disease a year.

The TB bacillus develops resistance through incomplete, erratic, or inadequate treatment and many TB patients do not complete their full 6-9 month drug regimen (TB Alliance, 2009). Treatment of TB is with multiple anti-microbial drugs this is because treatment with any single antibiotic always results in survival of a few organisms that have acquired spontaneous resistance mutations. Use of multiple drugs makes the occurrence of these simultaneous spontaneous mutations to be unlikely. The most widely used chemotherapeutic agents to combat mycobacterial infections are ethambutol, isoniazid, pyrazinamide and rifampicin (Grosset, 1989), **Table 2**.

Cases have been recorded however, whereby a strain resistant to one of the drugs has developed and proliferated and also acquired resistance to the other drugs through the same process. XDR-TB (extremely drug resistant TB) is a form of TB caused by bacteria that are resistant to the most effective anti-TB drugs. It has emerged from the mismanagement of multidrug-resistant TB (MDR-TB). Resistance to first line anti-TB drugs (isoniazid and rifampicin) defines MDR-TB and XDR-TB is defined as TB that has developed resistance to at least rifampicin and isoniazid as well as to any member of the quinolone family and at least one of the following second-line anti-TB injectable drugs: amikacin, capreomycin or kanamycin (World Health Organisation. 2006). Alternative treatments require a longer treatment course with less effective, more toxic, and more expensive second-line drugs. Surgery to remove localized areas of lung destruction plays an important role in the treatment of advanced cases of MDR-TB or XDR-TB but is not widely available in high-burden

regions. 27 countries account for 85% of all MDR-TB cases. The top five countries with the largest number of reported cases are India, China, Russian federation, South Africa and Bangladesh. XDR-TB has been found in 57 countries to date (WHO: TB Fact sheet, 2009). More than 5% or the world's TB cases may be multidrug-resistant and 10% of those may be extensively drug-resistant. Rifampicin is the focus of this study.

Table 2: Gene targets for anti-tuberculosis drugs and mutations associated with drug resistance (Adapted from Drobniewski and Wilson 1998).

Antimicrobial agent	**Probable targets**	**Mutation in genes conferring resistance**	**Function of gene**
Rifampicin	RNA synthesis	*rpoB*	DNA-dependent RNA polymerase (β subunit)
Isoniazid	Mycolic acid biosynthesis	*katG* *inhA/ mabA* *ahpC* *oxyR*	Catalase/peroxidise Fatty acid biosynthesis Alkyl hydroperoxide C reductase Oxidative stress regulator
Pyrazinamide	Pyrazinamide (Pzase)	*pncA*	Pzase nicotinamidase; loss of Pzase gives resistance
Streptomycin	Protein synthesis	*rrs* *rpsL*	16s rRNA Ribosomal protein S12
Ethambutol	Cell wall synthesis	*embA, B, C*	Lipoarabinomannan and arabinogalactan synthesis
Ethionamide	Cell wall synthesis	*inhA*	Cross-resistance associated with *inhA* mutations
Ciprofloxacin	DNA synthesis	*gyrA, gyrB, nor*	DNA gyrase subunit A and B (principally)

1.2 Antibiotics

Antibiotics are chemotherapeutic substance or compounds that inhibit the growth of micro-organisms, such as bacteria, fungi, or protozoa. They can be produced by micro-organisms or they can be made synthetically in the laboratory. There are a large number of antibiotic classes and the major ones are listed in **Table 3**. Antibiotics are relatively harmless to the hosts, they show selective toxicity and therefore can be used to treat infections.

Table 3: Antibiotic classes and their modes of action.

CLASS	EXAMPLES	TARGET
Aminoglycosides	streptomycin, kanamycin, spectinomycin	translation
Ansamycins	rifampicin, streptovaricin,	Transcription
Beta-lactams	penicillin, amoxillin	cell wall biosynthesis
Macrolides	erythromycin, tylosin	Translation
Tetracyclines	oxytetracycline, tetracycline	translation

1.2.1 The ansamycins

This family of antibiotics was first discovered in 1957 and introduced in 1968 (Sensi, 1983) as a major addition to the cocktail drug treatment of TB. Most ansamycins (**Figure 1**) are secondary metabolites of actinomycetes which are the producers of most antibiotics. The word, ansamycin refers to the group of antibiotics that are characterized by the presence of an aliphatic 17-membered ansa bridge connecting two non-adjacent positions of an aromatic nucleus. Ansamycins are natural compounds with a macrocyclic structure consisting of an aromatic ring system connected to an aliphatic chain that forms an amide linkage to the amino group of the aromatic moiety.

Ansamycins are classified into two groups based on the structure of their aromatic moiety. The first group has a naphthalene nucleus and is isolated from actinomycetes or higher plants. It includes the rifamycin antibiotics, tolpomycin Y, halomicin B, rifampicin and streptovaricin A. The second group has a benzene aromatic nucleus and includes the macrolide: geldamycin and ansamitocin, ansatrienin, geldamycin, herbimycin and mycotrienin. Ansamycins with a naphthalene nucleus are bacteriostatic and inhibit RNA polymerase while the benzoquinoid ones affect DNA replication in eukaryotic cells (Lancini and Zanichelli, 1977; Lancini, 1983) and are cytotoxic against eukaryotes. Rifamycins are used for the treatment of tuberculosis, leprosy, gonorrhoea, and biliary tract and respiratory infections. They have been described as having *in-vitro* activity against *Helicobacter pylori*.

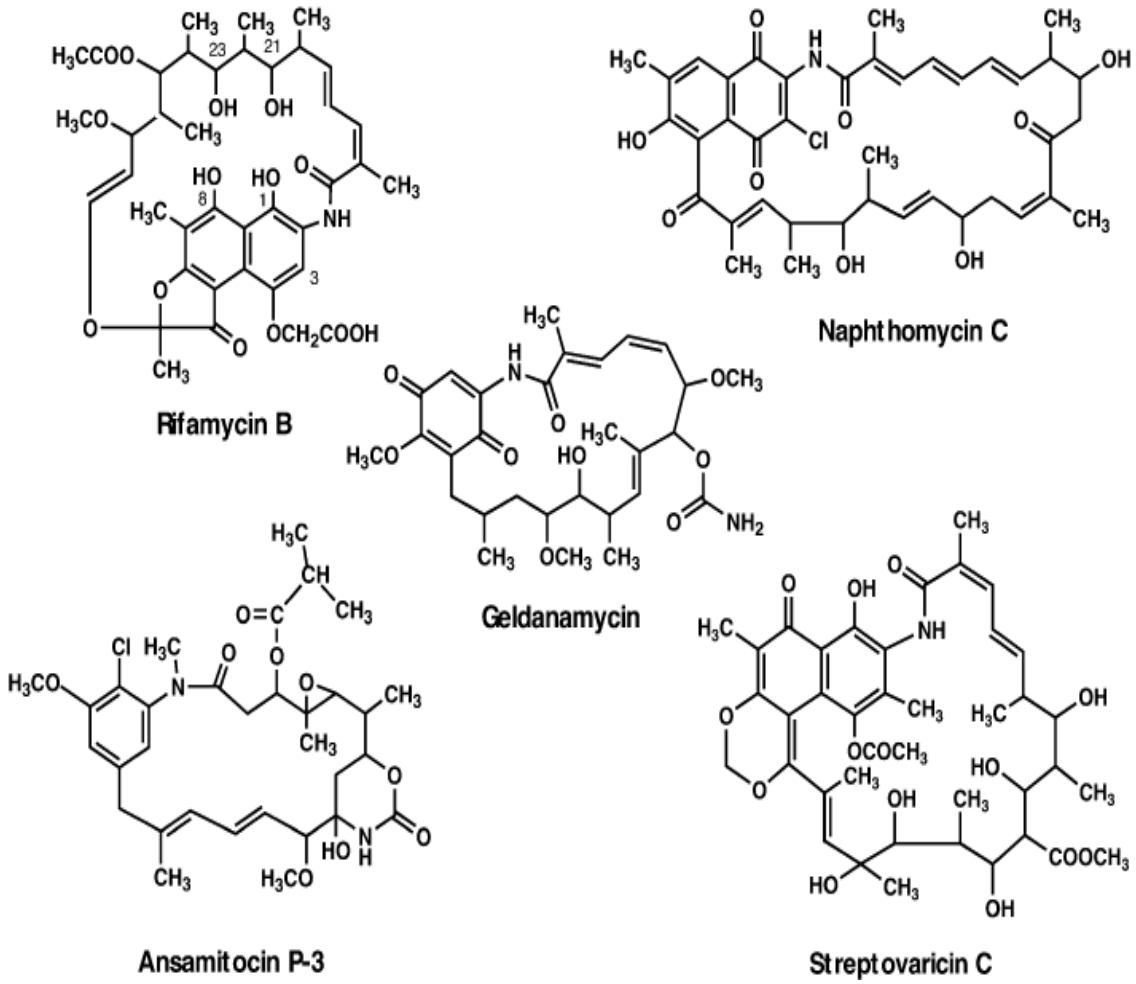


Figure 1: Structures of representative ansamycins (Adapted from Floss and Yu, 2004).

1.2.1.1 The rifamycins

Rifamycins are the naphthalenic ansamycin group of antibiotics and these were originally identified in 1957. They are secondary metabolites of the actinomycete *Amycolatopsis rifamycinica* (which was originally classified as *Streptomyces mediterranei*, (Margalith and Beretta, 1960) reclassified as *Nocardia mediterranei*, (Thiemann *et al.,* 1969), reclassified and reassigned to a newly defined genus as *Amycolatopsis mediterranei* (Lechevalier *et al.,* 1986) and most recently reclassified as *Amycolatopsis rifamycinica* **(**Bala *et al.,* 2004**)**. Despite its transfer to the new genus, *Amycolatopsis* is closely related to *Nocardia* species in various aspects of its physiological and biochemical nature. *A. rifamycinica* was found to be resistant to rifamycins and this is probably because it has a form of RNA polymerase which is not inhibited by rifamycins (Blanco, 1984 *et al.,*). It has been suggested that there may be a

close similarity between the resistance of a producing organism to its own antibiotics and such resistance in pathogenic bacteria (Benveniste and Davies, 1973).

The fermentation broth of *A. rifamycinica* led to the discovery of its derivatives which were five biologically-active compounds, designated rifamycin A, B, C, D and E. The only stable crystalline compound, rifamycin B, proved to be the least active antibiotic. A vast number of chemical modifications to the structures of the rifamycins have been made to obtain substances possessing greater activity. As such, the spontaneous expulsion of glycolic acid from rifamycin B yielded rifamycin S. Through subsequent reductions this resulted in the production of rifamycin SV (Sensi *et al.*, 1960). This compound is very active against Gram-positive bacteria such as *M. tuberculosis* and moderately active against several Gram-negative bacteria. However, rifamycin SV is poorly absorbed in the gastrointestinal tract, so oral administration did not give rise to effective levels in the blood stream of patients. Therefore, it was modified to create an antibiotic with improved antimicrobial efficacy by increasing its activity spectrum and absorption characteristics. A semi-synthetic derivative of rifamycin SV called rifampicin was then synthesized. This antibiotic was proven to have good oral absorption, and excellent distribution in body tissues and fluids. Furthermore, it had a low incidence of adverse reactions to daily administration (Sensi, 1983).

Other semi-synthetic rifamycin derivatives, such as rifabutin (Brogden and Fitton, 1994) and rifapentine (Mealy, 1979) were subsequently introduced for clinical use **Figure 2**. Rifabutin in particular is active against a number of rifampicin-resistant clinical pathogens ((Brogden and Fitton, 1994) and, is the main drug for treating MAC infection. It is used for patients taking drugs (particularly antiretroviral drugs) that have unacceptable interactions with rifampicin. Its action is similar to rifampicin, but it has been associated with uveitis when used with clarithromycin or fluconazole. Rifapentine on the other hand is used in one dose or one week regimens but is not used in children or patients with HIV (because of unacceptable treatment failure rates) or extrapulmonary TB.

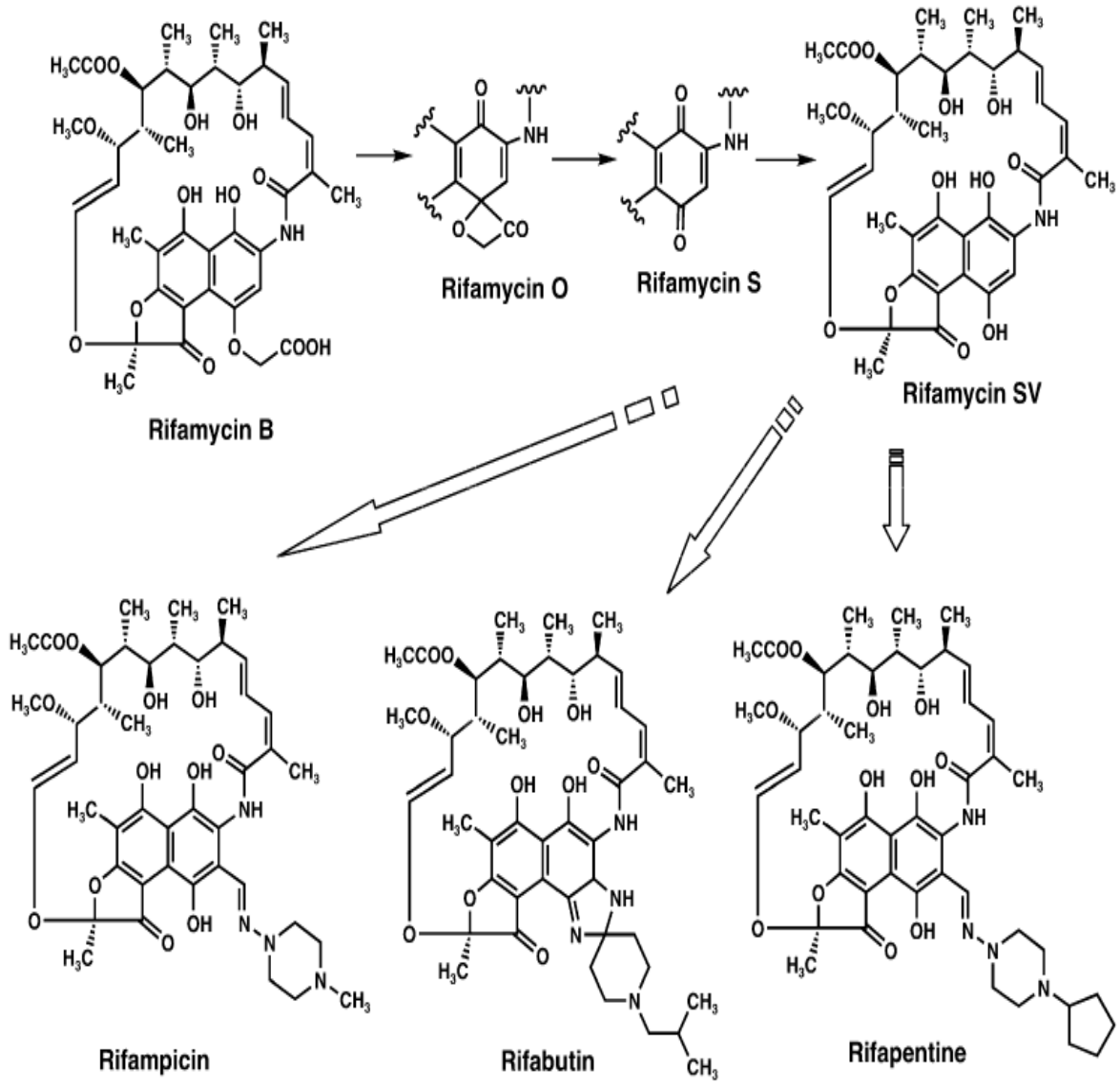


Figure 2: Conversion of rifamycin B into rifamycin SV and clinically used derivatives (Adapted from Floss and Yu, 2004).

1.3 Rifampicin

Rifampicin (Rif) is an odourless red-brown crystalline powder which readily dissolves in methanol, dimethyl sulfoxide and chloroform and is slightly soluble in water and acetone. Rifampicin ($C_{43}H_{58}N_4O_{12}$) has a molecular weight of 822.94 g\mol. It is an effective broad-spectrum bacteriostatic drug that is utilised predominantly as part of a cocktail-drug treatment of meningitis, AIDS associated mycobacterial infections and tuberculosis. In the treatment of

TB it is used in tandem with isoniazid, ethambutol and streptomycin for it to be effective as resistance to this antibiotic develops quickly during treatment (Long, 1991). It is also used in combination with dapsone and clofazimine in the treatment of leprosy. Rifampicin has many significant drug interactions. It accelerates metabolism of anticoagulants, oral contraceptives, clarithromycin, corticosteroids, digitoxin, warfarin and many other drugs. The interactions of rifamycins and many anti-retroviral drugs is particularly complex; combined use requires specialized expertise.

It is also employed in the treatment of infections caused by *Neisseria meningitides* (Carter *et al.*, 1994), *Haemophilus influenzae*, methicillin-resitant *Staphylococcus aureus* (MRSA) (Kapusnik *et al.*, 1984), *Legionella pneumophila*, *Streptococcus pneumoniae* and other pathogens (Abadi *et al.*, 1996). The lipophilic characteristics of rifampicin contribute considerably to its absorption and distribution throughout cellular tissues and body fluids. Therefore, it is a good candidate to treat the meningitis form of tuberculosis, which requires distribution to the central nervous system and penetration through the blood- brain barrier.

The most significant functional groups of rifampicin are the two free hydroxyl (OH) groups in positions 21 and 23 of the ansa chain and the presence of a napthoquinone ring carrying oxygen atoms at positions C_1 and C_8 in either the quinine of the hydroquinone form (**Fig 3**). These are the minimum requirements for antimicrobial activity as they are involved in the formation of a tight but reversible linkage to DNA-dependent-RNA-polymerase. Any modification involving a substitution, the molecular size of which is not important, or elimination of the 21- or 23-hydroxyl groups results in an inactive compound (Wehrli and Staehelin, 1971). Its most clinically effective derivative has an addition of a 4-methyl-1-piperazinaminyl group. The emergence of rifampicin-resistant strains has led to the use of structural analogues of rifampicin. Rifapentine, rifabutin, and rifalazil are new rifamycin derivatives and are tested in rifampicin-resistant isolates.

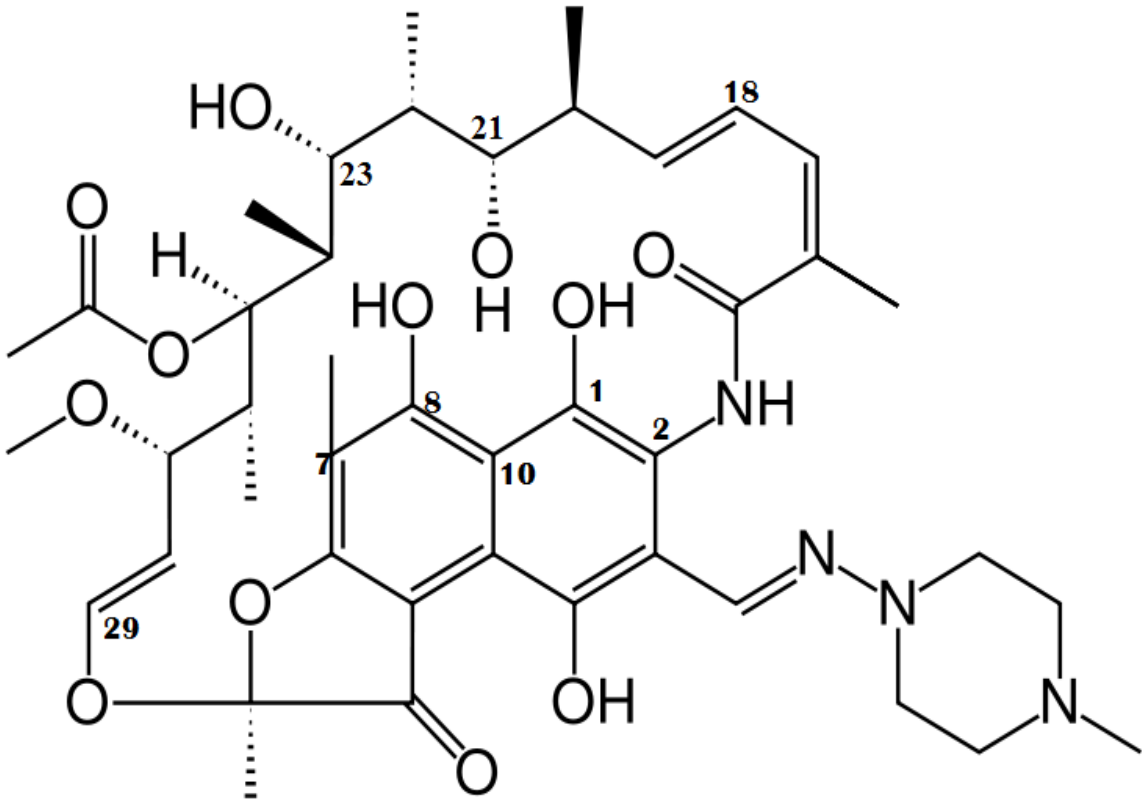


Figure 3: The chemical structure of rifampicin, (Adapted from Campbell *et al.*, 2001)

1.3.1 Mode of action

Rifampicin acts directly on messenger RNA (mRNA) synthesis. In 1967, Hartmann *et al.*, proposed that the rifamycins might act on RNA synthesis based on the observation that rifampicin inhibited uracil uptake in *S. aureus*. Experiments in *E. coli* showed that rifampicin inhibited transcription by binding to DNA-dependent RNA polymerase and preventing elongation of initiated RNA chains beyond a few nucleotides resulting in the accumulation of abortive initiation products (Johnston and McClure, 1976). Rifampicin was also shown to inhibit *M. smegmatis* RNA polymerase presumably by a similar mechanism to *E. coli* (Wiggs *et al.*, 1979). Levin and Hatfull in 1993 confirmed that RNA polymerase was the target for rifampicin and that the effect of the antibiotic is to inhibit the formation of full-length transcripts **Fig 4**.

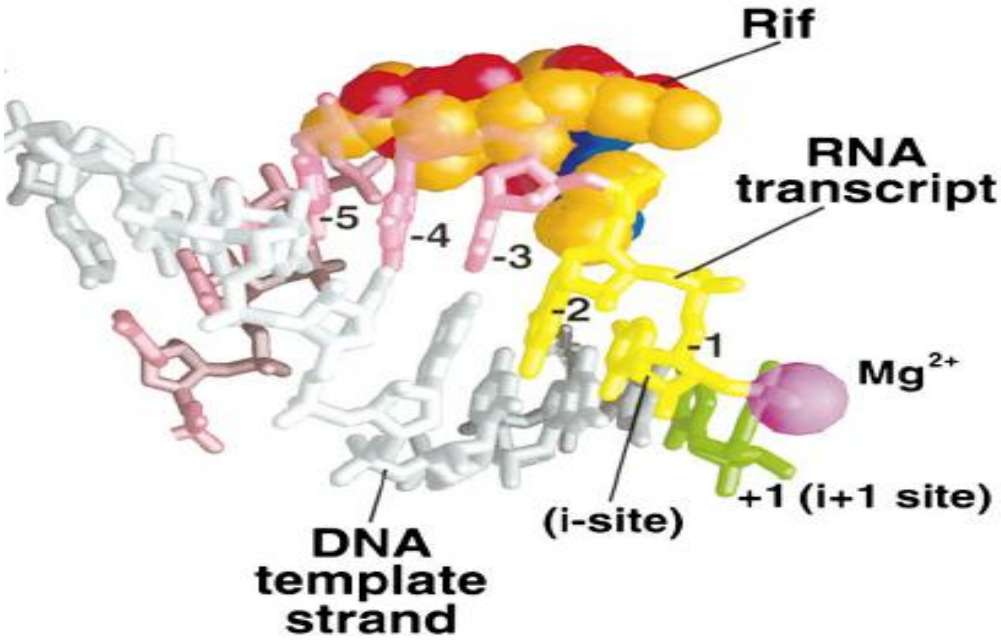


Figure 4: Mechanisms of RNA polymerase inhibition by rifampicin, (Adapted from Campbell *et al.*, 2001). The RNA itself and the rest of the nucleic acids have not been depicted for reasons of clarity. Mg^{2+} is represented by the **magenta** sphere. The incoming nucleotide substrate at the -1 position is shown in **green** and the -1 and -2 positions that can be accommodated in the presence of rifampicin are depicted in **yellow**. The RNA further upstream (-3 to -8) that cannot be accommodated in the presence of rifampicin is coloured **pink.** The DNA template strand is **grey**. Rifampicin is shown positioned in its binding site on the β subunit. The carbons atoms are coloured in **orange** while the oxygen in **red** and nitrogen in **blue**, (Nicholls et al., 1991). The rifampicin is partially transparent, illustrating the RNA nucleotides (at -3 to -5) that sterically clash.

Rifampicin inhibits DNA-dependent RNA polymerase (RNAP) in bacterial cells by binding its β-subunit, thus preventing transcription to RNA and subsequent translation to proteins. RNAP is responsible for the transcription of DNA into RNA, a process achieved by four enzymatic steps: DNA binding, chain initiation, chain elongation and chain termination. This enzyme consists of five polypeptides: namely, two α subunits, one β subunit, one β′ subunit and one σ subunit. The β subunit is an important target for anti-mycobacterial drugs such as rifampicin, which exerts its effects by binding to the RNA polymerase and prevents transcription by preventing the elongation of initiated RNA chains beyond a few nucleotides,

thus inhibiting bacterial growth (Wehrli and Staehelin, 1971; Zillig *et al.,* 1970). The β subunit of the DNA-dependent RNA polymerase is encoded by the *rpoB* gene of bacteria. Rifampicin interacts with the β subunit of RNA polymerase when it is in a $\alpha_2\beta$ trimer. This halts mRNA transcription, therefore preventing translation of polypeptides. However, it cannot stop the elongation of mRNA once binding to the template-strand of DNA has been initiated. The rifampicin-RNA polymerase complex is extremely stable and yet experiments have shown that this is not due to any form of covalent linkage. It is hypothesized that hydrogen bonds and π-π bond interactions between napthoquinone and the aromatic amino acids are the major stabilizers, though this requires the oxidation of napthohydroquinone which is found most commonly in Rif. It is this last hypothesis that explains the explosion of multi-drug-resistant bacteria: mutations in the *rpoB* gene that replace phenylalanine, tryptophan, and tyrosine with non-aromatic amino acids result in poor bonding between Rif and the RNA polymerase (Erlich *et al.,* 1973).

1.4 Antibiotic resistance mechanisms

Antibiotics like rifampicin are used in the control of infectious diseases. Unfortunately, the antibiotics can also select for microbes resistant to these drugs. Resistance to antibiotics has been around for as long as the antibiotics themselves, but they have become more serious. Some of the mechanisms of resistance are listed below:

- A reduced ability of the antibiotic to enter into the bacterium due to alterations in the structure of the outer membrane, as is the case for a number of mycobacteria (Carter *et al.,* 1994).
- rRNA methylation which prevents inhibition of protein synthesis by the drug.
- Efflux, (Bambeke, 2000).
- An increased production of a metabolite that antagonizes the antibiotic.
- Reduction of cell wall permeability. Abadi *et al.,* (1996) provided evidence that changes in cell wall permeability might diminish rifampicin susceptibility.
- The production of enzymes that inactivate the drug.
- Alteration of the target site e.g. alteration of PBP-the binding site of penicillin in MRSA.
- Alteration of the metabolic pathway.

1.4.1 Rifampicin resistance

There are a number of antibiotic resistance mechanisms as noted from the above discussion which occur for the various antibiotics, but in the following section only those which are applicable to rifampicin are discussed.

1.4.1.1 Rifampicin resistance by reduced cell wall permeability

Resistance to rifampicin, through the prevention of drug influx by modifications affecting the permeability of the cell wall has been observed in *Neisseria meningitis* (Abadi *et al.,* 1996). However, in Gram-negative bacteria the cell envelope is effective at restricting permeation by antibiotics and the first barrier in the envelope is the outer membrane. The extremely slow influx of lipophilic antibiotics is due to the ordering of water molecules within the membrane. Experiments done with Tween 80, a substance that alters membrane permeability, revealed high-level resistance shown by one strain was due to a decrease in membrane permeability. Consequently, the drug was unable to enter the cell. An earlier report also implicated changes in cell wall permeability as being responsible for rifampicin resistance in *M. avium* (Hui *et al.,* 1977).

1.4.1.2 Rifampicin resistance through active efflux

Efflux pumps are responsible for the removal of toxic substrates, including many classes of clinically important antibiotics from within the cell environment. This results in a decreased accumulation of the antibiotic within the cell. In 1997, Sánchez *et al.,* identified efflux pumps as a contributing factor to Rif resistance by the pathogen *Haemophilus influenzae*. These efflux pumps were actively transporting the drug out of the cell. The study identified a three-gene complex that was homologous to the *acrRAB* cluster of *E. coli*. These genes encode an efflux system that is responsible for the extrusion of toxic dyes, detergents (Nikaido, 1996) and lipophilic antibiotics (Okusu *et al.,* 1996) from the Gram-negative organism.

1.4.1.3 Rifampicin resistance through target-site modifications

Endogenous rifampicin resistance by mutation of the target moiety is ordinarily the most widespread. Extensive studies of the development of resistance by *M. tuberculosis* to Rif revealed that in most cases it develops as a result of mutations occurring in a region of the *rpoB* gene, thereby modifying the β-subunit of the RNA polymerase such that it is no longer recognizable by the antibiotic. However, the enzyme maintains its binding properties such that it can still function normally (Heym and Cole, 1997). Rifampicin resistance in *E. coli* was shown to result mostly from point mutations in four well-defined clusters of *rpoB*. Some strains of *M. tuberculosis* which are resistant to Rif have shown to possess mutations in the largest of these rifampicin resistance clusters. A sequence comparison in the four distinct regions of the *rpoB* gene showed a low level of conservation between prokaryotes and eukaryotes. This may be the reason why Rif is not active against eukaryotic RNAPs (Campbell *et al.,* 2001).

In *M. tuberculosis,* mutation in one of 3 loci in *rpoB* has been associated with Rif resistance. Several studies conducted on Rif resistant clinical isolates and multi-drug resistant isolates have shown that these mutations are missense mutations, deletions and insertions and occur in a 69 base pair (bp) conserved region encoding 23 amino acids (Musser, 1995). Most of these mutations are in this Rif resistance-determining region of the *rpoB* gene coding for amino acids 507 through 533. Codons 531, 526, and 516 are reported as the most frequent mutation sites, with codon 531 mutations being the most common (Ramaswamy and Musser, 1998).

In *E. coli* most mutations are clustered in a 225 bp region of the *rpoB* gene (Jin and Gross, 1988). Similar work done in *M. leprae* (Honore and Cole, 1993) and *M. tuberculosis* (Telenti *et al.,* 1993) identified the same section as the mutational hotspot for Rif resistance. Using the *E. coli* system of codon numbering (Ezekiel and Hutchins, 1968) the codons most prone to mutations were found to be 516, 526 and 531. Rifampicin-resistant bacteria therefore, produce RNA polymerases with subtly different β subunit structures which are not readily inhibited by the drug. Most Rif^R clinical isolates of *M. avium, M. intracellulare* (Guerrero *et al.,* 1994), *M. tuberculosis* (Ohno *et al*., 1996) and *M. smegmatis* (Hetherington *et al.,* 1995) do not have mutations in the *rpoB* gene suggesting other resistance mechanisms are involved in response to this challenge by Rif. One of which being the inactivation of the drug.

1.4.1.4 Rifampicin resistance by inactivation mechanisms

Analysis of clinical isolates that showed no mutation in the *rpoB* gene allowed for the identification of drug inactivation genes. Inactivation is due the modification of rifampicin by enzymatic activity and four mechanisms have been identified and these are decomposition, ribosylation, glucosylation and phosphorylation (**Table 4**). Major changes in the absorption spectrum of Rif which are readily visible to the naked eye occur during inactivation. The colour of the Rif solution turns from a bright orange to a dull red, as would be expected due to the disappearance of the principal absorbance peak in the visible part of the spectrum at 474nm (Dabbs, 1987).

1.4.1.4.1 Ribosylation

This mechanism of inactivation was first identified in 1995 when the inactivated compounds were isolated during studies conducted on fast-growing strains of mycobacteria. Ribosylation is limited to strains containing MK-9-type menaquinones such as *Gordona*, *Tsukamurella* and *Mycobacterium*. Ribosylation of rifampicin has been observed in the fast-growing *Mycobacterium* species: *Mycobacterium smegmatis*, *M. avium, M. parafortuitum, M. vaccae, M. flavescens* and the closely related slow-growing genera *Gordonia* and *Tsukamurella* (Dabbs *et al*, 1995). Further studies showed it is the only resistance mechanism in the closely related slow-growing genera *Gordona* and *Tsukamurella.* Interestingly, these strains were transferred from the genus *Rhodococcus* only due to differences in their menaquinone (Mk-9 type) compositions (Stackebrandt *et al.*, 1988).

Table 4: Rifampicin inactivation genes of mycobacteria and nocardioforms (Adapted from Dabbs, 2004).

Organism (strain)	Mechanism	Gene	Reference
R. equi ATCC 14887	decomposition	monooxygenase (*iri*)	Andersen and Dabbs, 1991
M. smegmatis DSM 43756	ADP-ribosylation (C_{23})	ADP-ribosyl transferase (*arr*)	Quan *et al.*, 1997
N. brasiliensis IFM 0236	glucosylation (C_{23})	glycosyltransferase (*rgt*)	Lephoto *et al.*, unpublished
N. otitidiscaviarum IFM0239	phosphorylation (C_{21})	not known	A. Rebić, unpublished

1.4.1.4.2 ADP-ribosylation

All the processes of ribosylation target the C-23 of the drug. The ADP-ribosylated compound is modified by the two-step removal of nicotinamide and ADP resulting in ribosylated rifampicin. This mechanism functions in many mycobacterial opportunistic pathogens and ADP-ribosylation usually occurs on a nitrogen atom of a protein. Here, the ADP-ribose moiety is attached to the C-23 of rifampicin via an O-glycosidic bond (**Figure 5**) and results in 23-O-ADP-ribosyl rifampicin (RIP-TA). The initial ADP-ribosylation step is followed by the removal of adenosine monophosphate (AMP) and thereafter dephosphorylation gives rise to ribosylated rifampicin, (Imai *et al.*, 2000).

Several ADP-ribosylation reactions have been described in both eukaryotes and prokaryotes. It is highly unlikely that it is present to combat rifamycins as it is more active against semi-

synthetics than the natural antibiotic (Gianniosis, 2006). More experimentation needs to be performed to elucidate the function of this protein within the cell.

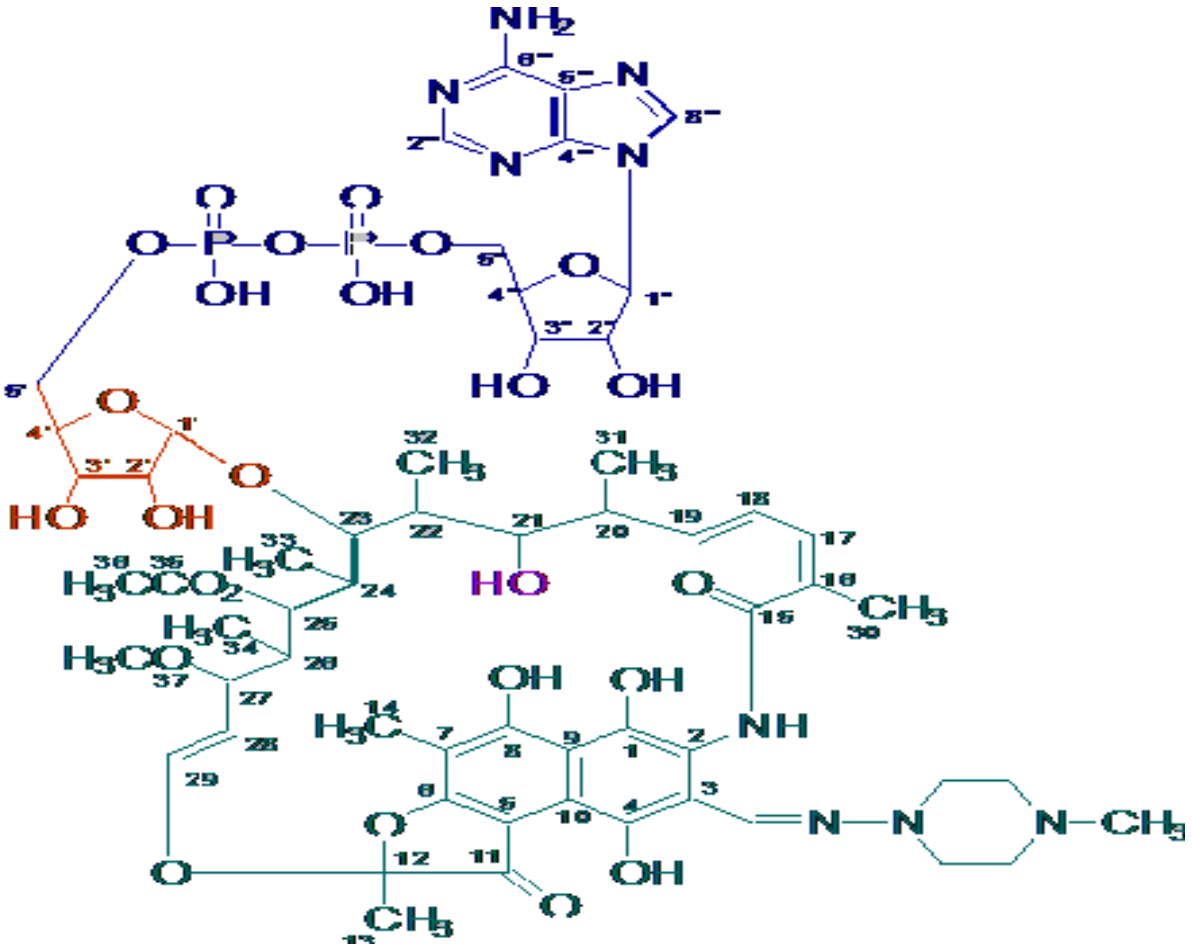


Figure 5: Chemical structure of ADP-ribosylated rifampicin (Adapted from Imai *et al.*, 1999). The adenosine diphosphate (ADP) group is shown in **blue** and the ribose sugar in **red**. Ribosylated rifampicin is achieved through two steps: the first involves the removal of adenosine monophosphate (AMP) and the second is dephosphorylation.

1.4.1.4.3 Glucosylation

Glucosylation is any process by which a glucose sugar moiety is added to another molecule. Glycosyl transferases are the enzymes responsible for catalyzing the transfer of sugar moieties from one compound to another. Glucosylation involves the replacement of the free hydroxyl group at position 23 of the ansa link with D-glucose by a glucosyl-transferase

enzyme (Yazawa *et al.,* 1993) thereby, rendering the drug inactive. Detailed studies of *Nocardia spp.* revealed that many *Nocardia spp.* were inherently resistant to rifampicin and this was reported to be as a result of an inactivation enzyme mechanism that was present in these bacteria (Yazawa *et al.,* 1993; Dabbs *et al.,* 1995). Glucosyl-transferases play a crucial role in a self-defence mechanism by organisms that produce this antibiotic. The chromatographic analysis of the inactive forms of Rif in *N. brasiliensis* revealed two compounds, RIP-1 and RIP-2. These compounds were identified using NMR Spectroscopy to be 3-formyl-23-(O-(β-D-glucopyranosyl)) rifamycin SV and 23-(O-(β-D-glucopyranosyl)) rifamycin respectively, the glucosylation products of Rif. The gene encoding the glucosylation, *rgt*, was cloned and characterized from the opportunistic pathogen *Nocardia brasiliensis*. Yazawa *et al.,* (1993) showed that 39/40 strains of *N. brasiliensis* they tested exhibited a minimum inhibitory concentration of >100F g/ml with Rif. Based on this they concluded that *N. brasiliensis* along with *N. otitidiscaviarum* are naturally resistant to Rif, which could be explained by glucosylation. However, from the family of the rifamycins, it was discovered that streptolydigin and streptovaricin are not inactivated by glucosylation and rifamycin SV is inactivated more slowly.

1.4.1.4.4 Phosphorylation

Phosphorylation results in the substitution of the free hydroxyl group at position 21 of the ansa link with a phosphate. This mechanism is dominant in the genera *Nocardia* and *Rhodococcus* (Yazawa *et al.,* 1994). *Nocardia otitidiscaviarum* as well as *R. rhodochrous* have been found to have the ability to inactivate rifampicin by phosphorylating the 21-OH group (Yazawa *et al,* 1993).

1.4.1.4.5 Decomposition

Decomposition is the most widespread of the four inactivation mechanisms and it has been detected in *Nocardia, Rhodococcus* and *Mycobacterium*, (Tanaka *et al.,* 1996). Decomposition was observed in *N. farcinica* and *N. nova*. In *Rhodococcus*, decomposition was observed in *R. erythropolis* and *R. globerulus*. Decomposition of rifampicin is believed to result from the transfer of a single oxygen atom to the naphthalenyl moiety of the drug

causing it to break apart (Anderson, 1996). The inactivation process results in the antibiotic being decolourized from its red-brown colour. This mechanism was discovered to be species-specific among mycolic acid-containing bacteria. The Rif decomposition gene was cloned from *R. equi* and showed high sequence similarity to the genes encoding the monooxygenases (hydroxylases), which act on phenolic compounds and may be involved in the biosynthesis of polyketides (Andersen *et al.,* 1996). The gene responsible for this Rif resistance was called the *iri* (***i***nactivation of ***ri***fampicin). This mechanism may also be present in other related streptomycetes such as *Arthrobacter oxydans* C1S and *M. smegmatis* DSM 43756. A survey of other organisms found no other groups were able to inactivate rifamycins, with the exception of *Bacillus*.

CHAPTER 2

JUSTIFICATION

Antibiotic resistance is on the rise globally and it is mainly driven by selective pressure imposed by inappropriate antibiotic use. In the case of tuberculosis which is caused by *Mycobacterium tuberculosis*, antibiotic resistance is due mainly to inappropriate usage of the prescribed drugs of treatment as well as to alterations of the prescribed drugs. Tuberculosis remains a global health problem of enormous proportions, as it is attributed to a 7% loss in the GDP of some countries (World Health Organization (2009): Fact sheet on Tuberculosis). This vastly affects the economies of most developing countries as it is most prevalent there. It has been estimated that there are 1 billion (20% of the world's human population) people infected worldwide, with 10 million new cases and over 3 million deaths per year.

Today's TB drug regimen takes too long to administer and requires too many pills. The current treatment courses, consists of four medicines: isoniazid, ethambutol, pyrazinamide and rifampicin. This regimen works for active, drug-susceptible TB, as long as patients complete the six- to nine-month treatment. If the treatment is not completed drug-resistant strains develop that take up to two years or more to treat with second-line drugs, which often cause severe side effects. In addition to this, it has been discovered that there has been an emergence of MDR and XDR strains of *M. tuberculosis* which are untreatable with any existing antibiotics (TB Alliance, 2009).

It has been over 40 years since a new TB drug has been developed, rifampicin which was first discovered in 1963 remains one of the important frontline antibiotics necessary for treating tuberculosis and leprosy. However, cases of the strain becoming resistant to it have been on the increase and it is therefore important that there is a detailed understanding of the mechanism(s) involved in conferring rifampicin resistance in this genus that will assist in the improvement of antimicrobials used in the treatment of infections caused by these organisms.

2.1 Aim of this work

My research followed the work of Susan Andersen (1996) on the inactivation of rifampicin in *Rhodococcus equi*. She identified a monooxygenase-like gene sequence in this bacterium

whose product confers increased resistance to rifampicin. The monooxygenase-like enzyme leads to the decomposition of rifampicin resulting in complete decolourisation of the antibiotic (Dabbs, 1987). The antibiotic is disrupted to a degree such that no information on the nature of the enzymatic attack could be obtained (Yazawa *et al.*, 1993). Therefore, my work was to: clone and characterise the corresponding gene from mycobacterial strain *M. smegmatis* 43756Km1, the parental *M. smegmatis* DSM 43756 believed to inactivate Rif through both ribosylation and decomposition.

2.2 Specific experimental objectives

• To make Rif^R mutants of *M. smegmatis* 43756Km1 (*arr* gene is disrupted) and to confirm whether they inactivate rifampicin by decomposition.
• To construct one or more genomic libraries of *M. smegmatis* 43756Km1.
• To clone the *iri*-like gene of *M. smegmatis* 43756Km1.
• To construct a restriction map and use this to subclone and identify the minimum necessary DNA.
• To sequence the gene.
• To characterize these mutants in terms of altered antibiotic MIC and altered kinetics of inactivation.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial Strains

Bacterial strains used in this work are listed in **Table 5** plasmids in **Table 6**

Table 5: Bacterial strains used in this work.

Strain	Relevant characteristics	Source
Escherichia coli		
MM294-4	*end*A1, *hsd*R17, *gyr*A	E. Dabbs
λMM294-4	λ lysogen of MM294-4	E. Dabbs
Bacillus subtilis		
1A3-1	Spc^R rifampicin assay organism	E. Dabbs
Rhodococcus		
Equi	Type strain	E. Dabbs
erythropolis 4277	Type strain	E. Dabbs
erythropolis SQ1	Highly transformable	S. Quan and E. Dabbs
erythropolis 1069	Type strain	DSM
rhodochrous Ri8	iri^-	S. Quan
rhodochrous 01	Type strain	E. Dabbs
opacus HP-LA1	Type strain	E. Dabbs
Mycobacterium		
smegmatis DSM 43756	Type strain	DSM
smegmatis 43756Km1	*arr* disrupted by a kanamycin cassette	S. Quan

3.2 Media and Growth Conditions

Luria-Bertani (LB) (Appendix A) media was used for growing *E. coli* and *Bacillus* strains and Brain-heart infusion (BHI) media was used to grow all mycobacterial and rhodococcal strains. Liquid cultures were obtained by inoculating single bacterial colonies into 5 ml LB medium and incubating at 37°C overnight. For short-term storage, *E. coli* strains were kept on LB-agar plates (Appendix A) at 4°C. *Bacillus* strains were kept on LB-agar plates at room temperature until they sporulated for long-term storage.

Table 6: Plasmids and phages used in this work.

Plasmid/ Phages	Relevant characteristics	Source
pDA71	*E. coli-Rhodococcus* shuttle vector	S. Quan and E. Dabbs
pDA71*	*E. coli-Rhodococcus* shuttle vector with *Eco* RI suicide gene inactivated	E. Dabbs
pUC 18/19	sequencing vector with multiple cloning sites	Fermentas
pGEM-T-Easy	*E. coli* high copy number vector, *lacZ′* gene, single 3′ thymidine overhangs, SP6 and T7 promoters	Promega
pNV18	*E. coli* and *M. smegmatis* shuttle vector	Promega
pNV19	*E. coli* and *M. smegmatis* shuttle vector	Promega
K04	*R. erythropolis* SQ1 phage	Y. Shibayama
W12	*R. erythropolis* SQ1 phage	Y. Shibayama

3.2.1 Generation of spontaneous mutants

Bacteria were grown to stationary phase and 100 µl of this bacterial culture was spread on LA plates containing a high concentration of the appropriate antibiotic. These were incubated

for 3 or more days, the resulting colonies were streaked onto the appropriate antibiotic containing plates.

3.3 Spot testing and the determination of minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) of antibiotics was determined on LB-agar plates for *E. coli* strains by the agar dilution method. Freshly grown colonies were inoculated into 200 µl of sdH_2O per well. This was replica plated onto LB-agar plates containing the dilutions of antibiotics starting from the highest concentration. Prior to use, the replicator was sterilized by flaming. *E. coli* spot tests were analysed after incubation at 37°C for 24 hrs. The antibiotic concentration at which there was no confluent growth was taken as the MIC. These tests were done in triplicates, the average result was used in drawing graphs and the standard deviation of the three results was used to incorporate error bars within these graphs.

3.4 Inactivation assays

The ability of different strains and plasmid containing mutants to inactivate Rif and related antibiotics was assayed using the plate assay method.

A pre-culture of each test organism was grown in 5 ml of BHI and grown at 37°C on the wheel overnight. An aliquot of 50 µl of each strain was inoculated into 5 ml of fresh BHI and grown for 3 hours at 37°C. After which, 20 µg/ml of Rif was added and left to grow overnight at 37°C. A control was made by adding 20 µg/ml of Rif to 5 ml of BHI and this was left on the wheel overnight as well. No cells were added to the control. The assay organism, *B. subtilis 1-A3* was also grown at 37°C in 5 ml of LB broth. The time when the antibiotic was added was taken as zero and assays carried out at various times after that (24, 48, 72 hrs). 100 µl of *B. subtilis* was diluted in sterile distilled water (1:10) and then spread on ½ LA streptomycin (200 µg/ml) plates. ½ LA plates contain half of the normal agar usually added to make LA plates. The lower concentration of agar allows for easier diffusion of Rif.

Wells where made in agar using the blunt end of a Pasteur pipette. The wells were then filled with 70 µl of the cultures being tested, care being taken not to spill any on the sides. The plates where then incubated at 4°C for 4 hrs, to allow the rifampicin to diffuse in the media. Subsequently, they were incubated at 37°C until zones of inhibition became visible.

3.5 Phage plaquing test

2 ml of LB was added into a test tube, to this 3 ml of sloppy agar (½ LA) and 20 µl of Hs7-1 was introduced, this was mixed by rolling gently between the hands. This mixture was then poured onto $CaCl_2$ and $MgCl_2$ plates and allowed to set in a 30ºC incubator for 15 min. The Ca^{2+} and M^{2+} ions are responsible for stabilising the phages such that the phage is effectively adsorbed onto the host cell (Hs7-1) surface. K04 and W12 phages were then spotted onto these plates, allowed to dry and incubated at 28ºC overnight.

3.6 DNA preparations

3.6.1 *E. coli* bulk plasmid DNA preparation

The plasmid being extracted was pDA71 which is a cloning vector. It is 8.8kb and has selectable markers (Amp^R in *E. coli* and Cam^R in *Rhodococcus*) and it has a multiple cloning site in *Eco*R, an endonuclease gene, which make it a suitable cloning vector. Plasmid pDA71 allows selection of transformants with inserts because of this "suicide" gene.

A culture of *E. coli* carrying pDA71 was grown in 100 ml of LB supplemented with 100 µg/ml of Amp. The culture was grown with gentle agitation at 37°C overnight. Cells were harvested by centrifugation in a Beckman JA-10 rotor at 6 000 rpm for 10 min and then re-suspended in 5 ml of solution I. 10 ml of solution II was then added to the cell suspension, mixed gently by inversion and left to stand at room temperature for 30 min. This allows for lysis of cells. Afterwards, 7.5 ml of chilled solution III was added and the tube was shaken vigorously and then left in ice-water slurry for 15 min. This was centrifuged in a pre-chilled JA-20 rotor at 15 000 rpm for 10 min.

The supernatant was decanted into a clean Beckman centrifuge tube and warmed at 37°C for 2 min. 12 ml of isopropanol was added and left to stand at room temperature for 5 min on the bench. The DNA was then precipitated by centrifuging in a warm JA-20 rotor at 15 000 rpm for 10 min. The resultant DNA pellet was washed with 2 ml of ethanol (96%) and re-centrifuged for 3-5 min at 15 000 rpm at room temperature, excess liquid was blotted on a paper towel. The DNA pellet was vacuum-dried for 20 min and then re-suspended in 4 ml TE buffer for 2 hrs at 30°C in a shaking water bath. TE buffer contains EDTA which chelates Mg^{2+} ions in the DNA solution hence protecting DNA from degradation by nucleases.

4.3 g of CsCl was added and dissolved by gently shaking. Thereafter, 400 µl of a 1% EtBr solution was added and the refractive index adjusted to between 1.387 and 1.389 (0.001 units = 100 mg CsCl if the index was below or 0.001 units = 100 µl TE if the index was over). The mixture was loaded into a Beckman Quick-seal tube using a Pasteur pipette. The tube was sealed, balanced and ultra-centrifuged overnight at 40 000 rpm in a Beckman vertical VTi 65.2 rotor for 22 hrs.

The tube with the DNA was then viewed under UV and the lower layer which is the plasmid DNA was extracted. EtBr was removed from the DNA by extracting three times with 200µl of butanol. Before the DNA can be used a salt-ethanol precipitation was performed.

3.6.2 Mycobacterial bulk genomic DNA preparation

The complex structure and low permeability of the cell wall of mycobacterial cells make them difficult to lyse and extract DNA. The cell walls are thick and contain large amounts of lipids, among which are mycolic acids which are resistant to simple lysis processes with strong alkali and acids. Therefore, commonly employed methods of isolating DNA yield either low quantity (due to incomplete lysis of bacterial cell wall) or poor quality DNA resulting in low sensitivity of the test. Successful extraction of mycobacterial DNA had to include the following steps as a result:

1) Harsh physical treatment to weaken the mycobacterial cell wall (i.e. freezing at 20°C or boiling bacterial suspension for 10 min).
2) Chemical treatment to lyse the mycobacterial cell wall.

3) DNA purification steps including treatment with proteinase K to remove proteins.

A culture of *Mycobacterium smegmatis* 43756Km1 was grown to stationary phase for about 2 days in 200 ml of BHI (0.5% glycine and 0.5 % Tween 80) on a shaker at 37°C. Mycobacterial cells tend to clump when growing in liquid media therefore, to reduce this clumping add 0.05% Tween 80 and/or (3-5) glass beads were added to the BHI, and the glycine weakens the cell wall. The BHI was supplemented with 200 µg/ml of Kan.

Cells were precipitated by centrifugation in a Beckman JA-10 rotor at 6 000 rpm for 10 min at room temperature. The cells were resuspended in 5 ml of TE and boiled for 10 min at 100°C. This was necessary for weakening the linkages between the lipid contents of mycobacterial cell wall, resulting in release of chromosomal DNA in solution. This was allowed to cool for 2-5 min and 5 mg/ml lysozyme was added and transferred to a fresh Sorval tube, which was incubated for 45 min at 37°C on a shaker. The cells were then centrifuged at 8 000 rpm for 5 min in a Beckman JA-20 rotor and resuspended in 4 ml of TE. 400 µl of solution B (TE + 15% SDS) was added to the cells to which a minute quantity of proteinase K was added. The proteinase K is responsible for inactivating nucleases which might otherwise degrade the DNA during purification. The tube was then incubated at 45°C for 30 min.

The viscous solution was transferred to a 50Ti tube and spun at 40 000 rpm for 30 min in a Beckman L55 ultracentrifuge. The supernatant was transferred to a clean centrifuge tube, 4.4g of CsCl was added, and the tube was inverted several times to mix. The solution was centrifuged at 15 000 rpm for 15 min and the liquid was decanted. 400 µl of (1%) EtBr solution was added and the refractive index adjusted to between 1.391 and 1.392.

The solution was loaded into a Beckman Quick seal tube and centrifuged for 22 hrs at 40 000 rpm in a VTi65.2 rotor in a Beckman L7-55 ultracentrifuge. The genomic DNA was extracted from the tube using a needle attached to a hypodermic syringe, the EtBr was removed by butanol extraction and the genomic DNA was precipitated with ethanol.

3.6.3 *E. coli* mini plasmid preparation

Individual bacterial colonies were inoculated into 1 ml of LB containing 100 µg/ml of Amp. This was incubated at 37°C on a shaker for at least 5 hrs or overnight. The culture was transferred to a sterile Eppendorf tube and the cells were harvested by microfuging for 1 min at room temperature. The supernatant was decanted off and the cell pellet re-suspended in 80 µl of solution I by vortexing. Then, 160 µl of solution II was added, mixed gently by inversion and left to stand on the bench for 15 min. Thereafter, 120 µl of solution III was added. The mixture was shaken vigorously and placed on ice for 5 min. Cellular debris was removed by microfuging at 4°C for 10 min. The supernatant was transferred to a clean sterile Eppendorf tube and warmed in a 42°C water bath for 2 min.

220 µl isopropanol was added and left to precipitate 5 min at room temperature. Isopropanol selectively precipitates DNA leaving RNA and polysaccharides in solution. This was followed by centrifugation for a further 5 min. The pellet was washed with 150 µl of 96% ethanol and dried in a speed vacuum for 20 min. The DNA was then re-suspended in 150 µl of sdH_2O with 10 mg/ml ribonuclease which digests any remaining RNA contamination. 13.5 µl was then analysed on an agarose gel.

3.6.4 *Rhodococcus* plasmid DNA mini-preparation

Cultures were inoculated in 300 µl of LBSG (LB + 3% glycine and 10% sucrose) supplemented with 40 µg/ml Cam and grown for 2-3 days at room temperature on the wheel. 200 µl of the cells were transferred to a sterile Eppendorf tube and the cells where harvested by microfuging for a minute. The pellet was resuspended in 200 µl of TE containing 5 mg/ml lysozyme. The suspension was then incubated in a 37°C water bath for an hour. After incubation 40 µl of TE-SDS (10%) was added to the suspension, mixed by inverting and left at room temperature for 10 min. 40 µl of 5.0M potassium acetate (pH 6.0) was added and the mixture was shaken vigorously. This was followed by incubation on ice for 5 min and microfuging at 4°C for a further 5 min. The supernatant was then transferred to a tube and further purified with a phenol-chloroform extraction. The DNA was ethanol precipitated and dried under vacuum for 20 min. The pellet was re-suspended in 50 µl of sdH_2O water.

3.7 DNA manipulations

3.7.1 DNA precipitation

3.7.1.1 Salt and ethanol precipitation

DNA was precipitated using a 0.1 volume of 1M NaCl and 2 volumes of 96% ethanol. The mixture was microfuged at 4°C for 20 min. The supernatant was decanted and the remaining liquid removed by blotting on a paper towel. The DNA pellet was vacuum-dried for 20 min and re-suspended in 100 µl of sdH_2O. Contamination with RNA was removed by incubating the plasmid DNA solution containing freshly boiled RNAse (10 mg/ml) for 15 min at 65°C.

3.7.1.2 Ethanol precipitation

CsCl is removed from a DNA solution by ethanol precipitation. The salt was removed by adding 200 µl of sdH_2O and 700 µl of 96% ethanol to 100 µl of DNA; this was left on ice for 10 min and precipitated by centrifugation for 20 min at 4°C. The DNA pellet was dried under vacuum for 20 min and re-suspended in 100 µl of sdH_2O.

3.7.2 DNA preparation from CsCl density gradients

Ethidium bromide (EtBr) was removed from the DNA by thorough mixing with a 0.1 volume of butanol. The procedure had to be repeated at least 3 times before all traces of EtBr were removed. This left the DNA in a CsCl solution. The DNA was stored at -20°C until required. It then undergoes ethanol precipitation before being used.

3.7.3 Phenol-chloroform extraction

DNA was extracted from aqueous solution by phenol and chloroform. Phenol removes restriction enzymes and other proteins including agarose. Chloroform facilitates the separation of aqueous and organic phases as well as removing phenol. Both phenol and

chloroform denature the proteins which can be removed easily as proteins partition into the organic phase, leaving the DNA behind in the aqueous phase.

50 µl of TE-saturated phenol (Appendix B) was added to 100 µl of DNA, mixed by inversion and microfuged at room temperature for 5 min to separate the organic and aqueous phases. The upper aqueous layer was then transferred to a sterile Eppendorf tube and where necessary, as in the case of extracting DNA from low gelling agarose, a further phenol step was performed until there was no visible protein at the interface. Then, a 100-120 µl of chloroform was added to the aqueous layer and mixed thoroughly by inversion. The organic and aqueous layers were separated by microfuging for 3-5 min at room temperature. The upper aqueous layer was removed to a fresh Eppendorf tube and the DNA precipitated with a 0.1 volume of NaCl as outlined above in section 3.6.1.1.

3.7.4 Restriction enzyme digestion

Enzymes were obtained from Boehringer Mannheim or Fermentas and used according to the manufacturer's instructions. The total volume of a digestion reaction was 15 µl (13.5 µl DNA and 1.5 µl 10x buffer). The mixture was tapped briefly to ensure even buffer distribution and spun down for a few seconds. 0.5 µl of restriction enzyme was added and the contents mixed and briefly re-spun. Digestions were incubated at the appropriate temperature for maximal enzyme activity for at least 4 hrs. For double digestions an appropriate buffer in which both enzymes showed suitable activity was selected, otherwise the digestions were performed sequentially starting with the enzyme that required the lower pH buffer. Where the procedure had been for determining fragment sizes or existence of enzyme sites these digestions were loaded onto agarose gels. Where the process had been carried out for subsequent ligation the enzyme was removed by the phenol-chloroform method.

3.7.5 Ligation of DNA

DNA ligases catalyze the formation of phosphodiester bonds by joining 3′-hydroxyl and 5′-phosphate ends of double stranded DNA. T4 DNA ligase (Fermentas) was used because it ligates not only sticky ends, but also blunt ends of DNA. The total volume for ligation was

kept minimal at 20 µl. Ligation buffer and the appropriate volume of sdH_2O were added to the DNA sample, mixed by tapping and microfuged for 5 sec. Subsequently, 1 µl of ligase was added, this was further remixed and re-spun. Ligation was performed in a water bath at 22°C for roughly 12 hrs. This DNA was then used in transformations.

3.7.6 Agarose gel electrophoresis

Agarose was prepared in 0.5x TBE at concentrations of 0.4; 0.8; and 1.2%. Gels were prepared by adding 2.5 µl of 1% EtBr to 25 ml of agarose. These were run in 0.5 TBE at 100V at room temperature. 0.4% gels were used for genomic DNA and the 0.8% gels for small DNA i.e. vector inserts, restriction fragments and plasmid DNA.

3.7.6.1 Freeze/ squeeze DNA extraction

This method was used to separate and extract DNA from the agarose gel after electrophoresis. Digested DNA was run on agarose gel and viewed under a UV illuminator and the DNA inserts excised using a scalpel into Eppendorf tubes. The samples were then incubated at -70ºC for 30 min to 2 hrs and then thawed at room temperature. Once thawed the resultant supernatant was collected into a fresh Eppendorf. The remaining agarose was crushed and microfuged and supernatant collected into the above Eppendorf. The thawing, crushing and microfuging was repeated once more and the resultant supernatant was collected and phenol-chloroformed to purify the DNA.

3.7.6.2 Low gelling agarose electrophoresis

Low molecular weight agarose gel electrophoresis was used to isolate DNA fragments from a 0.8% gel. Running buffer was allowed to chill at 4ºC for about 2 hrs before use. A gel was made with 8 wells and 35 µl of the *Pst* I digested DNA was added to each well. Electrophoresis was conducted at 95V and 23 mA for 2½-4 hrs at 4ºC or until the second band reaches the end of the gel. The gel was viewed under a UV illuminator for the shortest time possible and inserts were excised using a scalpel into Eppendorf tubes. This was melted

at 62ºC for 20-30 min. The DNA-melted agarose underwent 3 phenol extractions followed by a single chloroform extraction before the DNA was salt and ethanol precipitated to purify it. The DNA was resuspended in the lowest quantity (20-30 µl) of sdH_2O.

3.8 DNA calibration

This exercise is necessary to determine the optimal amounts of plasmid and genomic DNA that produce ligations that yield the maximum number of transformants during construction of a genomic library.

3.8.1 Vector calibration

Different (2-8 µl) volumes of the digested plasmid were religated back using T4 DNA ligase. These were then transformed into *E. coli* MM294-4 using the $CaCl_2$ method of transformation, spread on LA plates supplemented with 100 µg/ml of Amp and the plates were incubated overnight at 37ºC. A no DNA (0 µl) control was used as the negative control and the positive control was pDA71*. The volume that produces the least transformants, 0-5 are an acceptable range, was then used to make the genomic library. This procedure is done to ensure that during the genomic library construction the plasmid DNA being transformed carries the clone to be transformed considering that if pDA71 re-ligates back onto itself the suicide *Eco-R* gene is functional therefore cells with this DNA will not grow on the Amp plates.

3.8.2 Genomic DNA calibration

The volume of genomic DNA to be used with the volume of plasmid determined in 3.8.1 was also determined by first treating the genomic DNA with a fixed volume of plasmid DNA and different volumes of genomic DNA and setting up the ligations. The ligations were then transformed into *E. coli* MM294-4. A negative control (no DNA) and a positive control (pDA71) were set up as well. The volume that gave the highest transformants per plate was then chosen to be used in the construction of the genomic library.

3.8.3 Transformations and calculation of the number of transformants needed to complete the genomic library

$$\text{Number of clones} = \frac{\ln(1-P)}{\ln(1-a/b)}$$

where N = number of clones

P = probability of having the clone in the library

a = average insert size

b = genomic DNA size

3.9 Transformations

3.9.1 *Escherichia coli* $CaCl_2$ mediated transformations

A single colony of *E. coli* MM294-4 was inoculated into 5 ml of LB with 10-15 µl of 10 mg\ml Nal grown overnight on the wheel at 37°C. 200 µl of the pre-culture and 0.5% glucose was added to 20 ml of LB, and incubated for 1 hour and 45 minutes at 37°C. This was grown with vigorous aeration. The transformation buffer and the centrifuge tubes to be used were placed in an ice box at this stage and all subsequent procedures required all equipment and reagents to be ice cold when used. The centrifuge and rotor were cooled 15 min before use and kept cool for the next steps as well. After the incubation period cells were placed in an ice-water slurry for 5 min and centrifuged at 10 000 rpm for 5 min. The supernatant was decanted and discarded after centrifuging. The cell pellet was re-suspended in 10 ml transformation buffer and left in ice for 15 min or more.

This was centrifuged for 5 min at 10 000 rpm after the incubation period. The supernatant was discarded and re-suspended gently in one fifth volume transformation buffer and left on ice for 2-24 hrs. The DNA (ligations) to be transformed were placed on ice at least 10 min before being added to 100 µl of the competent cells. These were aerated gently and briefly by passing air through them. The cells were then left on ice for 10 min to allow diffusion and where then heat shocked at 44°C for 90 seconds.

0.5 ml of warm LB was added and incubated for an hour at 37°C with the Eppendorf caps open. This allows for phenotypic expression of antibiotic resistance. The cells were then spread on Amp 100 µg/ml plates and dried at 37°C for 24 hours with the plate lids upward.

3.9.2 *Rhodococcus* PEG-mediated transformations

The recipient strain *R. rhodochrous* Hs7 was inoculated into 5 ml of 3% glycine LBSG and grown for 2-3 days at 30°C on the wheel. After the culture reaches mid-log growth phase, cells were harvested from 1.2 ml of culture by microfuging for a minute, washed with basal buffer (B buffer) and microfuged for 30 seconds. The B buffer was decanted and the cells were resuspended in 1 ml B buffer containing 5 mg/ml lysozyme, freshly added. The tube was incubated for an hour in a 37°C water bath with inversion every 10 min.

Roughly 40 min into the above incubation period the PEG was sterilized under UV light for 10 min. During this time the protoplast buffer (P buffer) was made (Appendix B). This was used to make the P-PEG buffer, which was prepared by adding 1 ml of the P buffer to 0.5 g of PEG. After incubation the resultant protoplast suspension was microfuged for 15 seconds, washed with 1 ml B buffer and resuspended in 500 µl P buffer. Aliquots (100 µl) of the protoplast suspension were transferred to Eppendorf tubes and plasmid DNA, (10 µl) was added to each tube. The tube contents were mixed by gently bubbling air through the solution. The tubes were incubated for 10 min at room temperature and an equal volume of P-PEG was added to each tube while bubbling air through the contents so as to mix the two phases.

The content of the tubes were spotted on chilled regeneration media and spread using a 1 ml pipette. These were incubated for 10 hrs at 28°C, to allow phenotypic expression of the antibiotic resistance gene. 500 µl of 2 mg/ml Cam stock solution was used as an underlay. The plates were incubated for 3-5 days at 28°C.

4.0 Sequencing

The vector used for sequencing was pUC18. 3 µl of the vector was resuspended in 100 µl of sdH_2O, digested with *Pst* I and incubated at 37ºC overnight. So as to prevent the vector from religating back onto itself phosphatase was added to the reaction mixture. Addition of the dephosphorylation buffer was not necessary since phosphatase functions equally well in the digestion buffer. The reaction mixture was incubated further for 5 hrs or more at 37ºC. After incubation the enzyme was removed by phenol-chloroform and the DNA extracted was resuspended in triple the original volume of sdH_2O.

The DNA insert (from the clone) isolated by low gelling agarose electrophoresis was then ligated into pUC18. Ligations of the insert and plasmid were set up using 2 µl of digested pUC18, 15 µl of insert, 2 µl ligation buffer and 1µl of T4 DNA ligase and incubated at 22ºC for 2 hrs. This was transformed into *E. coli* MM294-4 using the $CaCl_2$ method. Control ligations included vector only (positive), no vector (negative) and insert only (negative). Single colonies from the sample plates were grown in 1ml of LA supplemented with 100 µg/ml of Amp, overnight and then miniprepped using the Fermentas plasmid kit. The miniprepped DNA was then further analysed by digesting with *Pst* I and run on a 0.8% agarose gel to confirm the presence of the insert. The DNA was sent off for sequencing at Inqaba Biotec using the M13 forward and reverse primers.

4. 1 Sequence analysis

This was done using the National Centre of Biotechnology Information online database (http://www.ncbi.nlm.nih.gov/) and the FASTA, BLASTN, BLASTX2 tools found on that website and NEBCUTTER tools (http://tools.neb.com/NEBcutter2/index.php).

5.0 Construction of a restriction map

DNA to be mapped was digested using various enzymes. Enzymes that cut the DNA once or twice were used to construct the map. Single digests were compared with double digests of

the DNA. All digestions were run on 0.4% agarose for larger fragments (>2kbp) and 0.8% gels for the smaller fragments (<2kbp).

CHAPTER 4

RESULTS

4.1 Selection of spontaneous mutants of *Mycobacterium smegmatis* DSM 43756 and *Mycobacterium smegmatis* 43756Km1

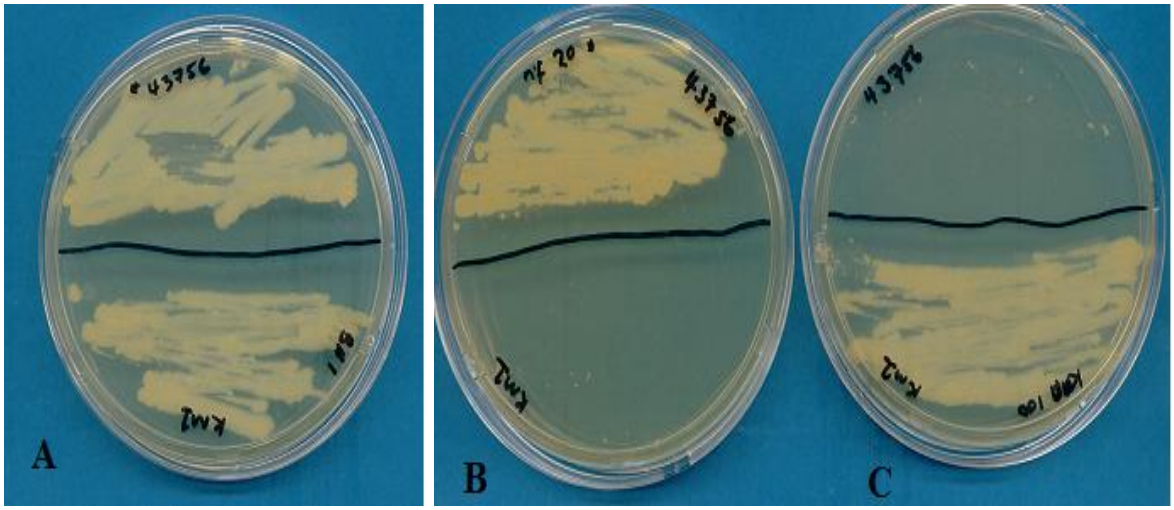


Figure 6: Growth of *Mycobacterium smegmatis* 43756Km1 and *Mycobacterium smegmatis* DSM 43756 on: **A**- BHI agar, **B**- BHI agar (20 µg/ml rifampicin) and **C**-BHI agar (100 µg/ml kanamycin).

The first step in this project was to generate spontaneous mutants of *M. smegmatis* 43756Km1 and *M. smegmatis* DSM 43756 which were resistant to high levels of rifampicin. This is because inactivation is more readily observable at high level rifampicin concentrations. Inactivation through ribosylation is not possible in *Mycobacterium smegmatis* 43756Km1, which is a mutant of *Mycobacterium smegmatis* DSM 43756 made by disrupting the *arr* gene with a Kan^R cassette (Quan *et al.* 1997). Therefore, *Mycobacterium smegmatis* 43756Km1 is sensitive to Rif but resistant to Kan **Figure 6B** and **6C** respectively. Once the spontaneous mutants of *M. smegmatis* 43756Km1 and *M. smegmatis* DSM 43756 were generated Rif inactivation assays were carried out.

4.2 *In-vitro* assays

The ability of the two strains to inactivate rifampicin was investigated through *in-vitro* assays. The *M. smegmatis* 43756Km1 and *M. smegmatis* DSM 43756 mutants were grown in BHI overnight at 37ºC. 50 µl of each culture was added to 5 ml of BHI and grown for 3 hrs after which Rif was added to final concentration 20 µg/ml. Aliquots were taken at 24 hrs, 48 hrs and 72 hrs and added to wells made on ½ LA plates, stored at 4ºC for 4 hrs and these were incubated overnight at 37ºC. The assay organism used was *Bacillus subtilis* 1A3. Two sets of assay tests were set up, to one 0.05 % Tween 80 was added to the cultures and to another 4 beads were added to cultures, this being done to minimise clumping of mycobacterial cells. Tween 80 is known to slow down growth, the test using beads was done as a control so as to check whether Tween 80 significantly affected the way in which rifampicin was inactivated by the microorganisms. The mutant strain, *M. smegmatis* 43756Km1, is expected to inactivate rifampicin faster than the parental strain because there is a higher probability that the inhibitory effects of rifampicin are absent in the mutant.

Samples of the supernatants were assayed at 24, 48 and 72 hrs to check for Rif inactivation. Two negative controls were set up, BHI with 20 µg/ml of Rif (no cells) and BHI with 20 µg/ml Rif inoculated with *Rhodococcus rhodochrous* Ri8.

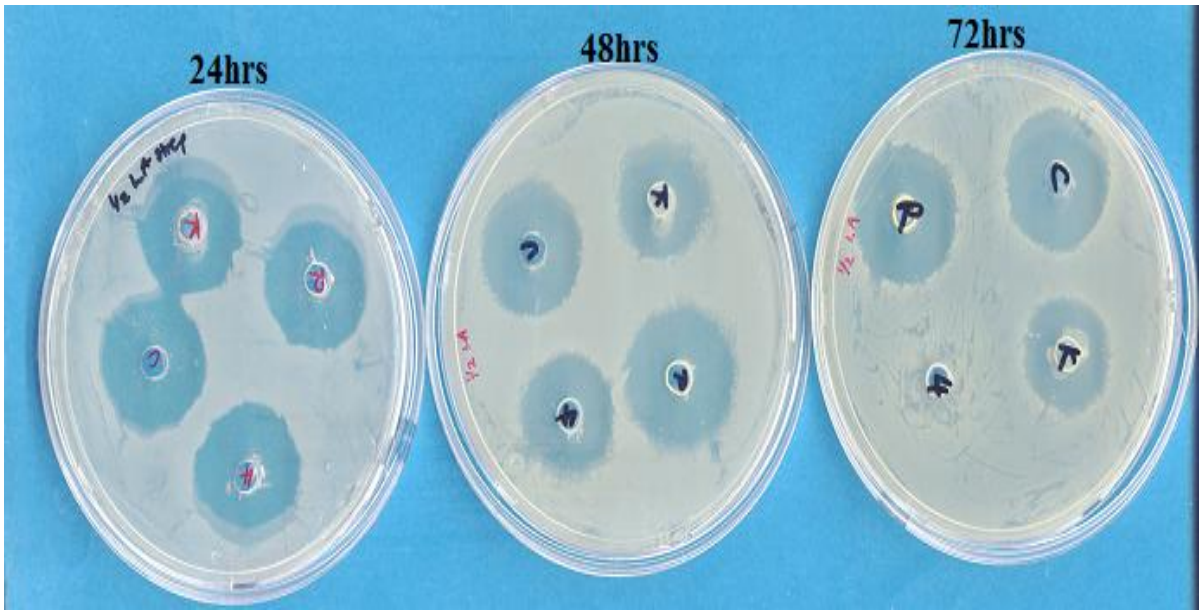


Figure 7: *In-vitro* assays; test organisms grown in the presence of Tween 80 (K- *M. smegmatis* 43756Km1, C- control, R- *R. rhodochrous* Ri8 and 4- *M. smegmatis* DSM 43756).

At 24 hrs both tests (**Figure 7** and 8) showed similar sized zones of inhibition, at 48 hrs in the bead test the zone of inhibition was almost completely gone around *M. smegmatis* 43756Km1 whilst in presence of Tween 80 test it was present but had reduced in size compared with that at 24 hrs. At 72 hrs in the bead test (**Figure 8**) the zones of inhibition were completely absent around both the *M. smegmatis* 43756Km1 and *M. smegmatis* DSM 43756 wells whilst in the Tween 80 medium only the *M. smegmatis* DSM 43756 was completely gone and the *M. smegmatis* 43756Km1 was reduced in size. Therefore complete inactivation of Rif occurred for both the *M. smegmatis* 43756Km1 and the *M. smegmatis* DSM 43756 strains at 72 hrs in the bead test. However inactivation was retarded by Tween 80 with complete inactivation for *M. smegmatis* DSM 43756 at 72 hrs and of *M. smegmatis* 43756Km1 at 80 hrs. *R. rhodochrous* Ri8 does not inactivate Rif at all so a zone of inhibition was expected, as well as around the cell-free negative control.

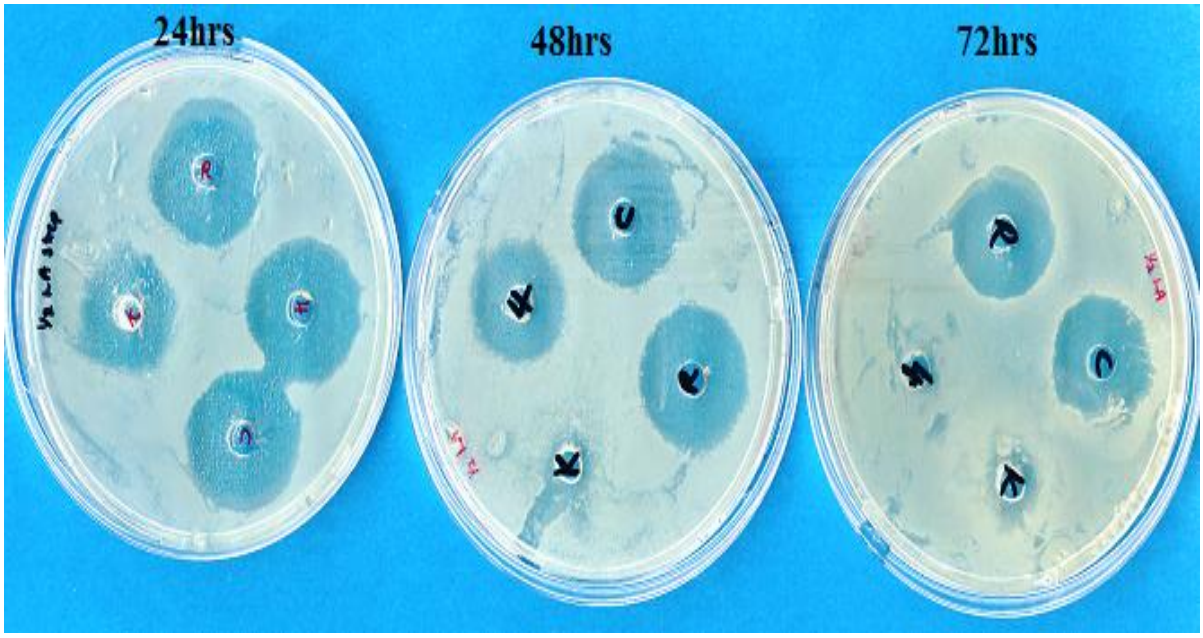


Figure 5: *In-vitro* assays; test organisms grown in the presence of beads (K- *M. smegmatis* 43756Km1, C- control, R- *R. rhodochrous* Ri8 and 4- *M. smegmatis* DSM 43756).

4.3 Genomic library construction in *E. coli*

A genomic library is a collection of cloned fragments of genomic DNA inserted into a cloning vector. These clones are stored and propagated in a population of micro-organisms

(host) through the process of molecular cloning. The purpose of creating a genomic library is to investigate entire structures of genes and to study regulation of gene expression. This is also essential for genetic transformation. In this project a genomic library was generated so as to study gene expression as regards to rifampicin resistance.

4.3.1 Determination of restriction enzyme for the genomic library

Twelve restriction enzymes were tested as candidates for the genomic library construction of *M. smegmatis* 43756Km1 as shown in **Figure 9**. The enzyme to be used was determined by its frequency of cutting genomic DNA as well as possessing a restriction site in the plasmid to be used which was pDA71-1. *Xba* I was determined to be the most suitable for construction of the genomic library. However, after ligating the digested *M. smegmatis* 43756Km1 genomic DNA to the *Xba* I digested pDA71-1 plasmid it was noted that the transformation and ligation efficiency was very low, as noted by the growth of very few transformants. Consequently, the cloning vector was changed to pDA71 and the restriction enzyme used was *Pst* I. Since *Pst* I was a frequent cutter of this genomic DNA a partial *Pst* I library was constructed.

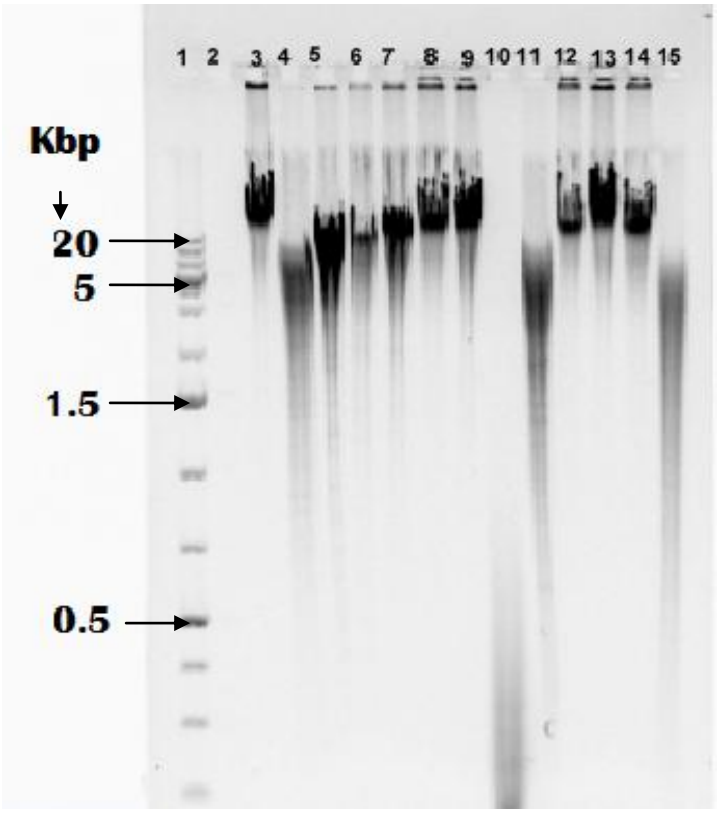


Figure 6: Determination of the restriction enzyme to be used for library construction; electrophoresed on a 0.4% agarose gel. *M. smegmatis* 43756Km1 after digestion with *X MA* JI (**Lane 4**), *Bam* HI (**5**), *Bgl* II (**6**), *Hind* III (**7**), *Sau* 3A (**8**), *Nhe* I (**9**), *Nsi* I (**10**), *Pst* I (**11**), *Sfu* I (**12**), *Xba* I (**13**), *Xho* II (**14**), *Xmi* I (**15**). **Lane 1**– molecular weight marker, **Lane 3**– undigested DNA.

The partial library was generated by diluting the *Pst* I enzyme ×4, ×16, ×64, ×128, ×256 and adding 0.5 µl of each dilution to 10 µl of genomic DNA and incubating at 37ºC overnight. From **Figure 10**, a dilution of between ×64 and ×128 was used for the library construction. At this dilution the genomic DNA will not be cut too frequently producing small fragments or cut infrequently producing larger fragments which were too large.

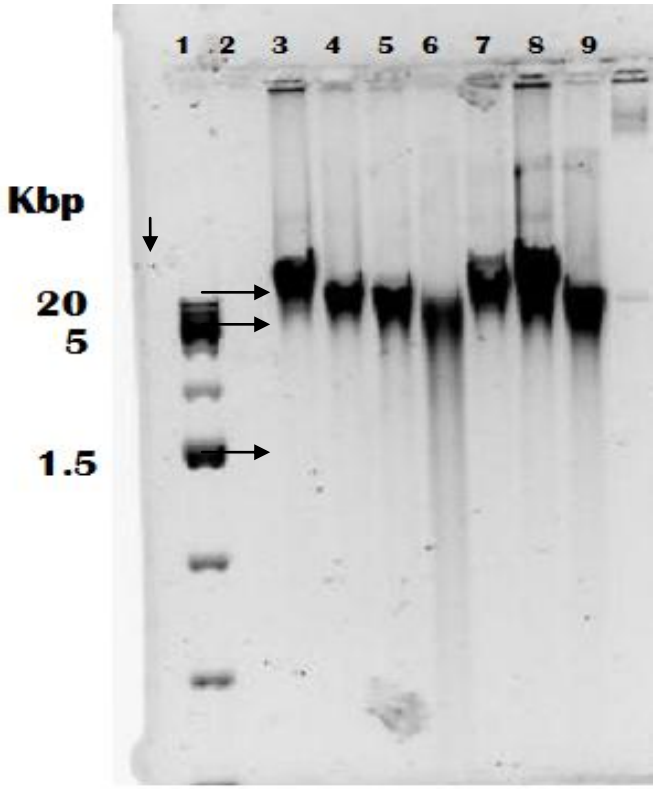


Figure 7: Determination of the dilution for a partial *Pst* I digestion of genomic DNA from *M. smegmatis* 43756Km1; electrophoresed on a 0.4% agarose gel. **Lane 1-** molecular weight marker; **Lanes 3-9**, *Pst* I dilution factor: ×4, ×16, ×64, undiluted Pst I, ×128, ×256 AND X96 respectively.

4.3.2 Calculation of number of clones

Screening minipreps of clones digested with *Pst* I the average insert size was found to be a ~2.9 kbp. The frequency of detectible inserts was determined to be ~60%, (**Figure 11).** According to the National Centre for Biotechnology Information (NCBI) the complete genome of *M. smegmatis* is 6 988 209 nucleotides. This number was used to calculate the number of clones required using the Clarke-Carbon formula.

Approximately 13 600 transformants with the recombinant vector DNA (pDA71 + genomic DNA) were washed off the plates using LB supplemented with 100 µg/ml of Amp and were pooled together to create a genomic library with a ≥95% probability of carrying a particular

DNA. Aliquots were stored at -20ºC and 200 µl was inoculated into 200 ml of LB in order to do a bulk extraction of the library.

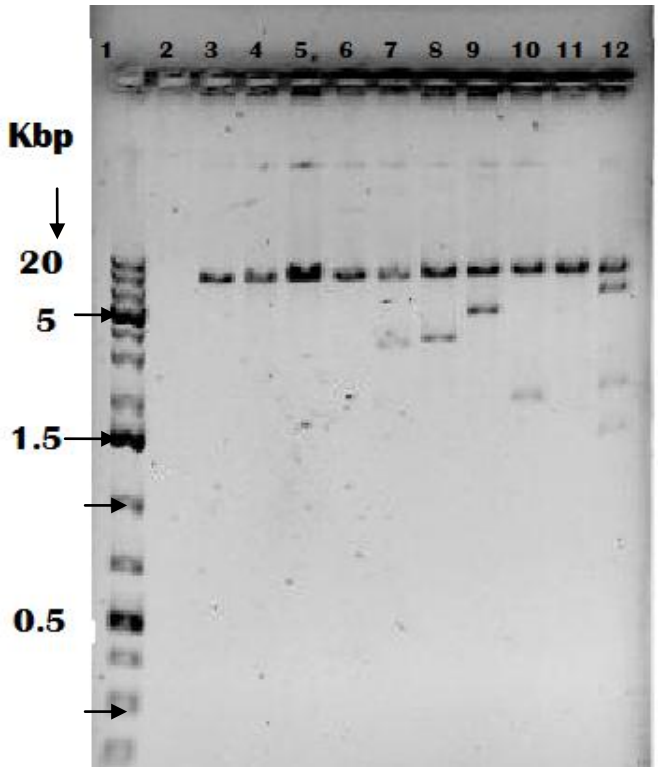


Figure 8: Plasmid screen of recombinant pDA71 vectors digested with *Pst* I; electrophoresed on a 0.8% agarose gel. **Lane 1:** molecular weight marker, **Lanes 3-12:** digested recombinant vectors.

4.4 Susceptibility of *Rhodococcus* strains to different antibiotics

A possible host strain for the expression of the genomic library was investigated from various *Rhodococcus* strains. The ideal strain to be used as a host or recipient of the library DNA would have to be sensitive to Rif (≥100 ng/ml) and sensitive to 40 µg/ml Cam. A *Rhodococcus* strain unable to inactivate rifampicin was used as the recipient strain since the objective of the experiment was isolating a clone which inactivated Rif based on it conferring Rif resistance. Once the recombinant pDA71 clone is transformed into this *Rhodococcus* strain it should be able to inactivate Rif if it contains the gene responsible for inactivation and vice versa. This recipient strain must be inhibited by 40 µg/ml of Cam as this is the

concentration at which pDA71 confers resistance in *Rhodococcus* strains. A number of *Rhodococcus* strains were tested for Rif and Cam sensitivity **Figure 12**.

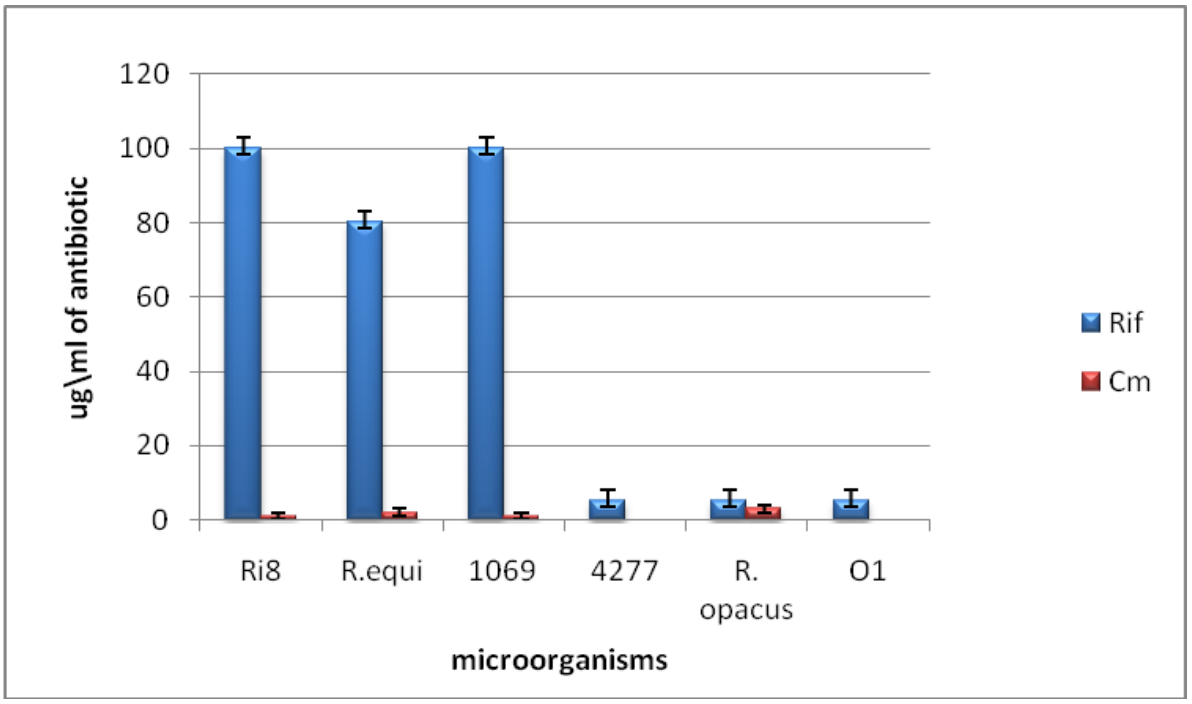


Figure 9: MICs of potential host strains of *Rhodococcus*

Unfortunately all six were insufficiently sensitive to Rif. However Susan Andersen in her work had made some highly sensitive (Hs) strains of *R. rhodochrous* to Rif; these were tested for their respective MICs to Rif and Cam, **Figure 13** and **14**.

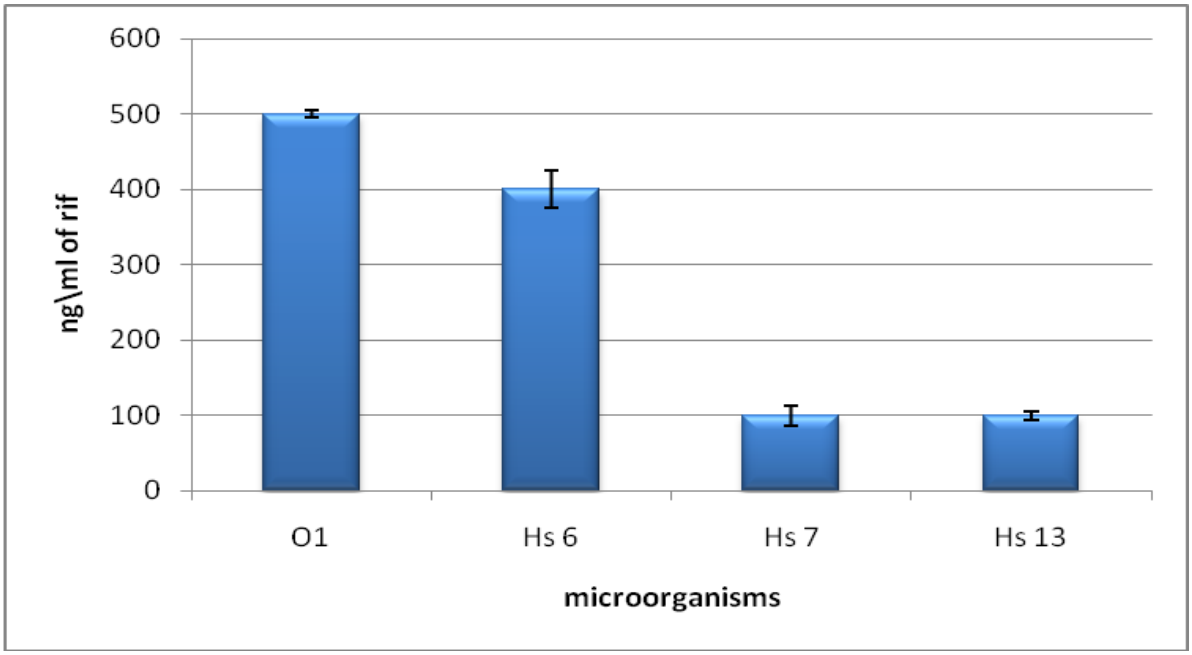


Figure 10: Determination of rifampicin MICs of *Rhodococcus erythropolis* strains.

Since Hs7 was more sensitive (100 ng/ml) to Rif than the rest of the strains it was chosen to be the recipient strain of the library. Cultures of Hs7 were grown in 1-4% of LBSG to determine which concentration of glycine was best suited to the growth of the organism. It was determined to be 3%.

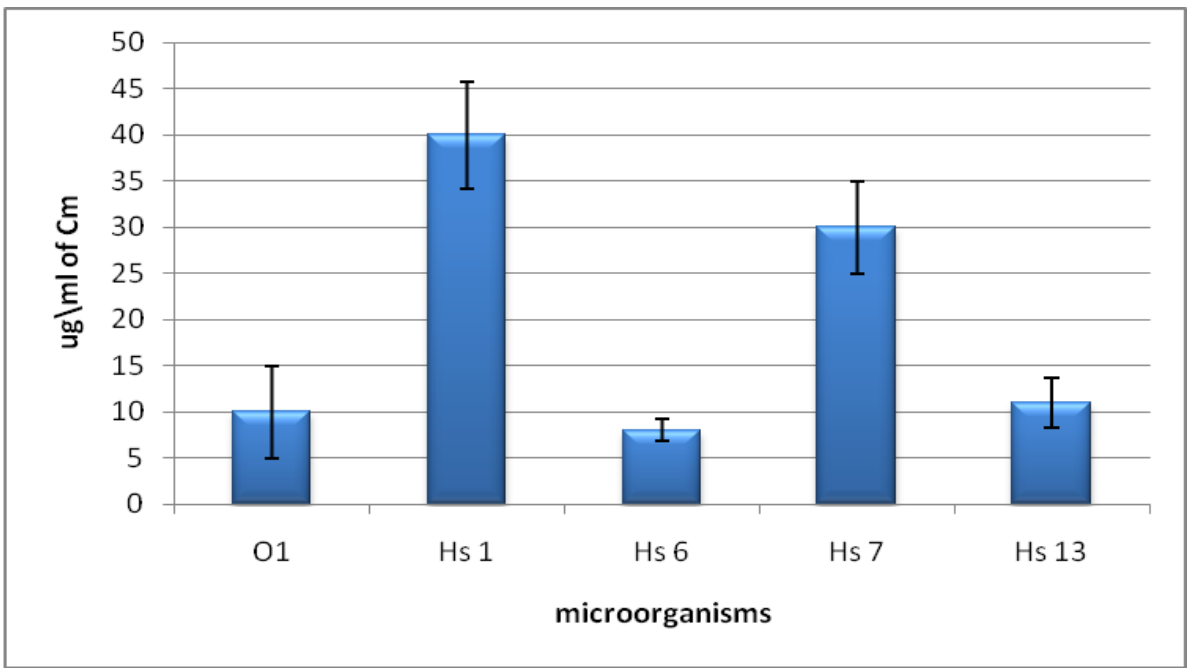


Figure 11: Determination of chloramphenicol MICs of *Rhodococcus* strains.

As an additional marker Hs7-1 was made resistant to 100 µg/ml Nal. This mutant was designated Hs7-1. Hs7-1 had very low transformation efficiency and to confirm it was actually a *Rhodococcus* species, a phage plaquing test was done (section **3.5**). The phages used, K04 and W12, were isolated from *R. erythropolis* SQ1 (Shibayama, 2006) and they only produce plaques in the presence of *Rhodococcus* species. Presence of plaques confirmed that Hs7-1 was indeed a rhodococcal strain, **Figure 15**.

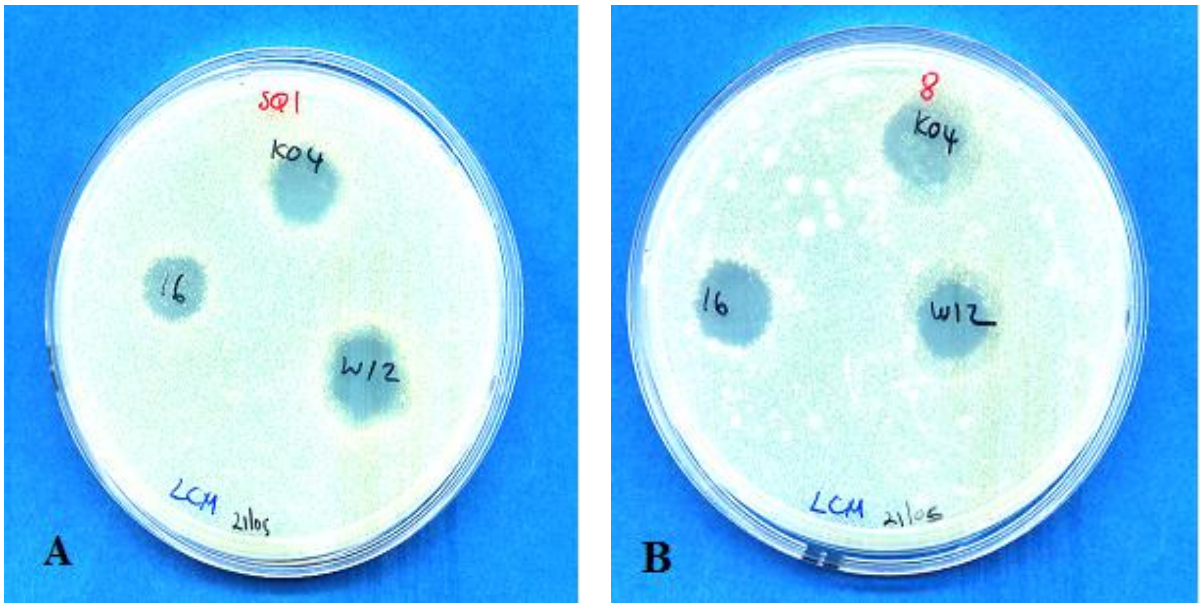


Figure 125: Phage spotting tests with **A**- *R. erythropolis* SQ1 and **B**- *R. erythropolis* Hs7-1m

4.4.1 Plasmid curing

To enhance transformation efficiency of Hs7-1 it was transformed with the pDA71*, selection being for chloramphenicol resistance. Resultant colonies were then grown in the presence of 1% Ethidium bromide (EtBr) but absence of Cam to cure the plasmid and streaked out into single colonies on LA plates. Plasmid curing is a process of completely removing or displacing plasmids from bacteria. This is usually achieved by introducing stress to the bacteria in some way, for example by growing at: high temperatures, or in the presence of a detergent, mutagens or some other DNA modulating agents, such as intercalating agents (ethidium bromide, acriflavin or acridine orange). Plasmids are non-essential, except under certain conditions, such as when an antibiotic is present. However, naturally occurring plasmids tend to be very stable as they have managed to survive selective pressure over

millions of years. Hence, to find out what property they confer on the bacterium that carries them, it is essential to displace them to produce bacteria that lack the plasmid so that bacteria with and without the plasmid can be compared.

The single colonies of the EtBr treated Hs7-1 were then patched onto an LA Cam 40 µg/ml plate and on LA. The untreated Hs7-1 without the cloned DNA and Hs7-1 that contained the cloned DNA were used as controls. All of these were expected to grow on LA but only the Hs7-1 with the cloned DNA is expected to produce growth on Cam 40 µg/ml. Which was what was observed with the results obtained. Of the colonies of the Hs7-1 that had undergone EtBr treatment, those which were able to grow on Cam plates were named Hs7-11. Upon transformation with the cloned DNA these cells had an increased ability to take up the cloned DNA as shown by the increased number of transformants.

Plasmid curing is usually stressful to the host, and most bacteria respond by increasing the mutation rate. This can result in alteration of the host's phenotype irrespective of whether the plasmid has been displaced or not, thereby seriously undermining the conclusions that can be drawn. Therefore, six mutants of Hs7-11 were patched onto Rif 100-700 ng/ml to check whether the procedure had affected the Rif MIC. Two of the mutants (#2 and #3) had an MIC of 200 ng/ml, mutant # 4 an MIC of 700 ng/ml and the rest 100ng/ml (**Figure 16**). A Hs7-11 mutant that still had the same MIC as the parental Hs7, i.e. 100ng/ml of Rif, was used as a host for the library of *M. smegmatis* 43756Km1-*E. coli*.

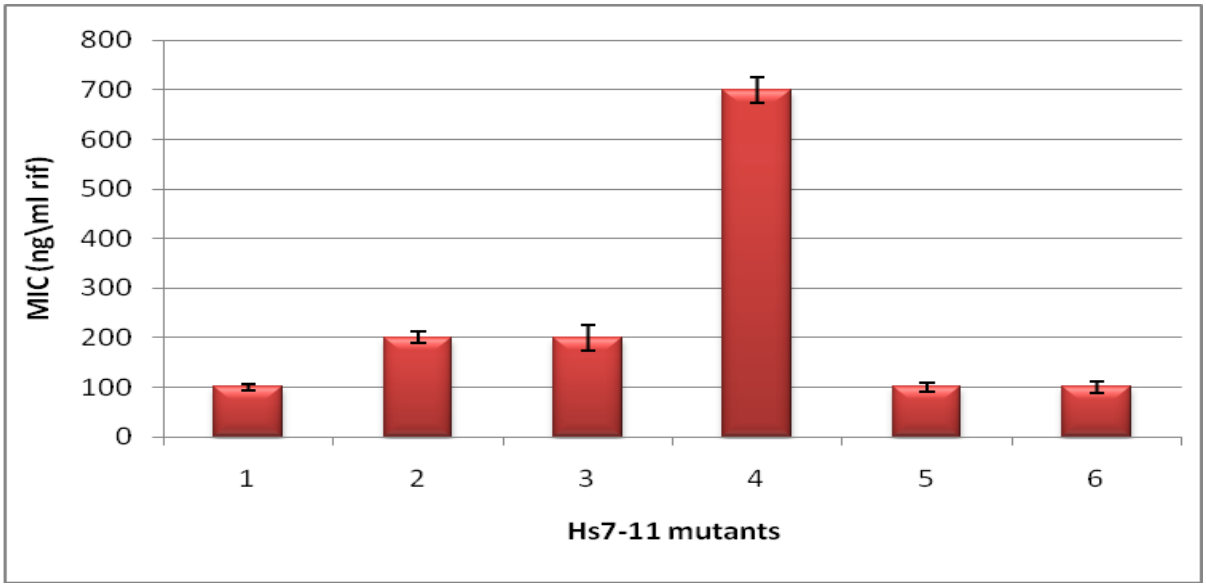


Figure 136: Determination of rifampicin MICs for the *R. rhodochrous* Hs7-11 mutants.

4.4.2 Screening of the Hs7-11 library

After transformation into Hs7-11 this library was pooled into LA and grown in the presence of 40 µg/ml Cam. Aliquots were tested for the presence of clones with an increased Rif MIC however in no case was plasmid-borne resistance detected.

4.5 Rifampicin resistance in *E. coli*

The reason why a clone conferring rifampicin resistance could not be found in *R. rhodochrous* Hs7-11 could probably be because this strain expressed very low levels of the heterologous protein responsible for resistance. An alternative approach which was done in this project was to screen the library for Rif resistance heterologously expressed in *E. coli*. This bacterium was decided upon as an alternative host because it is the most widely employed host used in heterologous expression systems. Its popularity is due to the vast body of knowledge about its genetics, complete genomic sequence and high growth rates combined with its ability to express high levels of heterologous proteins, i.e. strains producing up to 30% of their total protein as the expressed gene product, result in high volumetric productivity (Rai and Padh, 2001). It however poses significant problems in post-translational modifications of proteins, as *E. coli* has no capacity to glycosylate proteins in either N- or O-linked conformation. This fact is not relevant in this project so it will not be discussed further. A number of clones with Rif resistance were detected this way (**Figure 17)**.

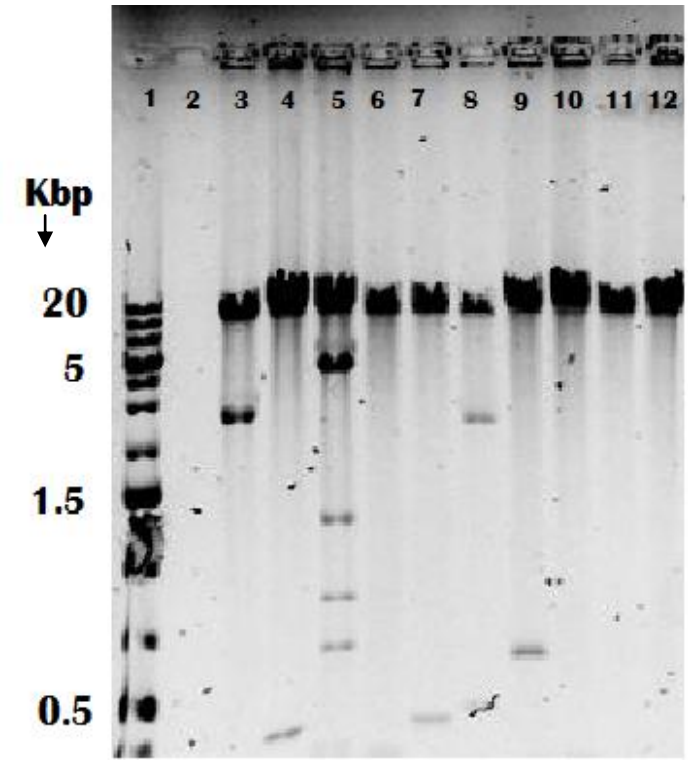


Figure 17: Plasmid screen of recombinant pDA71 vectors of *E. coli* clones digested with *Pst* I; electrophoresed on a 0.8% agarose gel. **Lane 1:** molecular weight marker, **Lanes 3-12:** digested recombinant vectors.

Ten of these clones were digested and six of the ten digested recombinant vectors had inserts (**Figure 17**) and these were named p6, p7, p8, p9, p10 and p11 respectively. These clones were different in size as noted by their different restriction patterns. However, some of the clones had inserts of the same apparent size. A common 3.0 kbp fragment was observed for both the p6 and p10 clones, but p10 had another 0.5 kbp fragment as well. A fragment of 0.7 kbp was also observed in both p8 and p11 although p8 had two more fragments (5.0 and 1.0 kbp) which it did not share with p11. These clones were then retransformed into *E. coli* MM294-4 and selected for on Amp 100 and Rif 20 µg/ml plates. This marker rescue procedure was to check whether the resistance to Rif was plasmid borne or not. *E. coli* carrying pDA71* was used as a control and had a Rif MIC of ≤7 µg/ml.

4.6 Determination of the recombinant *E.coli* rif-resistant clones rifampicin MICs

The 6 clones together with pDA71* were grown on LA Amp overnight and individual colonies were picked up and inoculated into a replica plating plate containing 250 µl of LB per well. A replicator was used to plate these on Amp 100 and Rif (20-400 µg/ml) plates from the higher antibiotic concentration to the lowest. These were incubated for 24 hrs at 37ºC. Five (p6, p7, p8, p9, p10 and p11,) had varying MICs ranging from 20-50 µg/ml and only p7 (renamed pTM1), had an MIC of >400 µg/ml of Rif, **Figure 18**.

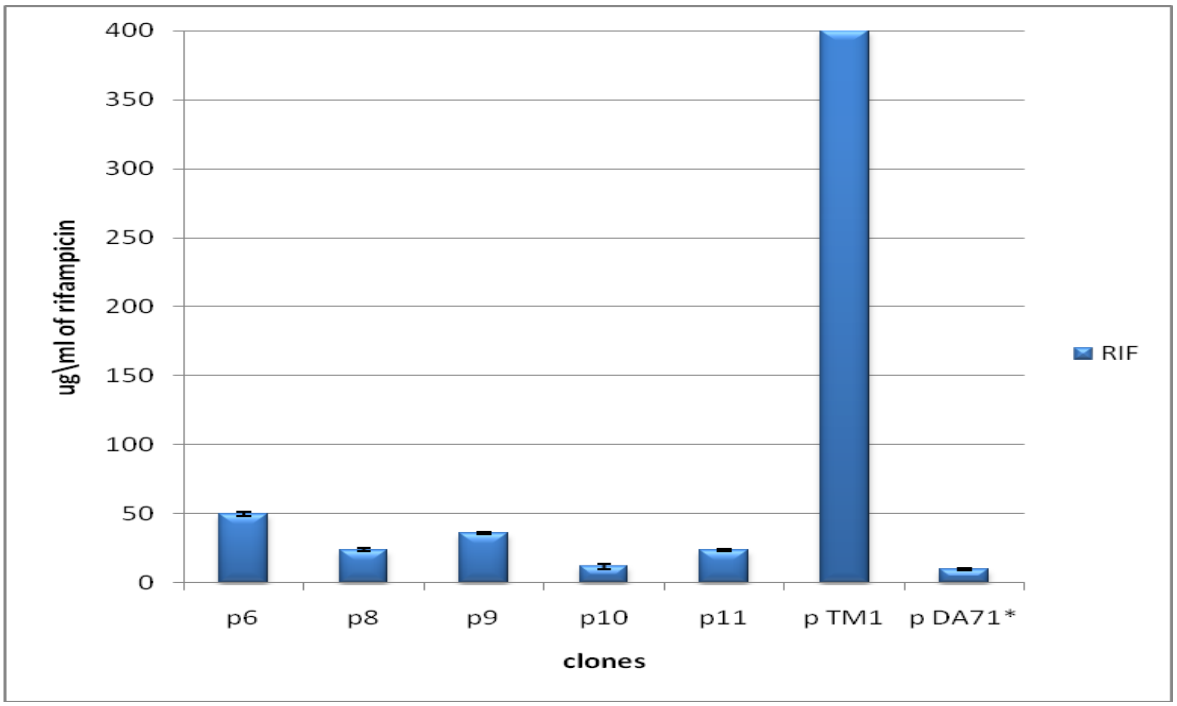


Figure 14: MICs of the 6 clones identified by marker rescue.

Clones pTM1 and p6 which had the highest Rif MICs were also tested on other antibiotics to check whether they had altered MICs. Plasmid pDA71* was again the control. These were tetracycline, kanamycin, chloramphenicol, spectinomycin, streptomycin, and erythromycin, **Figure 19**. For Kan, Spc and Tet the MIC of both pTM1 and pDA71* was the same and p6 was either slightly higher or lower as compared to them. A modest difference in MIC was found with Ery: the MIC was ~40% higher for pTM1.

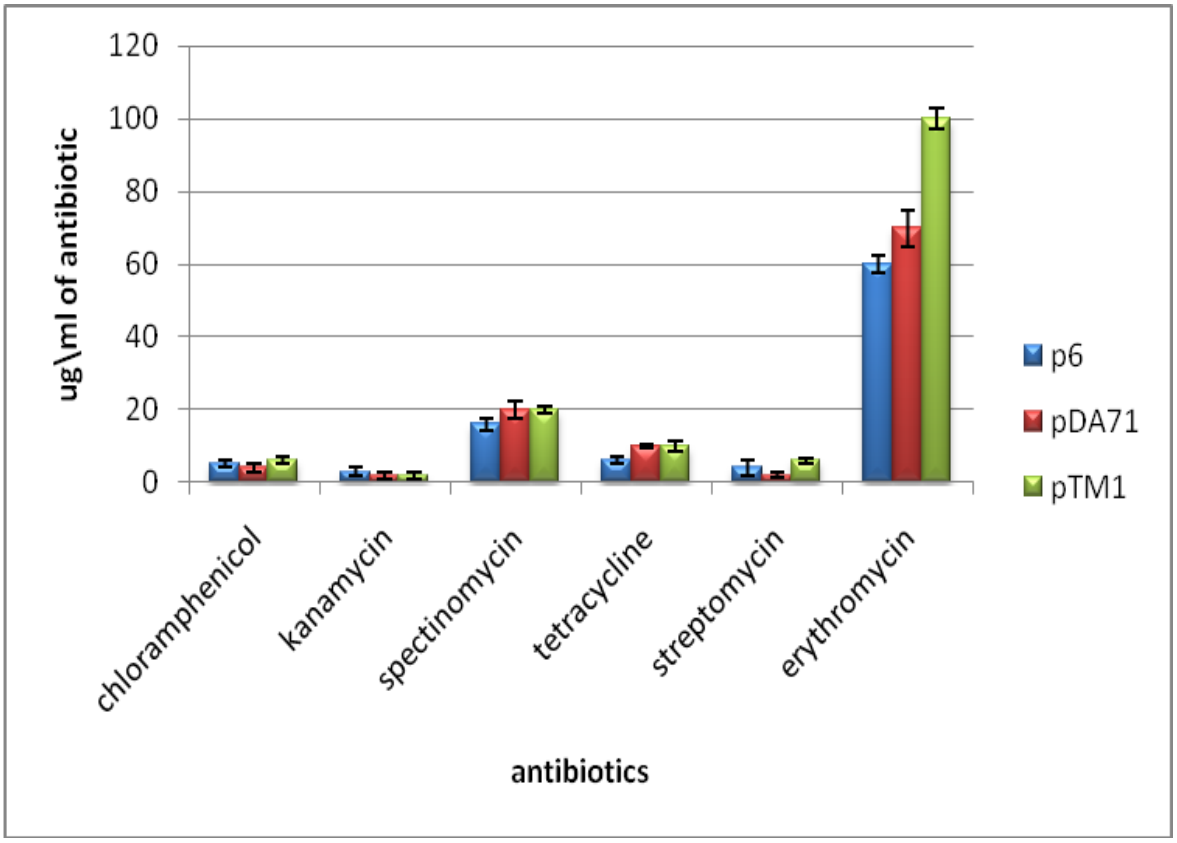


Figure 15: MICs with respect to other antibiotics.

4.7 Phenotypic characterization of *M. smegmatis* rifampicin resistant clone

The effect of temperature and pH was tested so as to characterize the best growth conditions of the clone. *E. coli* MM294-4 transformants were used for determining effect of pH on rifampicin resistance.

4.7.1 Effect of temperature on rifampicin resistance

E. coli MM294-4 cells transformed with pTM1 was spotted onto LA Rif plates (50-400 µg/ml) and incubated at 28ºC, 37ºC and 42ºC for 24 hrs. The results indicated that at lower temperatures resistance was reduced, **Table 7**. At 28ºC its MIC dropped to 300 µg/ml of Rif. But at 37ºC and 42ºC MIC was ≥400 µg/ml of Rif though at 42ºC it there was slightly less growth compared to that at 37ºC.

Table 7: Effect of temperature on Rif resistance.

MIC ug/ml rif	T (ºC)		
	28	37	42
50	++++	++++	++++
100	++++	++++	++++
150	+++	++++	++++
200	+++	++++	++++
250	++	+++	+++
300	-	+++	+++
350	-	+++	++
400	-	+++	+

++++ confluent growth +++ pronounced growth ++ growth
+ sparse growth - no growth

4.7.2 Effect of pH on rifampicin resistance

Plates used in these tests were standard LA plates whose pH had been adjusted to 5.5 using HCL or 8.5 with NAOH. The control being standard LA plates whose pH has not been altered, these have a more neutral pH. The results indicated that resistance was highest at neutral pH and lowest at acidic pH. There was no significant difference on the growth of *E. coli* MM294-4 cells transformed with pTM1 on Rif 150-400 µg/ml of Rif at both pHs. However, at a pH of 8.5 the growth was a bit more pronounced than at 5.5, **Table 8**. At alkaline pH, the vector only control grew at higher concentrations of rifampicin than it did at acidic or neutral pH.

Table 8: Effect of pH on Rif resistance.

Rif µg\ml	pH		
	7.0	5.5	8.5
100	++++	+++	++++
200	++++	+++	+++
300	+++	++	+++
400	++	+	+

++++ confluent growth +++ pronounced growth ++ growth
+ sparse growth - no growth

4.8 Cloning and sequencing

Since pTM1 conferred a Rif MIC of above 400 ug/ml it was selected for sequencing and cloned into pUC18 and pUC19. Both the clone and the vectors had very few compatible restriction sites. Since the *Pst* I site was in both, it was used for cloning. Upon digestion with *Pst* I it produced 2 fragments with a total size of 2.5 kbp, **Appendix A**. Partial *Pst* I digestions of the clone were done so as to produce a larger single fragment that possessed both the 2 smaller ones as one piece. Once pTM1 was ligated into these 2 plasmids it was sent for sequencing at Inqaba Biotech. M13 forward and reverse primers were used for sequencing and the sequence below was shown to be derived through processing with the M13 reverse primer.

4.8.1.1 Analysis of sequenced DNA

This sequence showed a 63% G-C content which is in line with expected results of currently documented complete genomic sequences of mycobacterial DNA which is high in G-C nucleotides as well. When the nucleotide sequence was subjected to a BLAST analysis, it showed 100% Max identity to *M. smegmatis* mc^2 155 and had an E value of 0.00.

4.8.1.2 Detection of ORF

For the detection of ORFs, 2 programmes were utilised: NEBCUTTER and GeneMark. Both of these programs identified the same ORF. Using the NEBCUTTER program the ORF identified (**Figure 20**), was 394 aa long, flanked with *Xho* I and *Eco* RV sites whilst using the GeneMark program, it was shown to be 369 aa long. To further verify the result of these translation programs, a comparison of the above ORF's was done using BLASTn (NCBI) and the ExPASy Proteomics server (Swisprot). They showed significantly similar results and that the ORF was 394 aa. The ExPASY server had a score of 589 and an E value of 2×10^{-166} for 2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase whilst that of the BLASTn was 599 and E-value of 2×10^{-169} respectively. Both confirming that 2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase from *M. smegmatis* was encoded by this clone. This enzyme has been reported by KEGG (Kyoto Encyclopedia of Genes and Genomes) to take part in the catabolism of aromatic compounds by acting on carbon-carbon bonds in ketonic substances. The gene for this enzyme was named *rdn* (**r**ifampicin **d**ecompostio**n**).

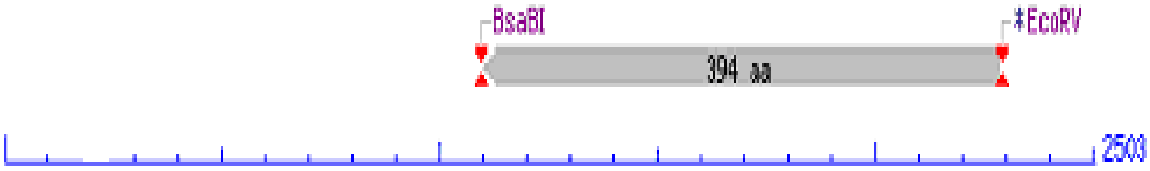


Figure 16: *rdn* predicted ORF.

4.8.1.3 Comparison between *rdn* and *iri*

The inactivation mechanism of Rif through decomposition was first discovered in *R. equi* and since a similar gene was being tested for in *M. smegmatis* 43756Km1 a comparison was done between the *iri* gene and the *rdn* gene sequences. The amino acid sequences of both genes were inputted into BLASTp and compared, only one hit was found. The blast results showed that there was a significant similarity between the two gene sequences over a query coverage of 60% (with an E value of 0.50). Unfortunately, that one hit was for an unnamed protein 106 amino acids long (**Appendix B**) and search on NCBI and Swiss-Prot revealed no further information concerning this protein. Therefore we can conclude that though these two genes

products have a similar property of inactivating rifampicin they show no major shared features as regards sequence. A similar disparity was previously shown between *iri*, *arr*, and *grt* (Quan *et al*, 1997; C Lephoto, unpublished).

4.8.1.4 Comparison of *rdn* and *arr*

A similar BLAST as above was done as well with the *rdn* and *arr* genes. *M. smegmatis* 43756Km1 is a mutant of *M. smegmatis* 43756 but it has the *arr* gene disrupted by a $Kana^R$ cassette. This prevents a functional ADP-ribosyltransferase being produced and therefore inactivation of Rif by ribosylation is prevented. The alternative inactivation method may still be utilised, in this case is decomposition. The comparison between *arr* and *rdn* showed that there was no significant similarity between the two.

4.8.2 Analysis of protein parameters

The translated sequences were used as the input sequences to conduct an analysis of the protein parameters using the ProtPar (protein parameters) and ProtScale (protein scale) programmes from the ExPASy engine. From the data shown in **Table 9**, the total number of positively charged residues, arginine and lysine was 53 (13.3%) while that of negatively charged residues, glutamic acid and aspartic acid was determined to be 41 (10.4%).

Table 9: Protein parameters of the *rdn* protein (hydrolase) as determined by the ProtPar programme

Number of amino acids: 394. Molecular weight: 43187.3. Theoretical pI: 9.68

Amino acid composition:

Amino Acid	Symbol	Number	% composition
Alanine	(A)	43	10.9%
Arginine	(R)	41	10.4%
Asparagine	(N)	9	2.3%
Aspartic acid	(D)	20	5.1%
Cysteine	(C)	6	1.5%
Glycine	(Q)	16	4.1%
Glutamic acid	(E)	21	5.3%
Glycine	(G)	42	10.7%
Histidine	(H)	15	3.8%
Isoleucine	(I)	12	3.0%
Leucine	(L)	39	9.9%
Lysine	(K)	12	3.0%
Methionine	(M)	8	2.0%
Phenylalanine	(F)	16	4.1%
Proline	(P)	23	5.8%
Serine	(S)	17	4.3%
Threonine	(T)	17	4.3%
Tryptophan	(W)	4	1.0%
Tyrosine	(Y)	6	1.5%
Valine	(V)	27	6.9%
Pyrrolysine	(O)	0	0.0%
Selenocysteine	(U)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 41

Total number of positively charged residues (Arg + Lys): 53

The N-terminal of the sequence considered is M (Met).

These protein parameters were used to investigate some properties of the protein. The first of which was the protein's hydrophobic\ hydrophilic characteristics. This information can be used to suggest the cellular localization of this protein. The Kyte and Doolittle scale of hydropathic characteristics of the twenty amino acids was used to plot a hydrophobicity plot (**Figure 25**) of *rdn* protein. In the plot, hydrophobic amino acids have a positive value whilst the hydrophilic a negative value.

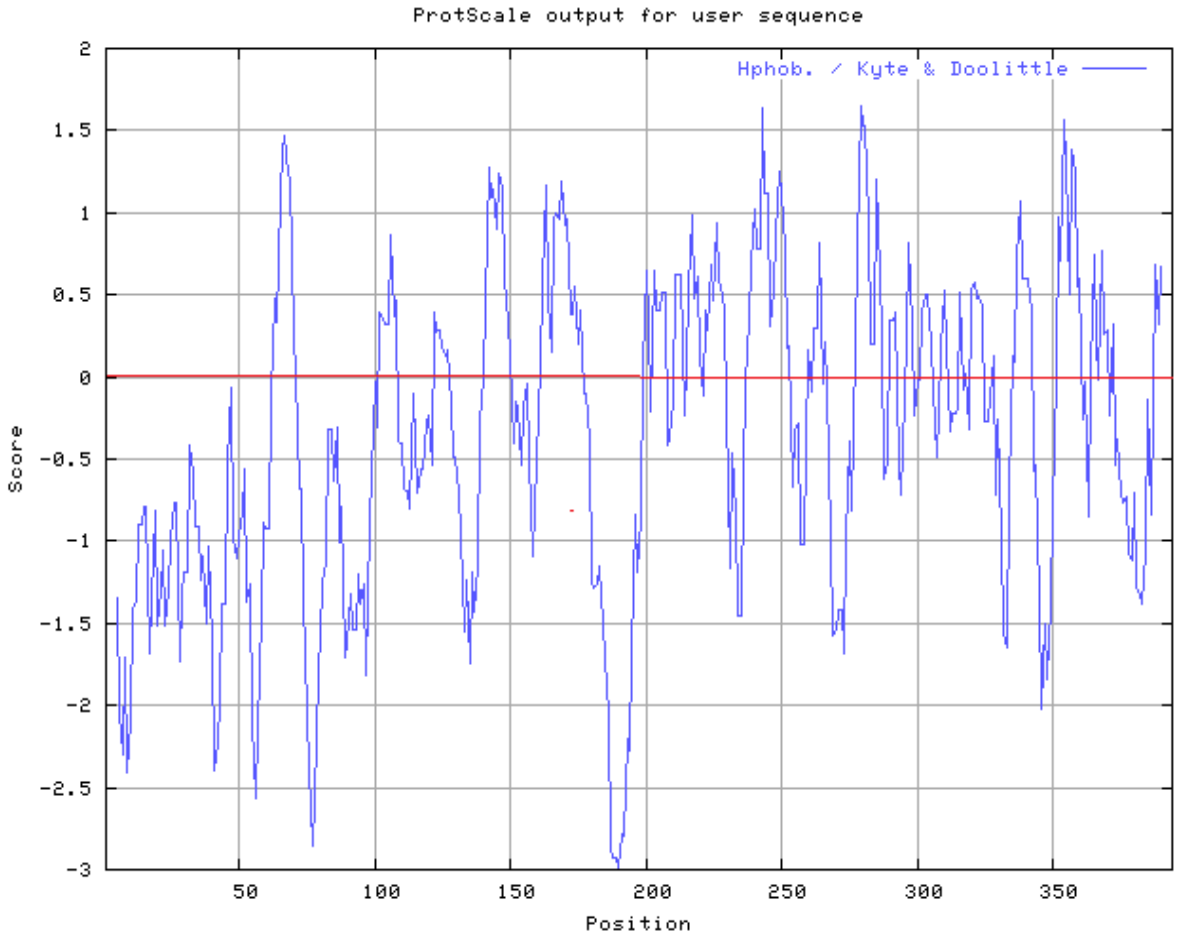


Figure 17: Kyte-Doolittle hydrophilicity plot for the hydropathic characteristics of *rdn* protein. The x-axis shows amino acid numbers.

An amino acid window of 50 was used to plot the graph and showed a maximum hydrophobic protein of 1.6 and a minimum hydrophilic protein of -3. According to **Figure 21**, the *rdn* protein is primarily hydrophobic in nature with one large hydrophilic region spanning amino acids ~5- 60.

4.8.3 Gram staining

Dabbs *et al.*, (2003) discovered through light microscopy studies that *E. coli* cells transformed with a plasmid carrying the *arr* gene are filamentous rather than coliform. This could be because the *arr* gene product is involved in bacterial wall biosynthesis and when over-expressed leads to an altered cell morphology. Whereas those transformed with the vector only were normal. This was not the case when *rdn* was transformed into *E. coli*; it did not show any altered cell morphology **Figure 22**.

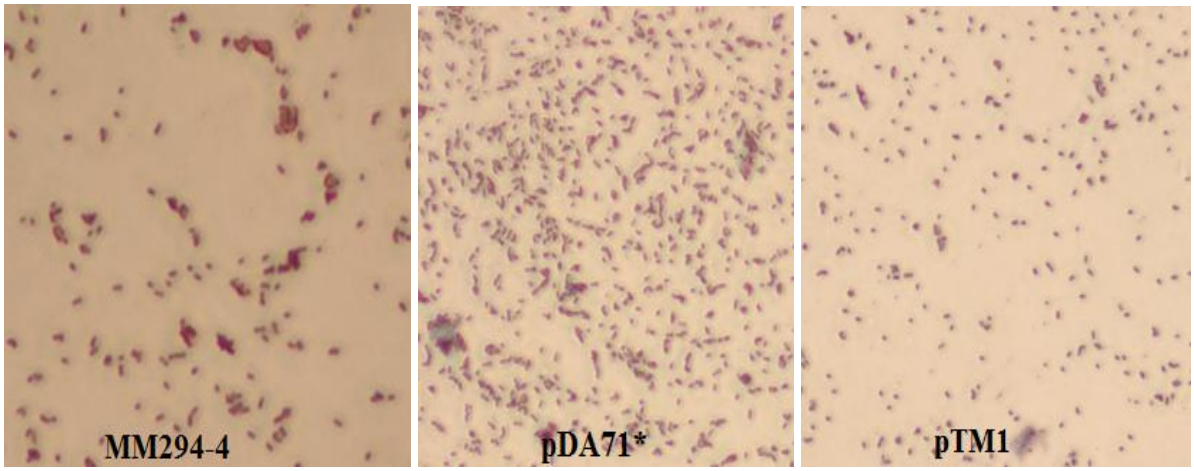


Figure 182: Gram stains of transformants.

4.8.4 Inactivation assays with *rdn*

The clone was transformed into *M. smegmatis* mc^2 155 and *in-vitro* assays were conducted to check whether *rdn* was responsible for the inactivation of rifampicin, since this strain lacks the ability to inactivate Rif. This was done by first digesting pTM1 with *Pst* I and ligating the resultant fragment into *M. smegmatis* shuttle vector, pNV18. The resultant clone was named pTM2 and it was then transformed into *M. smegmatis* mc^2 155. The controls were *M. smegmatis* 43756Km1 (K) and *M. smegmatis* DSM 43756 (4) and BHI with 20 µg/ml Rif. At 24 hrs zones of inhibition could be detected around all of the test assays but at 48 hrs *M. smegmatis* 43756Km1 had inactivated rifampicin as seen by the absence of the zone of inhibition. At 48 hrs as well, there was a reduction in the zones of inhibition around the rest except the negative control and *M. smegmatis* DSM 43756 showing that inactivation was occurring though at a slow rate. At 72 hrs no zones of inhibition could be detected around *M.*

smegmatis mc^2 155 (mc4 and mc7). These results showed that the *M. smegmatis* mc^2 155 (mc4 and mc7) when transformed with pTM2 inactivated the antibiotic but at a very slow rate as compared to the rate of inactivation in *M. smegmatis* 43756Km1, **Figure 23**.

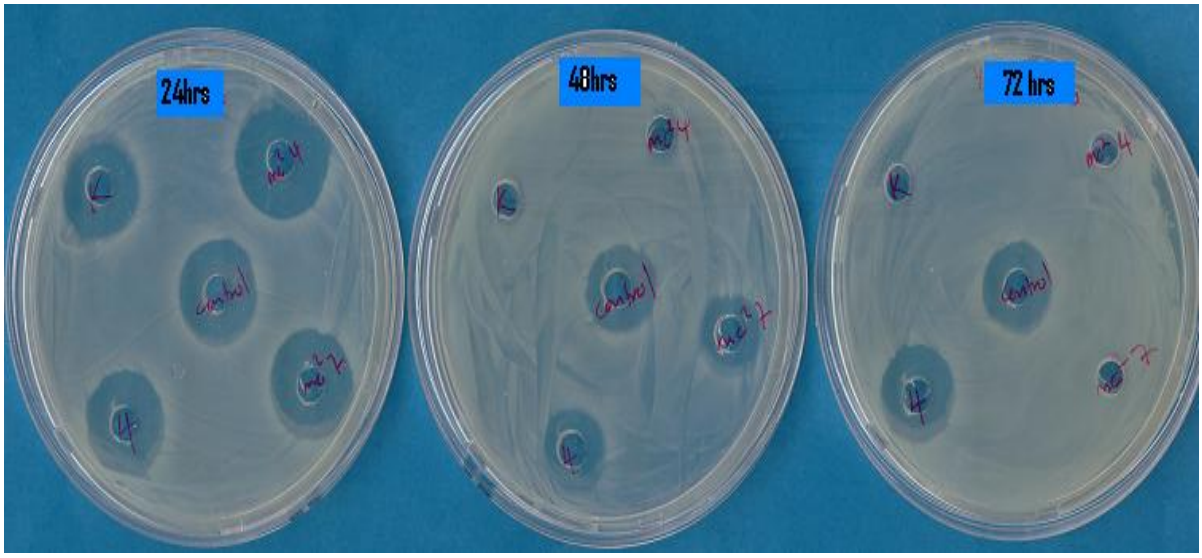


Figure 193: Assays of *Mycobacterium smegmatis* mc^2 155 transformed with pTM2. **K-** *Mycobacterium smegmatis* 43756Km1, **4-** *Mycobacterium smegmatis* DSM 43756, **mc4-** *Mycobacterium smegmatis* mc^2 155+pTM2 and **mc7-** *Mycobacterium smegmatis* mc^2 155+ pTM2.

4.8.5 Decolourisation tests with *rdn*

The decolourisation of rifampicin is the main characteristic observed in the inactivation of this drug through decomposition. The purpose of this test was to check if *rdn* decolourised this antibiotic as well. Spectrophotometric tests were also carried out in tandem with the above assays to test for the decolourisation of Rif. The absorbance of the supernatant of the above cultures was measured at the time (48 hrs) where complete inactivation was seen, **Figure 23**, at a wavelength of 560 nm. Percentage decolourisation was calculated using the following formula:

$$\% \text{ Decolourisation} = \frac{A_0 - A}{A_0} \times 100$$

The test showed that the *rdn* product decolourised Rif by 75% unlike *iri* protein which completely decolourised Rif. The slower inactivation noted in the assay test above could be because Rif is not totally broken down by the *rdn* enzyme.

4.9 Location of the rifampicin enzyme

Tests were done to investigate the location of the enzymatic activity responsible for the inactivation of rifampicin. To do this the extracellular fraction (culture broth and EDTA treatments), and the cell bound fraction were separated. At 72 hrs the zones around the cell bound fraction of *M. smegmatis* 43756Km1 and *M. smegmatis* DSM 43756 had not significantly reduced in size suggesting that the enzyme responsible for the inactivation of rifampicin was absent from this fraction, **Figure 24**. But the zone of inhibition was abolished for the extracellular fraction of *M. smegmatis* 43756Km1 (**K**) and the one around *M. smegmatis* DSM 43756 (**4**) had reduced in size. This indicated the enzyme may be released from the cells into the growth media so this 2-hydroxy-6-ketonona-2, 4-dienedioic acid hydrolase may have an extracellular function.

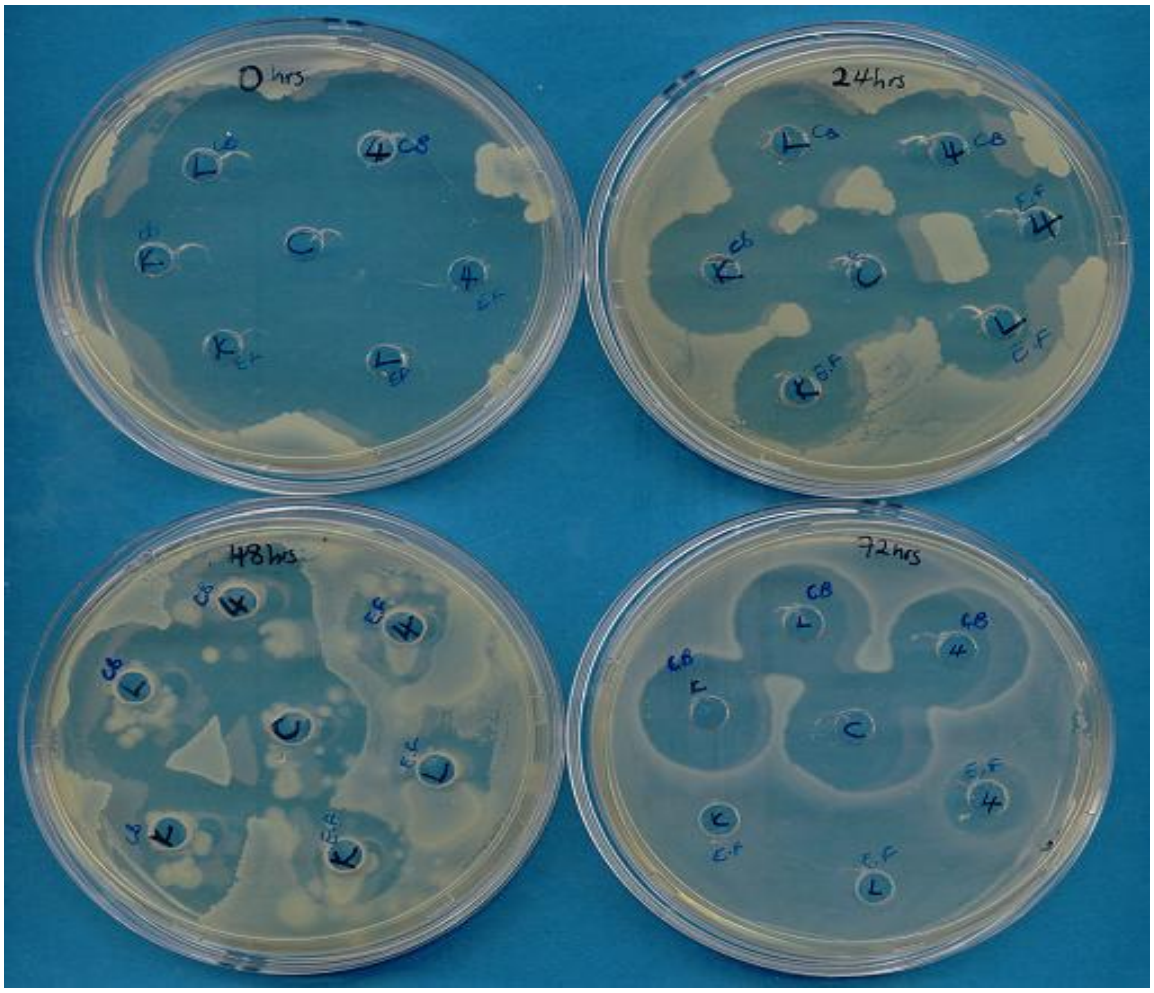


Figure 20: Enzyme distribution assays, CB- cell bound fraction, EF- extracellular fraction

CHAPTER 5

DISCUSSION

5.1 Hydrolases

The enzyme, 2-hydroxy-6-ketonona-2, 4-dienedioic acid hydrolase, discovered to be responsible for rifampicin resistance belongs to a family of enzymes called hydrolases. Hydrolases are related to monooxygenases in that they both catalyze oxidoreductase reactions however; monooxygenases are cofactor–dependent enzymes whilst hydrolases are not. Hydrolases are part of the monooxygenase family.

$S + O_2 + XH_2 = SO + X + H_2O$ or $SH_2 + O_2 = SO + H_2O$ (monooxygenases)

$A–B + H_2O \rightarrow A–OH + B–H$ (hydrolases)

Hydrolases can be further classified into several subclasses, based upon the bonds they act upon; i.e. esterases, DNA glycosylases, proteases and helicases. These enzymes catalyze the hydrolysis of proteins, nucleic acids, starch, fats, phosphate esters and other macromolecular substances or of various chemical bonds. In simpler terms they catalyze the hydrolysis of chemical bonds (-O, C-N, C-C and phosphoric anhydride bonds) with the addition of H_2O. The enzyme, 2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase is believed to play a part in the hydrolysis of phenylpropionate by hydrolysing C-C and –O bonds.

5.1.2 2-Hydroxy-6-ketonona-2, 4-dienedioic acid hydrolase

The 394 amino acid long ORF (*rdn*) discovered on the pTM1 clone from *M. smegmatis* 43756Kml codes for the enzyme 2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase [EC: 3.7.1-]. It was discovered that in *E. coli* it takes part in the phenylpropionate degradation pathway and that it is a 62 kDa homodimeric enzyme. This hydrolase belongs to the *mhpC* alpha/beta hydrolase family, *mhpC* gene codes for a number of enzymes which can degrade m-hydroxyphenyl propionic acid. The enzyme 2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase is part of the multidomain esterase-lipase superfamily.

The gene for this enzyme is found from position 6 103 557 - 6 104 435 on the complete genome of *M. smegmatis* mc^2 155. It lies between a gene for biphenyl-2,3-diol 1,2-dioxygenase and for a pigment production hydroxylase. All 3 genes as well as for nitrilotriacetate monooxygenase component B share the same operon. Biphenyl-2,3-diol 1,2-dioxygenase plays a role in the degradation of biphenyl whilst nitrilotriacetate monooxygenase component B and the pigment production hyroxylase are thought to take part in xenobiotics degradation and metabolism. 2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase is involved in aromatic compound catabolic processes meaning its chemical reactions and pathways result in the breakdown of any substance containing an aromatic carbon ring and in phenylalanine metabolism.

I hypothesize that this enzyme attacks the naphthoquinoid ring of Rif which results in its inactivation (**Figure 25**). Since 2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase is known to hydrolyse ketoic compounds I postulate that it might break down Rif at the C11.

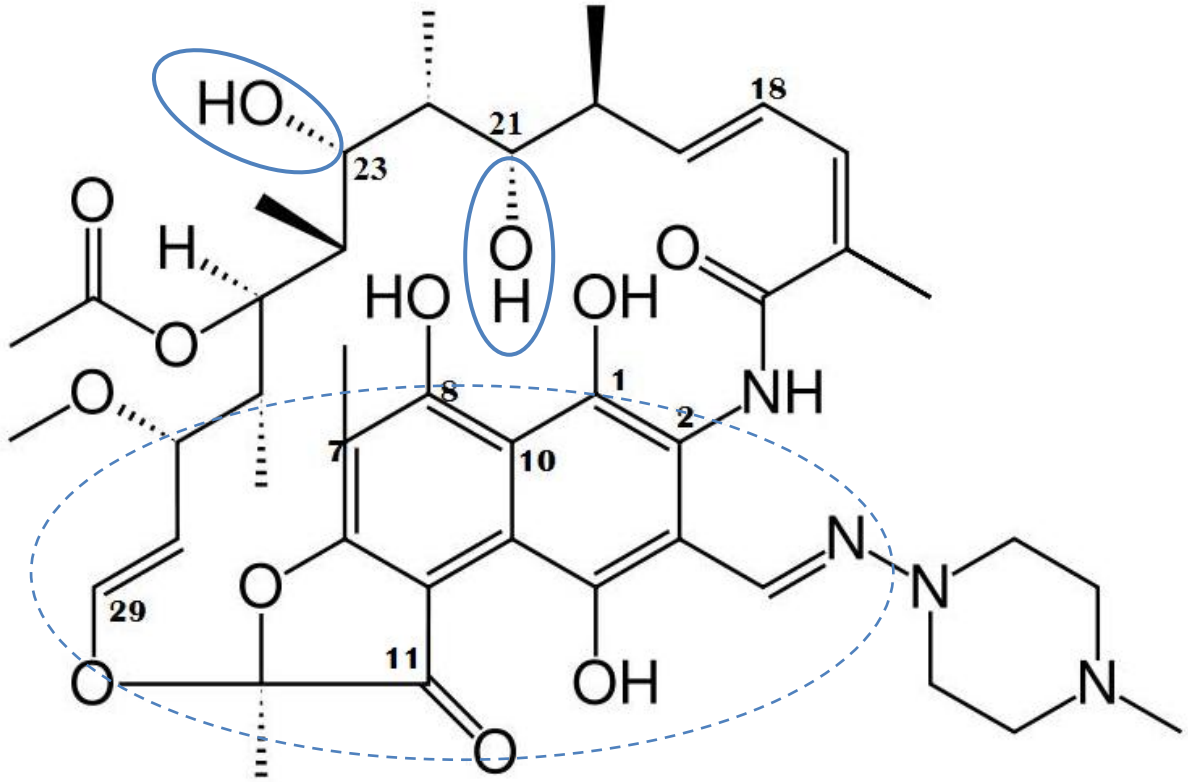


Figure 25: Rifampicin, positions of inactivation; C21, C23 and the naphthalene moiety

This enzyme takes part in a number of different reactions some of which are shown below:

2-Hydroxy-2,4-pentadienoate + Succinate <=> 2-Hydroxy-6-oxonona-2,4-diene-1,9-dioate + H_2O

6-Oxo-2-hydroxy-7-(4'-chlorophenyl)-3,8,8-trichloroocta-2E,4E,7E-trienoate + H_2O <=> 2-(4'-Chlorophenyl)-3,3-dichloropropenoate + 2-Hydroxy-3-chloropenta-2,4-dienoate

6-Ketoxycyclohex-1-ene-1-carboxyl-CoA + 2 H_2O <=> 3-Hydroxypimeloyl-CoA

2,6-Dihydroxypseudooxynicotine + H2O <=> 2,6-Dihydroxypyridine + 4-Methylaminobutyrate

Some of the mechanisms of action are shown below, **Figure 26**. These reactions as well as well as some of the above show possible hydrolysis of –O bonds in various molecules which are similar in structure to rifampicin.

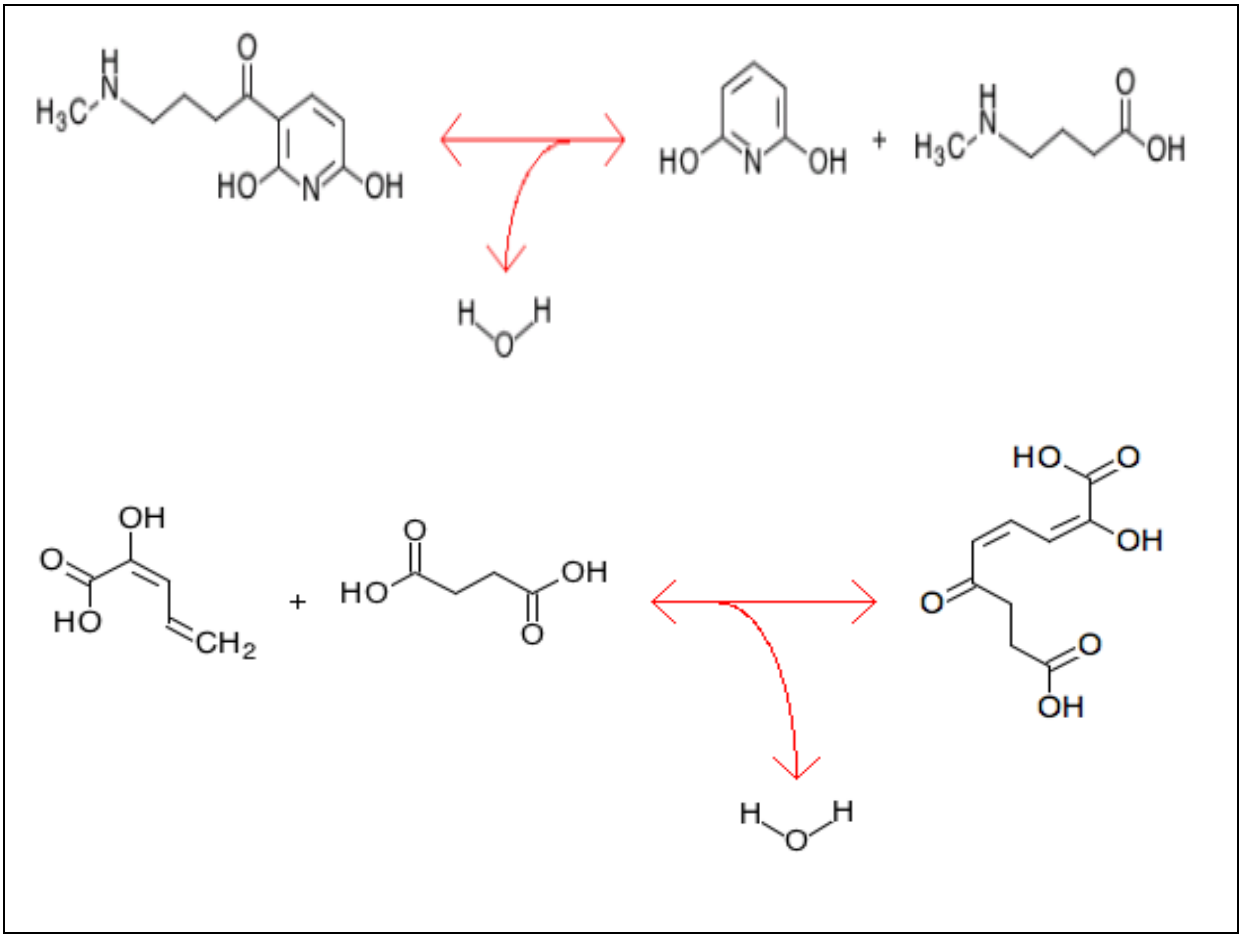


Figure 21: Possible mechanisms of action of 2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase

Also amongst the substrates catalysed by this enzyme are: glycolate; pyruvate; 2-hydroxy-2,4-pentadienoate; succinate; 4-carboxy-2-hydroxymuconate semialdehyde; acetate; 6-oxo-2-hydroxy-7-(4'-chlorophenyl)-3,8,8-trichloroocta-2E,4E,7E-trienoate; 26-ketoxycyclohex-1-ene-1-carboxyl-CoA 4-Isopropenyl-2-oxy-cyclohexanecarboxyl-CoA; 2-hydroxy-6-ketononatrienedioate 2,6-Dihydroxypseudooxynicotine and 3D-(3,5/4)-trihydroxycyclohexane-1,2-dione; showing that it has a broad spectrum of activity.

5.5 Cell morphology

According to Dabbs *et al.*, 2003, it was discovered that if the *arr* protein is involved in bacterial wall biosynthesis, its over-expression alters cell morphology. Light microscope visualization of *E. coli* cultures revealed those transformed with plasmid with the *arr* gene

became filamentous whilst those with the vector only were normal. When *rdn* was transformed into *E. coli* in this work there was no evidence of the *E. coli* becoming filamentous. Apart from altered Rif phenotype (and to a much lesser extent Ery) no other phenotypic differences were observed between cells transformed with pTM1 and those of the parental strain.

In conclusion of this work, 2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase was found to be responsible for the inactivation of rifampicin. However, unlike the monooxygenase -like enzyme discovered in *R. equi* this enzyme slowly breaks down but does not completely breakdown rifampicin as evidenced by the incomplete decolourisation. Future projects can look into the actual mechanisms of action of 2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase in the inactivation of rifampicin as well as the products produced in the process. Comparison between these 2 related enzymes can also be done to determine how their enzymatic functions differ from each other.

CHAPTER 5

APPENDICES

5.1 APPENDIX A: pTM1 clone complete sequence.

The brown sequence codes for 2- hydroxy-6-ketonona-2,4-dienedioic acid hydrolase.

GTCCCGGGGATCCTCTAGAGTCGACCTGCAGGCCACCCCACTGCTCGGGCTGCGKCTGTCGAGCCGTG
GCTGCCCAGGGGCTCATCAGTCACGGGGGTGACCCTTCAACAGCTTCTCTTCTTGAAGAAGCCGAGCTC
ATCGAGTTCGTCGATCGACTCCTGCGGGATCTGACGGCAAATCAGCCAGAACTAGAACAGGTTACAAA
ACTACAGTTGCGCAGGTCCACAGCTCCTGCCGATGTCCGCGGCCTGGGCGCGTTCCCGCAGCTTCGGC
AGCAGATCATCAACGCCGGCTTTGCAGCGCTACTCGACACAAATCGTAACGTGTTCTAATCTCTGTCT
AGTTGGAGGACCGTGGATCTCCTGCACGTCACGCTGTTCAATGGACGTCACTGAATTGCCTCCCGGAT
CGAGCGATCCGGGAGGCAATTCAGTGACGTCCATTGAACAGCGTGACGTGCAGGCGATCCACAAGTCC
TCGCCTAGACAGAGATTAGAACACGTTACGATTTGTGTCGAGTAGCGCTGCAAAACCGGCGTTGATGA
TCTGCTGCCGAAGCTGCGGGAACGCGCCCAGGCCGCGGAGATCGGCTGGAGCTGCGGACCTGCGCAAC
TGTAGTTTTGTAACCTGTTCTAGTTCTGGCTGATTTGCAGATCAGATCCCGCAGGAGTCGATCGACGA
ACTCGATGAGCTCGGCTTCTTCAAGAGAGAAACTGTTGCAGGGTCACCCCGTGACTGATGAGCCCCTG
GGCAGCCACGTGCTCGAACTGCAGGCCCGAGCAGTGGGGTGGCCTGCAGGTCGACTCTAGAGGATCCC
CGGGTACCGCATGCAAGAGCTCGAGCTTGGCACTGGCGATTCGTAATCRRGGTCATAGTKGKTTTCCT
AAGTNCCCGGGGATCCTCTAGAGTCGACCTGCAGACGGTCGTGTTCACCGGGGACGATCACCAGCCGC
GCCGGGAACTCGTCCATACGCAGATAGAGCGCACCGTCGGTGTTGCCCTTGCCCTCGACCATGCCGAG
GACCTTGAGGCCGTACTCGCGCCAGGCCGCGACGTCGGTGGCCTCGATGCGCAGATAACCCAACGACT
TGATGCTCATCTAGCCTCCCAGGAAGTCGATGGTGAGCTTGTTGAATTCGTCGAACTTCTCGACCTGT
GCCCAGTGTCCACACTGCCCGAACACGTGGAGCTGAACCCGTGGAATCTGTTTGACGGCCACGAGCGC
GCCGTCGAGCGGGTTCACGCGGTCCTCGCGGCCCCAGATCAGCAGCACCGGCTGACGCAGCTTGTAGA
CCTCGCGCCACATCATGCCGAGCTCGAAGTCCGCTCCGGCGAAGGACTTCCCCATCGCCCTGGTGGCG
GCCAGCGATTCCGGCGTGCTGGCGATCGCGAACCGCTCCTCGACCAGCTCCGGCGTGATGAGCTTCTG
GTCGAACACCATGATCCGCAGGAAGGCTTCGAGATTCTCGCGCGTCGGTTCGACGTTGAACTTCGCGA
GCGCCTTGACGCCCTCGGTGGGATCGGGCGCGAACAGGTTGACGCTGAGCCCACCGGGACCCATCAGC
ACGAGCTTGCCGGCCCGGTCGGGGTAGTCGAGCGCAAACCGCACCGCCGTACCGCCGCCGAGTGAGTT
GCCCAGCAGCGGAACTCGGCCCTCGACACCGAGGTGGTCGAGCAGGCCGCGCACGGCCTTGGCGCTGT
AGCGGTTGTACTGCTCGTGTTCGGTGTGCTTGTCCGACAGCCCGTAACCGGGCTGATCGACGGCCAGC
ACGTGGAAATGCTCGGCGAGCACCGCGATGTTGCGGCCGAAGTTCGACCAGCTGGCCGCACCGGGACC
GCCGCCGTGCAGCAGCACGATGGTCTGCGCGGACGGGTCACCGGCCTCGTGGTAGTGCAACCGCATGG
CCAGCTCGCCGGCCTGCACATCGGCGAAGCGCGAGGTGGATTCGAACGTGATCTCCTGTGTGGCAGTC
ACCATCAGACCATCGTGTCCGCGGGCGGAAGACCGAATTCGTGGTTGCCGAAGATCAGGTAGGCACGC
TCGGGTTCGTTGGCGGCGTGCACGCGACCCGCATGCGCATCGCGCCAGAACCGTTGCAGCGGTGCGGT
GTTGATCAACGCCGTGGCGCCGGCCGATTCGAAGAGCAGGTCGATCGAGGCGATGGCGCGGCCCGTGG
CACGCACCTGATCGCGGCGGGCACGGGCGCGCAGGTCGAACGGGATCTCCTTGCCCGCCTTGAGCAGT
TCGTATTCCTCGCCCACGTTTCCGATCAGCTGACGCCACGCGGCGTCGATATCGCTGGCGGCCTCGGC
GATACGGACCTTGGCGAACGGATCGTCCTTGGACTTCTCACCGGCGAACGCCGCACGCACACGCTTGC
CCTGATGCTCGACGTGGGCCGCGTACGCGCCGTAGGCCATGCCCACGATGGGTGCCGAGATGGTCGTG
GGATGCACGGTGCCCCAAGGCATCTTGTACACCGGCGCGGTGTTGGTCGCATATC

5. 2 APPENDIX B: BLASTp comparison of *rdn* and *iri*.

Sequences producing significant alignments:

Accession	Description	Total score	Query coverage	E value
62581	unnamed protein product	117	60%	0.50

Alignments

unnamed protein product of length=479

Score = 18.5 bits (36), Expect = 0.50, Method: Compositional matrix adjust.
Identities = 19/107 (17%), Positives = 37/107 (34%), Gaps = 20/107 (18%)

```
Qry89       QPRIRSSARGHDGLMVTATQEITFESTSRFADVQAGELAMRLHYHEAGDP    138
             Q  + S+A G   +    ++ + FE   R+   +   +++R  + E  D
Sbjct                                                            339
QAELISTAAGPQAVRRLISELMEFEDVKRYLTEKITAISIRYDFGEGDDLLGRRLRNIAL
398

Query  139  ----------SAQTIVLLHGGGPGAASWSNFGRNIAVLAEHFHVLAV  175
                     S + ++L  GG     WS+   +I   +     AV
Sbjct  399              TRGNLYDLMRSGRGLLLDQGGQLSVDGWSDRADHIVDTSTELEAPAV
445
```

Score = 18.1 bits (35), Expect = 0.64, Method: Compositional matrix adjust.
Identities = 7/13 (53%), Positives = 9/13 (69%), Gaps = 0/13 (0%)

```
Query  7    KRGRGIRTAQGGQ  19
            + GRG+   QGGQ
Sbjct  408  RSGRGLLLDQGGQ  420
```

Score = 17.7 bits (34), Expect = 0.74, Method: Compositional matrix adjust.
Identities = 10/22 (45%), Positives = 10/22 (45%), Gaps = 0/22 (0%)

```
Query  201  RGLLDHLGVEGRVPLLGNSLGG  222
             RGLLD    GR   LG    G
Sbjct  56    RGLLDKFLAHGRKYPLGGFFAG  77
```

5.3 APPENDIX C: MEDIA

Table 1: Composition of bacterial culture media

Medium	Components	Components per 100 ml (Grams)
LB	Tryptone Yeast extract NaCl	1 0.5 0.5
LB-agar	Tryptone Yeast extract NaCl Agar (1.5% LB agar)	1 0.5 0.5 1.5
½ LA	Tryptone Yeast extract NaCl Agar	1 0.5 0.5 0.75
BHI **BHI agar**	Brain heart media Brain heart media Agar	3.7 3.7 1.5
LBG	LB agar (100 ml) + Glycine	2% or 3%
LBSG	LBG agar (100 ml) + Sucrose	10

Medium	Components	Components per 250 ml (grams)
Regeneration media	Tryptone Yeast extract NaCl	3 1.5 0.9

	Sucrose	30.9
	Glucose	1
	Technical agar	5.5
		After autoclaving the solution was cooled to 60ºC and the following were added:
	0.25 TES	10 ml
	1M $CaCl_2$	6 ml
	0.5% KH_2PO_4	3 ml
	Nystatin	1.5 ml
		Plates were dried at 37ºC for 2 days before use.

100 ml of distilled water was added and this was mixed and autoclaved at 121°C for 20 min. Media was stored in the cold room at 4°C.

5.4 APPENDIX D: SOLUTIONS

Table 2: Composition of solutions used for mini-plasmid preparations of *E. coli*

Solution	Composition of working solution
Solution I	50 mM glucose 25 mM Tris-HCl (pH 8) 10 mM EDTA
Solution II	0.2 M NaOH 1.0% SDS
Solution III	60 ml 5M potassium acetate pH 4.8 11.5 ml glacial acetic acid 28.5 ml sterile dH_2O
Ribonuclease	10 mg/ml solution in sterile dH_2O, heated for 10 min at 95°C before use

Distilled water was added to solutions I-III and autoclaved at 121°C for 20 min. Solutions I, II, and ribonuclease were stored at -20°C and solution III in the cold room at 4°C.

Table 3: Composition of solutions for *E. coli* $CaCl_2$ transformation

Solution	Composition of working solution
$CaCl_2$ transformation buffer	10 mM Tris-HCl 100 mM $CaCl_2$ pH 7.5
20% glucose	4 g glucose 20 ml dH_2O

100ml of distilled water was added, mixed and the solutions were autoclaved at 121°C for 20 min. Solutions were stored in the cold room (4°C).

Table 4: Composition of solutions for Rhodococcus PEG mediated transformation

Solution	Composition of working solution
Basal buffer (B)	10.3 g sucrose 25 mg K_2SO_4 202 mg $MgCl_2.6H_20$ 10 ml 0.25M pH 7.2 87.5 ml distilled water
Protoplast buffer (P)	5 ml B buffer 50 µl 0.5% KH_2SO_4 125 µl 1M $CaCl_2$
1M $CaCl_2$	21.9 g $CaCl_2$ 100 ml distilled H_2O
0.5 % KH_2SO_4	0.5 g KH_2SO_4 100 ml distilled H_2O

The basal buffer, 0.5 % KH_2SO_4 and 1M $CaCl_2$ were autoclaved at 121°C for 20 min. Only the basal buffer was stored at -20°C and the rest of the solutions were stored in the cold room (4°C).

Table 5: Composition of agarose gel electrophoresis buffers for analysis of DNA

Solution	Composition of working solution
Agarose gels	0.4 g (0.4%), 0.8 g (0.8%) or 1.2 g (1.2%) agarose 100 ml 0.5x TBE

TE buffer	2 ml 0.5 M EDTA pH 8.0 1.0 ml 1M Tris-HCl pH 8.0 97 ml dH_2O Autoclaved at 121°C for 20 min.
5x TBE	54.0 g Tris base 27.5 g boric acid 20 ml 0.5 M EDTA pH 8.0 Added distilled water to 1 litre, mix and autoclaved at 121°C for 20 min.
Running Buffer (0.5x TBE)	25 ml 5x TBE 225 ml sterile dH_20 25 µl EtBr

Table 6: Composition of other solutions used in this work

Solution	Composition
0.5M EDTA pH 8.0	18.6 g EDTA Adjust to pH 8 with 10 M NaOH Add 1 L water Autoclave (121°C, 20 min)
1.0M NaCl	5.8 g NaCl 100 ml sterile dH_20 Autoclave (121°C, 20 min)
Sodium Acetate pH 5.2	408 g sodium acetate Adjust pH with 3M glacial acetic acid Add 1 L water Autoclave (121°C, 20 min)
TE-saturated Phenol	14 g phenol 10 ml TE buffer

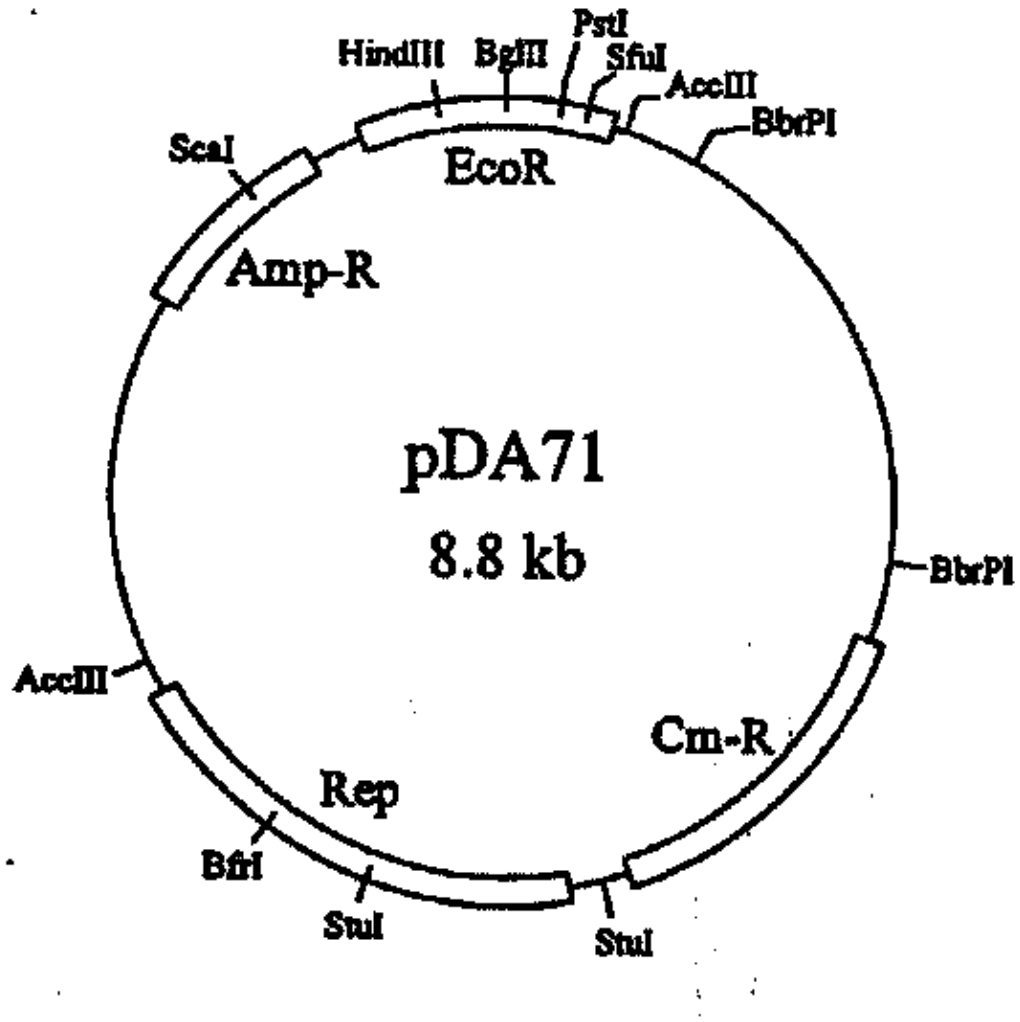
1.0M Tris-HCl **pH 8.0**	24.2 g Tris base 160 ml sterile dH_2O Adjust to pH 8.0 Top up to 200 ml Autoclave (121°C, 20 min)

5.5 APPENDIX E: ANTIMICROBIAL AGENTS STOCK SOLUTIONS

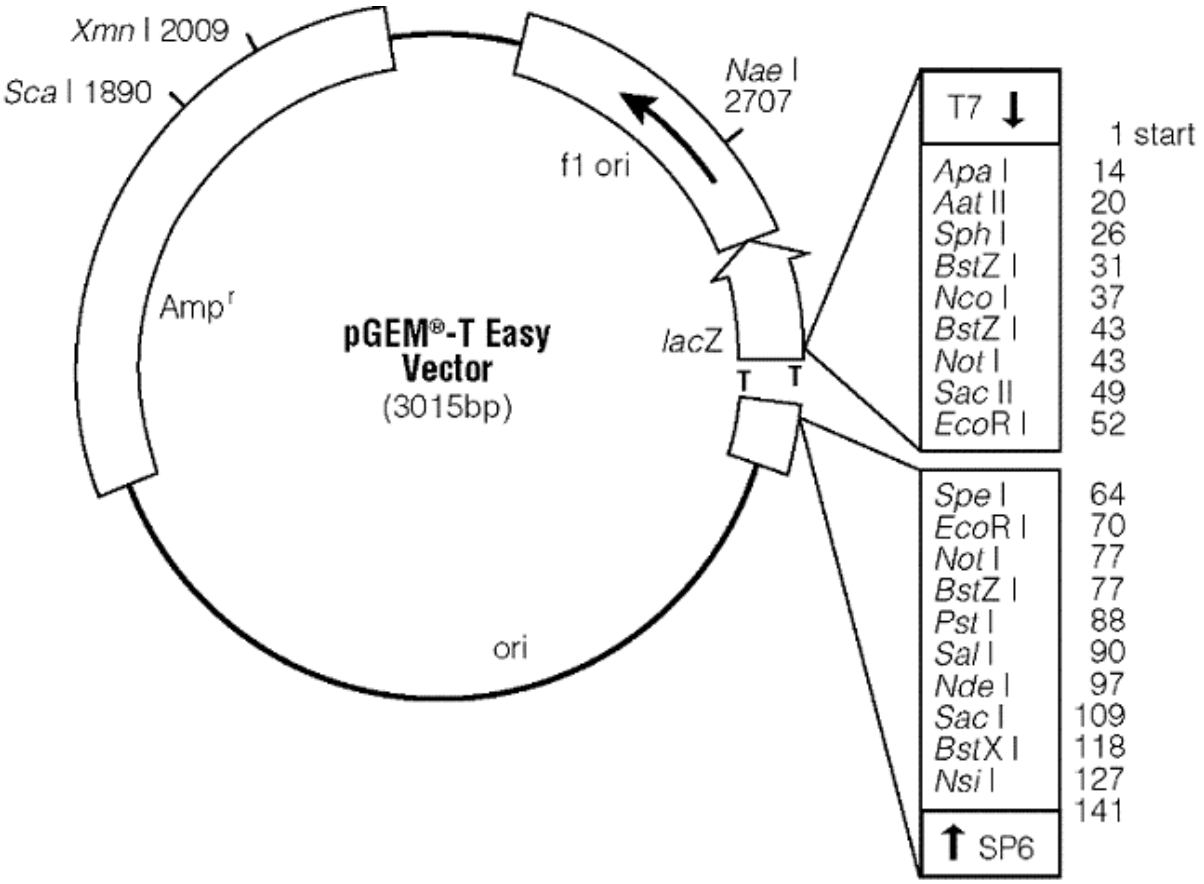
Antibiotic	Stock Concentration (mg/ml)	Solvent
Ampicillin	100	30% dH_2O:70 % ethanol
Chloramphenicol	20	ethanol
Erythromycin	5	30% dH_2O:70 % ethanol
Kanamycin	10	sdH_2O
Nalidixic Acid	10	30% dH_2O:70% ethanol
Rifampicin	10	methanol
Spectinomycin	20	sdH_2O
Streptomycin	20	sdH_2O
Tetracycline	10	methanol

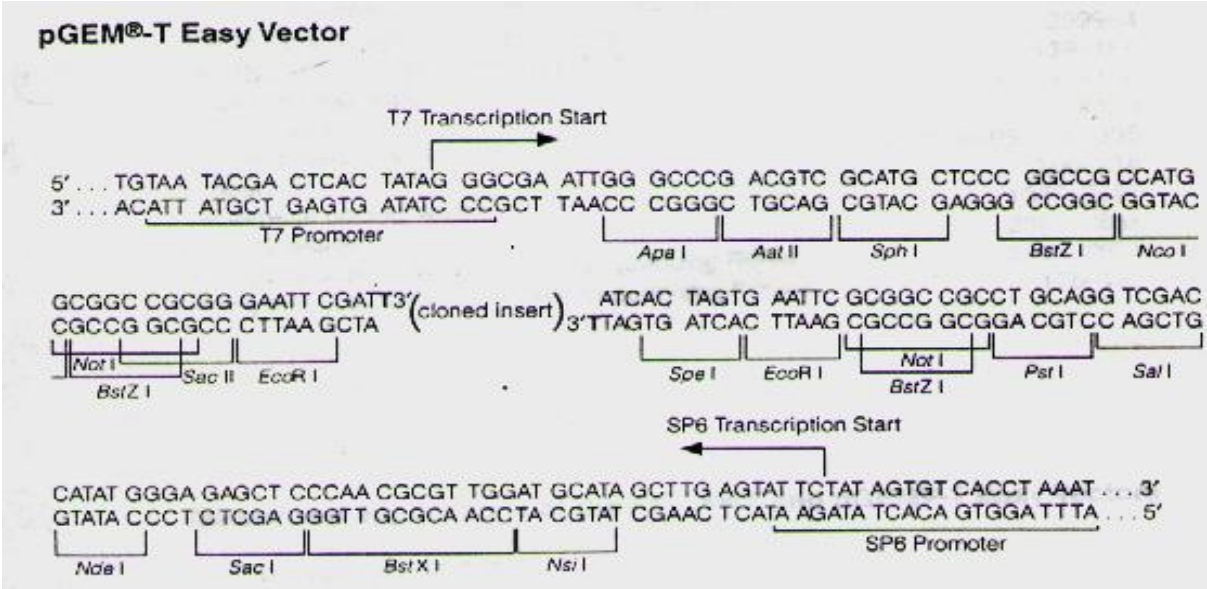
5.7 APPENDIX F: PLASMIDS USED IN THIS WORK

Restriction maps of plasmid pDA71 a Rhodococcus/ *E. coli* shuttle vector (adapted from Dabbs *et al.*, 1995a).

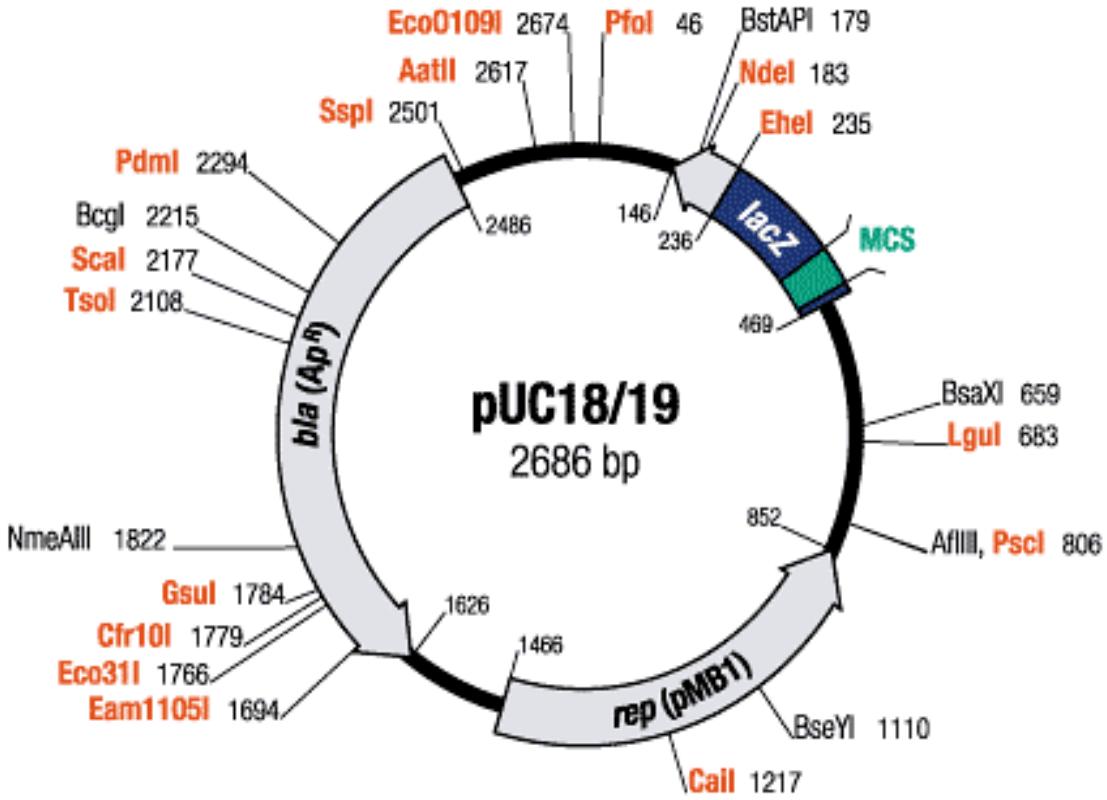


Restriction map of plasmid pGEM-T-Easy (adapted from www.promega.com, Technical manual No. 042)



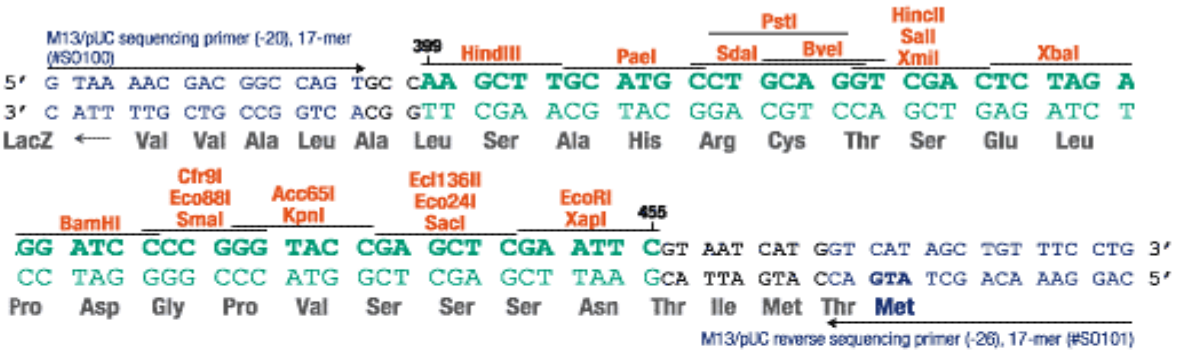


Restriction map of pUC 18\19 (adapted from www.fermentas.com/en/products/all/molecular-cloning/vectors-phage/sd005-puc18-puc19).

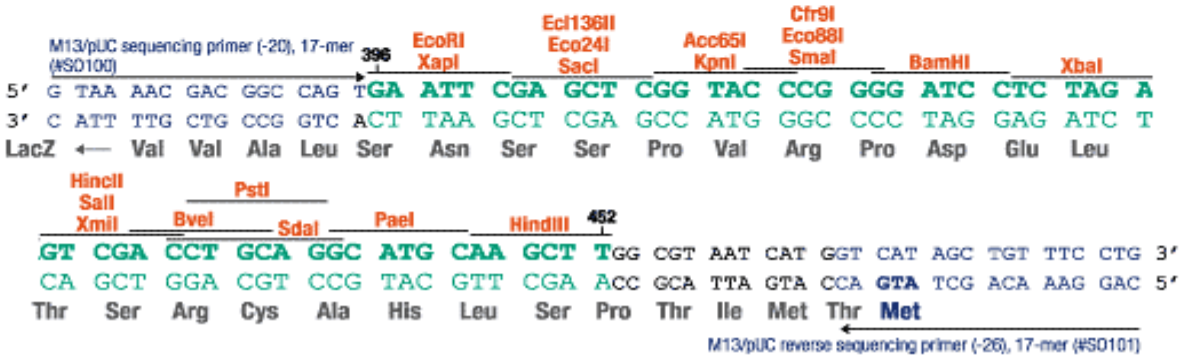


\Call 1217

Multiple cloning sites of pUC18



Multiple cloning sites of pUC19



CHAPTER 6

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