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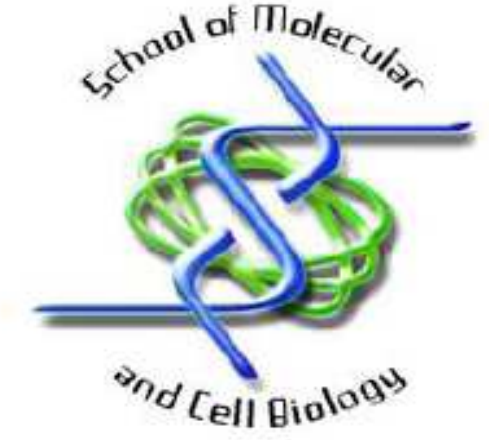
Ribosomal RNA mutations to rifampicin resistance

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RIBOSOMAL RNA MUTATIONS TO RIFAMPICIN RESISTANCE

RULANE GLENDA MACHEKE

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

October 2011

DECLARATION

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

Signature ……..

Signed at …….. on …….. day of …..... 20…….

ABSTRACT

In prokaryotes, transcription and translation are coupled and as a result, the beginning of the messenger RNA is translated by the ribosome while the 3' end is still synthesized. How exactly this occurs is still not clear. One possibility is that RNA polymerase and the ribosomes may be in physical contact with each other at some stage during gene expression or RNA polymerase has a binding site in the ribosomes. Mutational analysis is one method to explore how coordination between these two moieties occurs in bacteria.

An *Escherichia coli* strain with all seven chromosomal ribosomal RNA operons deleted, replaced by a single *rrnB* plasmid-borne operon, was used to isolate ribosomal RNA mutants with increased rifampicin resistance, two of which were studied further. The altered *rrnB* operon in pGM1 was obtained by spontaneous whilst in pGM2 by EMS mutagenesis. The mutated *rrnB* operon in pGM1 conferred resistance to 25µg/ml of rifampicin while in pGM2 resistance of 30µg/ml was observed. A base substitution of T to A at position 355 of the 23S rRNA was detected in pGM1and no nucleotide change was detected in pGM2. The successful isolation of ribosomal RNA mutants with rifampicin resistance is consistent with the hypothesis of interaction between the RNA polymerase and the ribosomes and suggests the part of this interaction is with the large ribosomal subunit.

DEDICATIONS

To my family at large but, most importantly to my husband, Alister Ngobeni, my son Ntwanano Ngobeni, and my mother Magdeline Macheke.

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I like to give my deepest gratitude to the following people:

The Lord Almighty for keeping me safe and giving the strength to go on when times were tough.

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TABLE OF CONTENTS

DECLARATION **I**

ABSTRACT **II**

DEDICATIONS **III**

ACKNOWLEDGEMENTS **IV**

TABLE OF CONTENTS **V**

TABLES **IX**

FIGURES **X**

ABBREVIATIONS **XII**

CHAPTER 1 **1-14**

INTRODUCTION

1.1. The central dogma 1

1.2. Coupled transcription-translation 2

1.3. RNA polymerase 3

1.3.1. Action of RNA polymerase 4

1.4. The ribosome 5

1.5. Antibiotics 6

1.6. Rifampicin 8

1.6.1. Mode of action 10

1.7. Resistance to rifampicin 11

1.7.1. Resistance by mutation of target 12

1.8. The Delta (Δ) 7system 12

1.9. Aim of the study 13

CHAPTER 2 15-29

MATERIALS AND METHODS

2.1. Bacterial strains and plasmids 15

2.2. Media and growth conditions 17

2.3. Determination of minimum inhibitory concentration (MIC) 17

2.4. Plate patching 17

2.5. Marker rescue 18

2.6. DNA preparations 18

2.6.1. *E. coli* bulk plasmid preparations 18

2.6.2. *E. coli* mini plasmid preparations 19

2.7. DNA manipulation and cloning techniques 20

2.7.1. DNA precipitation 20

2.7.1.1. Salt and ethanol precipitation 20

2.7.1.2. Isopropanol-mediated DNA precipitation 20

2.7.1.3. DNA precipitation from CsCl gradient 20

2.7.1.4. Phenol-chloroform extraction 21

2.7.2. Restriction enzyme digestion 21

2.7.3. Ligation of DNA 22

2.7.4. DNA extraction using a kit 22

2.7.5. The freeze-squeeze method 22

2.7.6. Determination of DNA concentration 22

2.7.7. Alkaline phosphatase treatment 23

2.8. Gel electrophoresis 23

2.8.1. Agarose gel electrophoresis 23

2.8.2. Low-gelling agarose gel electrophoresis 24

2.9. Transformations 24

2.9.1. *E. coli* $CaCl_2$ transformation 24

2.9.2. Electroporation 25

2.10. Mutagenesis 26

2.10.1. Ethyl methyl sulfonate (EMS) mutagenesis 26

2.10.2. PCR-mediated site-directed mutagenesis 26

2.11 Preparation of DNA for sequencing 28

CHAPTER 3 **30-52**

RESULTS

3.1. Generation of an *E. coli* strain with a single *rrnB* operon 30

3.2. Determination of sucrose concentrations lethal to *E. coli* SQZ10 31

3.3. Selection of rifampicin-resistant SQ170 mutants 32

3.3.1. Selection by spontaneous selection 32

3.3.2. Selection by EMS mutagenesis 33

3.4. Identification of plasmid-borne mutations by marker rescue 34

3.5. Phenotypic characteristics of rRNA mutants 37

3.5.1. Rifampicin phenotype 37

3.5.2. Effects of temperature on rifampicin resistance 38

3.5.3. Effects of pH on rifampicin resistance 39

3.5.4. Effects of phosphate buffered media on rifampicin resistance 40

3.5.5. Effects of plasmid-borne mutations on other antibiotics 41

3.6. Restriction fragment exchange 45

3.7. DNA sequence analysis of rRNA mutants 49

3.7.1. Nucleotide sequence analysis 49

3.8. Site-directed mutagenesis of pKK3535 50

CHAPTER 4 **53-58**

DISCUSSION

4.1 Isolation of rifampicin rRNA mutants 53

4.2 Mutational analysis of rRNA 54

4.2.1. Mutational analysis of pGM1 54

4.2.2. Mutational analysis of pGM2 55

4.3. Identification of r-proteins associated with RNA polymerase 55

4.4. Conclusions 57

4.5. Future work 57

CHAPTER 5 **59-65**

REFERENCES

CHAPTER 6 **66-76**

APPENDICES

Appendix A: Media 66

Appendix B: Solutions 67

Appendix C: Plasmids and molecular weight markers 73

TABLES

1.1: Composition of bacterial RNA polymerase 4

1.2: Selected antibiotics and their targets 7

2.1: Bacterial strains used in this study 15

2.2: Plasmids used in this study 15

2.3: Components of mutagenesis PCR 27

2.4: Components of control PCR 27

2.5: PCR conditions used to amplify the *rrnB* gene 28

2.6: Internet addresses used for sequence analysis and database access 29

3.1: Transformation of *E. coli* SQZ10 with pKK3535 using optimized $CaCl_2$-mediated transformation and electroporation techniques 30

3.2: Rifampicin resistant clones obtained from spontaneous selection 33

3.3: Rifampicin resistant clones obtained from EMS mutagenesis 34

3.4: Transformation of SQZ10 with plasmid DNA (derived from a pool of Rif-R clones generated by spontaneous mutagenesis) from a Fermentas GeneJet Miniprep Kit using optimized electroporation conditions 36

3.5: Transformation of SQZ10 with plasmid DNA (derived from a pool of Rif-R clones generated by EMS mutagenesis) from a Fermentas GeneJet Miniprep Kit using optimized electroporation conditions 37

3.6: Summary of the effects of mutations on tested antibiotics 45

3.7: Mutagenic primers used to construct 23S rRNA mutants 51

A6.1: Microbial agents 70

A6.2: Chemical suppliers 71

FIGURES

1.1: Central dogma of molecular biology 2

1.2: A prokaryotic cell showing an mRNA transcript being translated into protein by the ribosome 3

1.3: Mechanism of transcription by RNA polymerase 5

1.4: The chemical structure of rifampicin 9

1.5: Mechanism of RNA polymerase inhibition by rifampicin 11

3.1: Inhibition of *E. coli* SQZ10 growth by different concentrations of sucrose 32

3.2: Rifampicin MIC of rRNA mutants obtained from spontaneous selection and EMS mutagenesis in *E. coli* SQ170 38

3.3: Rifampicin MIC of rRNA mutants at varying temperatures of 18^{o}C, 25^{o}C, 37^{o}C, and 42^{o}C in *E. coli* SQ170 39

3.4: Rifampicin MIC of rRNA mutants at varying pH conditions ranging from pH 5.5, 7.0 and 8.5 in *E. coli* SQ170 40

3.5: Rifampicin MIC of rRNA mutants at varying pH conditions ranging from pH 5.5, 7.0 and 8.5 in buffered L(A-N) media 41

3.6: Streptomycin MIC of rRNA mutants in *E. coli* SQ170 42

3.7: Chloramphenicol MIC of rRNA mutants in *E. coli* SQ170 43

3.8: Tetracycline MIC of rRNA mutants in *E. coli* SQ170 43

3.9: Kasugamycin MIC of rRNA mutants in *E. coli* SQ170 44

3.10: Erythromycin MIC of rRNA mutants in *E. coli* SQ170 44

3.11: Purified *Bam*HI DNA fragments on 0.8% agarose. 46

3.12: Restriction fragment exchange between the wild-type and the mutant recombinant plasmids 47

3.13: Plasmid DNA of pKK3535 digested with *Bam*HI to release inserts 48

3.14: Nucleotide alignment of the known *rrnB* operon sequence (wt) compared to its mutated pGM1 counter-part using BLASTN search programme 50

3.15: Amplified *rrnB* gene 52

A6.1: Restriction map of pDA71 73

A6.2: Restriction map of pKK3535 74

A6.3: Restriction map of pGEM-T-Easy 75

A6.4: 1Kb DNA molecular weight marker 76

ABBREVIATIONS

Amp	ampicillin
Amp-R	ampicillin resistance
AP	alkaline phosphatase
bp	base pairs
°C	degrees Celsius
Cm	chloramphenicol
Cm-R	chloramphenicol resistance
Cm-S	chloramphenicol sensitivity
CsCl	cesium chloride
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylene diamine tetraacetic acid
EMS	ethyl methyl sulfonate
EtBr	ethidium bromide
f-Met	formyl-methionine
g	grams
hr	hours
H	hydrogen
Kan-R	kanamycin resistance
kb	kilobases
kV	kilovolt

LA	Luria Bertani agar
LB	Luria Bertani broth
µg	microgram
µl	microlitre
µM	micromolar
mA	milliAmper
mg	milligram
ml	milliliter
min	minutes
mM	millimole
M	molar
MIC	minimum inhibitory concentration
MW	molecular weight
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
Nal	nalidixic acid
ng	nanogram
N	nitrogen
OH	hydroxyl group
Ω	ohm
OD	optical density
O	oxygen
P-site	peptidyl tRNA-binding site
Rif-R	rifampicin resistant
RNA	ribonucleic acid
RNAP	DNA-dependent RNA polymerase
RNaseA	ribonuclease A
rpm	revolutions per minute
rRNA	ribosomal RNA

sdH_2O	sterile distilled water
SDS	sodium dodecyl sulphate
sec	second
TB	tuberculosis
Tet	tetracycline
Tet-R	tetracycline resistance
TBE	tris base, boric acid, EDTA
Tris	tris (hydroxymethyl)-aminomethane
tRNA	transfer RNA
Ts	temperature-sensitive
V	volt

CHAPTER 1

INTRODUCTION

1.1. Central dogma

In all living organisms, gene expression is the process by which information stored in nucleic acid is used in the synthesis of a functional gene product. These products are often proteins, but in non-coding genes such as ribosomal RNA genes or transfer RNA genes, the product is a functional RNA. The process of gene expression is used by eukaryotes, prokaryotes and viruses to generate macromolecular components, usually proteins, used to sustain life. The central dogma of molecular biology governs the storage and transfer of information in DNA, RNA, and proteins. It is the frame of understanding the flow of information between genetic elements in living organisms. Since its formulation by Francis Crick in 1958, (Burian *et al*., 1998) it has been used as a keystone in molecular biology.

The transfer of information described by the central dogma is sequential, wherein one sequence of one biopolymer is used as a template for the construction of another biopolymer. Firstly, DNA is replicated by a complex group of proteins that unwinds the double-stranded helix and DNA polymerase to make more copies of the DNA. The replicated DNA is then used as a template to synthesize messenger RNA (mRNA) by RNA polymerase during a process called transcription. Transcription is followed by translation of the mRNA transcript into proteins by the ribosomes (**Figure 1.1**).

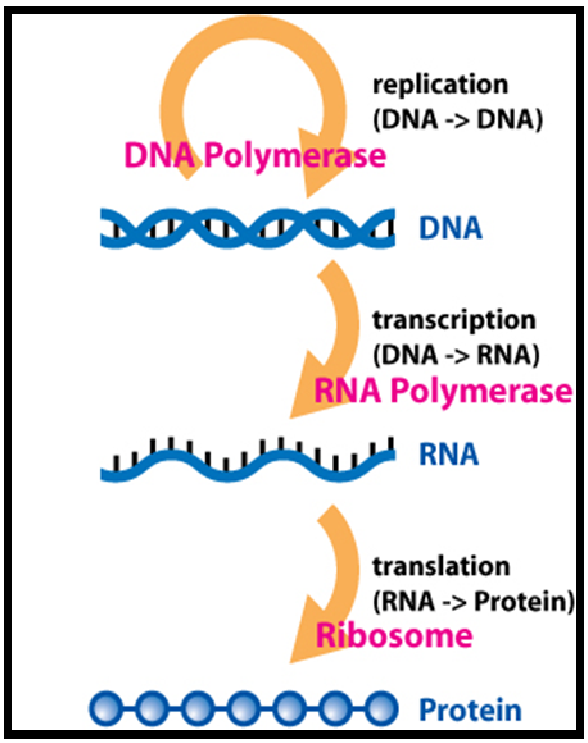


Figure 1.1: Central dogma of molecular biology and the flow of information indicating that genes are expressed by being transcribed from DNA to RNA with subsequent translation of the RNA transcript into a functional protein (Adapted from Crick, 1958).

1.2. Coupled transcription-translation

Transcription is the first major step of the expression of genes. In transcription, RNA polymerase synthesise an mRNA transcript from the coding strand of DNA. The second major step is translation, the process of making a polypeptide chain from an mRNA transcript. In eukaryotic cells, the nucleus and cytoplasm are separated by a nuclear membrane which means that after the mRNA is transcribed it has to be transported from the nucleus to the cytoplasm where it can be bound by ribosomes for translation.

In contrast to eukaryotic cells, prokaryotic cells do not have a nuclear membrane separating the two cellular compartments. As a result, gene expression is the result of a coupled process between transcription by RNA polymerase and translation by the ribosome and its cofactors. RNA polymerase reads the three nucleotides for a codon in the DNA to produce mRNA at a similar speed as the translating ribosome decodes these three bases for extension of the

growing polypeptide by one amino acid. This means that the translation machinery that reads the mRNA follows the RNA polymerase as it extends the mRNA chain (Martin and Koonin, 2006). Therefore prokaryotic transcription is coupled to translation (**Figure 1.2**).

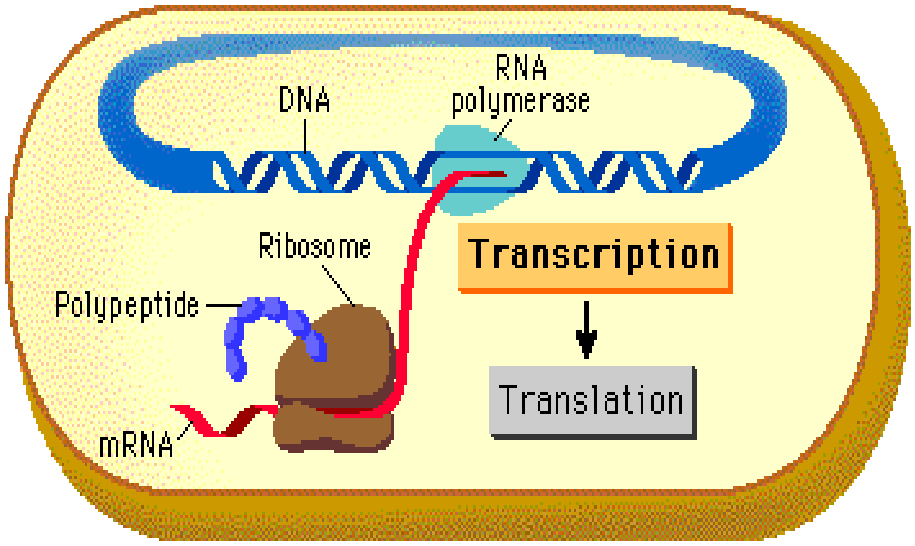


Figure 1.2: A prokaryotic cell showing an mRNA transcript being translated into protein by the ribosome (Adapted from French *et al*., 2007).

How this occurs, if and how coordination happens, is not clear. In certain cases, depending on an efficient translation initiation frequency, the translation machinery can be close enough to the transcription machinery so one can anticipate transient physical interaction between the two machineries.

1.3. RNA polymerase

The RNA polymerase of *Escherichia coli* is a multisubunit enzyme that exists in two forms, namely the core and holoenzyme. The core enzyme has four polypeptide subunits: alpha (α), beta (β), beta′ (β′) in the stoichiometry $\alpha_2\beta\beta'$ (Zhang *et al*., 1999). RNA polymerase holoenzyme has the same subunits as the core enzyme with an additional sigma (σ) subunit. The two α subunits assemble the enzyme and bind regulatory factors. Each subunit has two domains, namely α CTD (C-Terminal domain) which binds upstream of the extended

promoter and, α NTD (N-Terminal domain) which binds to the rest of the RNA polymerase. The β-subunit has the polymerase activity and is mainly involved in the synthesis of RNA. β′ binds non-specifically to DNA while ω restores disassociated RNA polymerase to its functional form and it has been reported to offer chaperone function in *Mycobacterium smegmatis* (Mukherjee and Chatterji, 2008). The σ-subunit helps to reduce the affinity of the core enzyme for non-specific DNA and greatly increases the affinity for the promoters during the initiation of transcription (Murakami *et al*., 2002).

Table 1.1: Composition of bacterial RNA polymerase

Subunit	**Molecular weight (kDa)**	**Stoichiometry**	**Genes**
α	40	2	*rpoA*
β	145	1	*rpoB*
β'	160	1	*rpoC*
σ	85	1	*rpoD*
ω	10	1	*rpoZ*

1.3.1. Action of RNA polymerase

RNA polymerase catalyzes the synthesis of mRNA during transcription. Prokaryotic transcription is divided into three stages; initiation, elongation and termination. During initiation RNA polymerase core enzyme, associated with initiation factor (σ), forms an initial closed promoter complex by recognizing two hexamers of consensus DNA sequences: the Pribnow box (-10 element), centered at about -10 with respect to the transcription start site (+1), and the -35 element (Murakami, 2002). After binding to the DNA, transcription factors

unwind the DNA strand and allow RNA polymerase to transcribe only the antisense strand of DNA into mRNA. Transcription elongation involves the further addition of ribonucleotides as RNA polymerase moves along the DNA until it reaches a terminator sequence. At that point, RNA polymerase releases the mRNA polymer and detaches from the DNA.

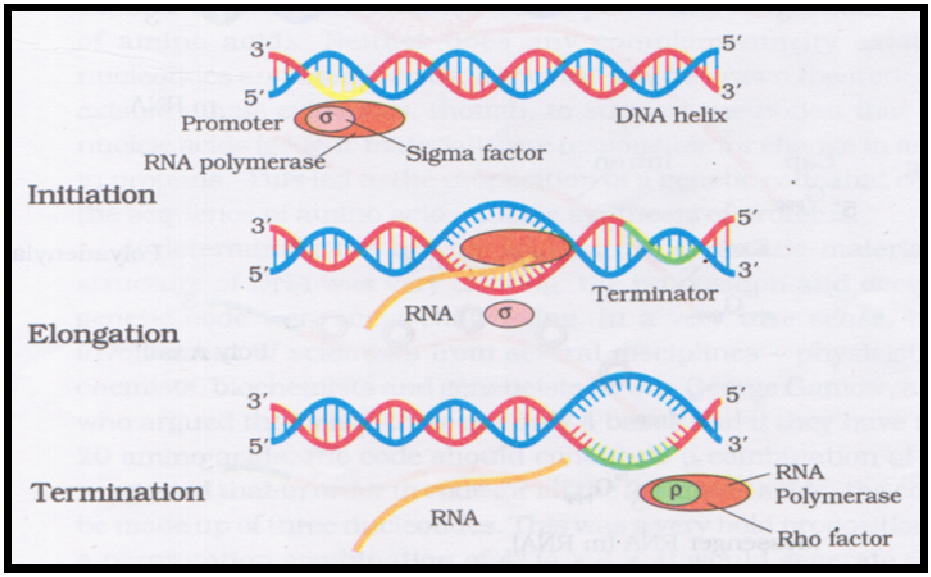


Figure 1.3: Mechanism of transcription by RNA polymerase. The anti-sense strand to be transcribed is depicted in blue. The incoming RNA polymerase holoenzyme (orange) binds to the promoter depicted in yellow. The terminator sequence is depicted in green (Adapted from Orphanides and Reinberg, 2002).

1.4. The ribosome

The ribosome is a macromolecular complex of – in prokaryotes – three ribonucleic acid molecules (RNAs) and over fifty ribosomal proteins on which the second step of gene expression, translation, takes place (Elliott and Elliott, 2002). Ribosomes have been described as ribozymes since the rRNA seems to play an important role in the peptidyltransferase activity that links together amino acids during translation. In particular, the coupling of amino acids to form peptides (peptidyl-transferase reaction) is catalyzed by domain V of the 23S rRNA, known as the peptidyltransferase centre (Douthwaite, 1992). This structure has been highly conserved through evolution due to its role in translation.

The prokaryotic ribosome (70S) is made of two subunits referred to as the large (50S) and small (30S) subunits. In *Escherichia coli* the large subunit is composed of about 34 proteins and two rRNA components – the 5S and 23S of 120 and 2900 nucleotides in length respectively. The small subunit is composed of 21 proteins and 16S rRNA of 1540 nucleotides in length (Yusupov *et al.*, 2001). The rRNA component of the prokaryotic ribosome will be the main focus of this study. Hence, a more detailed discussion follows on the specifics of the rRNA components and their roles in translation.

1.5. Antibiotics

Antibiotics are chemical substances that kill, or inhibit the growth of other microorganisms. They are derived from *Streptomycetes,* which are predominantly found in soli and on decaying vegetation.These substances are secondary metabolites since their production is not required for the growth of the microorganism producing them. Large scale production is carried out using fermentation in an aerobic environment. Antibiotics are mainly classified as bactericidal; those that kills bacteria or bacteriostatic; those that inhibits the growth of bacteria (Blacheford and Cegange, 2006).

The first antibiotic to be isolated was penicillin from *Penicillium notatum* by Alexander Fleming in 1929 (Diggins, 2003; Fleming, 1980). Penicillin was produced on a large scale using a culture of *Penicillium notatum* during the 1940s. The high demand for chemotherapeutic agents to treat wound infections during World War II led to the development of a production process for penicillin and this marked the beginning of an era of antibiotic research and continues to be a most fascinating area of microbial biotechnology even today.

Antibiotics differ chemically and in the way they inhibit microorganisms. Certain antibiotics destroy bacteria by affecting the structure of their cell walls and cell membranes. Another way is by interfering with the organism′s metabolism. A large number of antibiotics (**Table**

1.1); such as macrolides, tetracyclines, and aminoglycosides are known to target the ribosome. This may be useful in the study of interaction between RNA polymerase and the ribosome.

Table 1.2: Selected antibiotics and their targets

Antimicrobial agent	**Target**
Rifampicin	β subunit of RNA polymerase
Chloramphenicol	Peptidyltransferase activity, 23S rRNA
Erythromycin	Exit tunnel, 50S subunit
Kasugamycin	P-site bound fMet from 30S subunit
Streptomycin	Formyl-tRNA of the 30S rRNA
Tetracycline	Docking of the amino-acylated tRNA in the 16S rRNA

(Vannuffel, and Cocito, 1996; Recht *et al*., 1999; Lambert, 2005).

rRNA is the target of several antibiotics such as chloramphenicol (Mankin and Garrett, 1991), erythromycin (Lucier et al., 1995; Gregory *et al*., 2001), kasugamycin (Vila-Sanjurjo, 1999), spectinomycin (O′Connor and Dahlberg, 2002; Criswell *et al*., 2006), streptomycin (Honore *et al*., 1996), and thiostreptone (Thompson and Cundliffe, 1996). In most cases, a single mutation in the rRNA may confer resistance to a particular antibiotic relating to the fact that they interact with the ribosome.

In early functional studies of ribosomal mutations, much focus was on ribosomal proteins rather than the rRNA components. This was due to several reasons and amongst that was

because it was assumed that enzymatic activities had to be a function of proteins and mutants resistant to ribosomally-targeted antibiotics had alterations in the ribosomal proteins. Secondly, ribosomal proteins are relatively stable, and amenable to purification as compared to their rRNA counterparts. Lastly, isolation of rRNA mutants has been difficult due to the presence of multiple *rrn* operons in most experimental organisms. This probably reflects the need for high levels of protein synthesis in rapidly growing bacteria. For example, the *E. coli* (Asai *et al*., 1999) and *Bacillus subtilis* (Javis *et al*., 2000) genomes contain seven and ten *rrn* operons respectively.

Resistance of many antibiotics, including streptomycin, spectinomycin and erythromycin, is "recessive" or weakly co-dominant to sensitivity when the mutation conferring resistance alters ribosomal proteins (Gregory and Dahlberg, 2009). Dominance of sensitivity is best documented for streptomycin-resistant mutations, in which streptomycin-resistant/sensitive merodiploids contain ribosome of both normal and altered types and are still nearly as sensitive to the bactericidal and bacteristatic activities of streptomycin as are streptomycin-sensitive cells (Springer *et al*., 2001). Therefore, a mutation in an rRNA gene also might go undetected due to dominance of sensitive ribosomes that contain rRNA synthesized from non-mutant *rrn* operons in the same cell. This was observed in streptomycin, spectinomycin and erythromycin in early ribosomal mutation studies.

1.6. Rifampicin

Rifampicin ($C_{43}H_{58}N_4O_{12}$) has a molecular weight of 823 $g.mol^{-1}$ (Tupin *et al*., 2010). This compound is an odorless red-brown crystalline powder which readily dissolves in methanol. The lipophilic characteristics of rifampicin contribute considerably to its ability to cross the cell wall barrier by passive diffusion. It is a broad-spectrum bacteriostatic drug that is predominantly used as the first-line antibiotics in the treatment of tuberculosis (TB), leprosy and a growing number of Gram-positive bacteria such as multidrug-resistant *Staphylococcus aureus*. Much of the membrane of this acid-fast Gram positive bacteria is mycolic acid complexed with peptidoglycan, which allows easy movement of the drug into the cell.

Rifamycins were discovered in 1959 by Sensi *et al*., produced by a strain of *Amycolaptosis mediterran*ei (previously known as *Streptomyces mediterranei* and *Nocardia mediterranei*) and were introduced in therapy in 1962. Rifamycins are active against a large variety of bacteria and some eukaryotes (Gardner *et al*., 1991). They have a broad activity against Gram-positive bacteria and, to a lesser extent, against Gram-negative bacteria (Pukrittayakamee *et al*., 1994). Ansamycins are structurally characterised by their planar naphthoquinone ring in which positions 3 and 4 have been extensively modified by hemisynthesis to yield commercial antibiotics such as rifampicin (**Figure 1.4**).

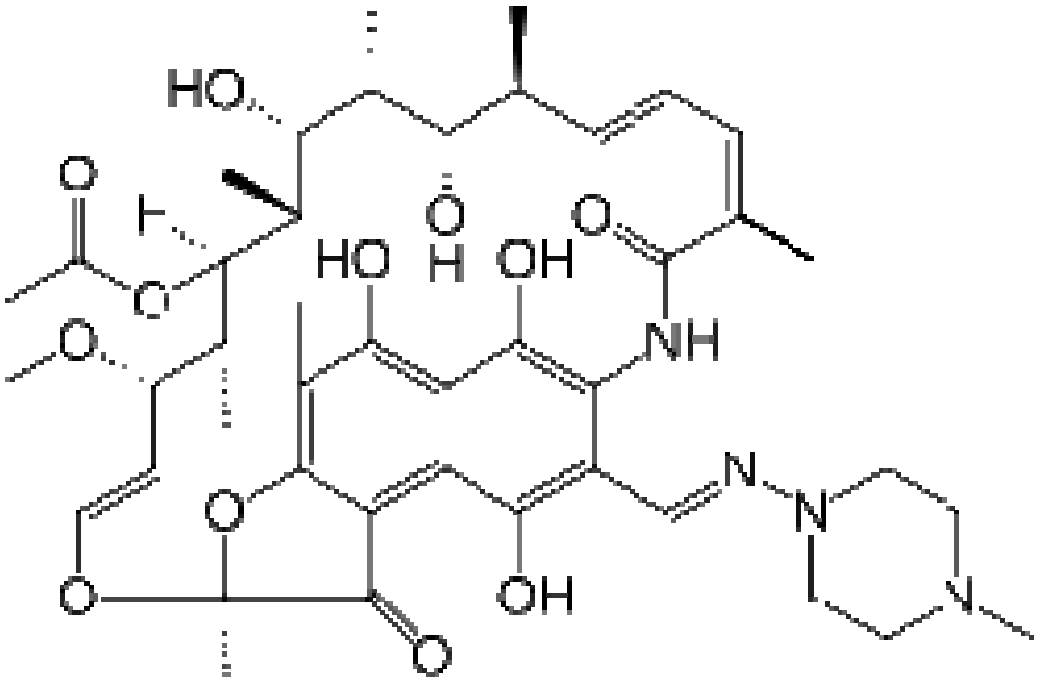


Figure 1.4: The chemical structure of rifampicin (www.bikudo.com/rifampicin).

The most important functional groups of rifampicin are the two free hydroxyl (OH) groups at positions 21 and 23 of the ansa ring , the two oxygen atoms (O_1 and O_2) at positions C_1 and C_8 and the unsubstituted hydroxyl groups (O_{10} and O_9) at positions C_{21} and C_{23} of the naphthoquinone ring (**Figure 1.4**). These groups are considered to be essential for antimicrobial activity as they are involved in the formation of a tight but reversible linkage between rifampicin and RNA polymerase. Modification of the ansa bridge or any modification that alter the conformation of the ansa bridge reduces antimicrobial activity of

this compound. Any modification involving a substitution, change in molecular size of which is not important or elimination of the hydroxyl groups results in an inactive compound (Wehri and Staehelin, 1991).

1.6.1. Mode of action

The antimicrobial activity of rifampicin is due to it inhibiting DNA-dependent RNA polymerase by binding its β-subunit. This drug blocks the pathway of the elongating RNA transcript when it is 2 or 3 nucleotides long. The initiating nucleotide binds the RNAP i-site (i = initiation site at -1 position), while the second nucleotide binds at the i+1 site. After a phosphodiester bond has been formed between the two nucleotides, RNAP is translocated. Consequently, the i+1 nucleotide occupy the i-site at the -1 position and the i-site nucleotide moves into the -2 position (Campbell *et al*., 2001).

The physical association of rifampicin to the β subunit of RNAP results in a severe steric clash with the 5′ triphosphate of the initiating nucleotide at the -2 position. Owing to the conformational change in the enzyme, the synthesis of the second phosphodiester bond is inhibited (Wehrli and Staehelin, 1991). Consequently, RNAP remains at the same template position, the 2 nucleotide transcript is released and this futile cycle resumes. The mechanism of RNAP inhibition by rifampicin is shown in **Figure 1.5**. If the third phosphodiester bond of the RNA transcript has been formed the drug is no longer able to exert its effect (Campbell *et al*., 2001). A possible explanation may be that the complex comprising the DNA template, RNAP and the RNA transcript, has undergone a conformational change, eliminating binding sites for rifampicin.

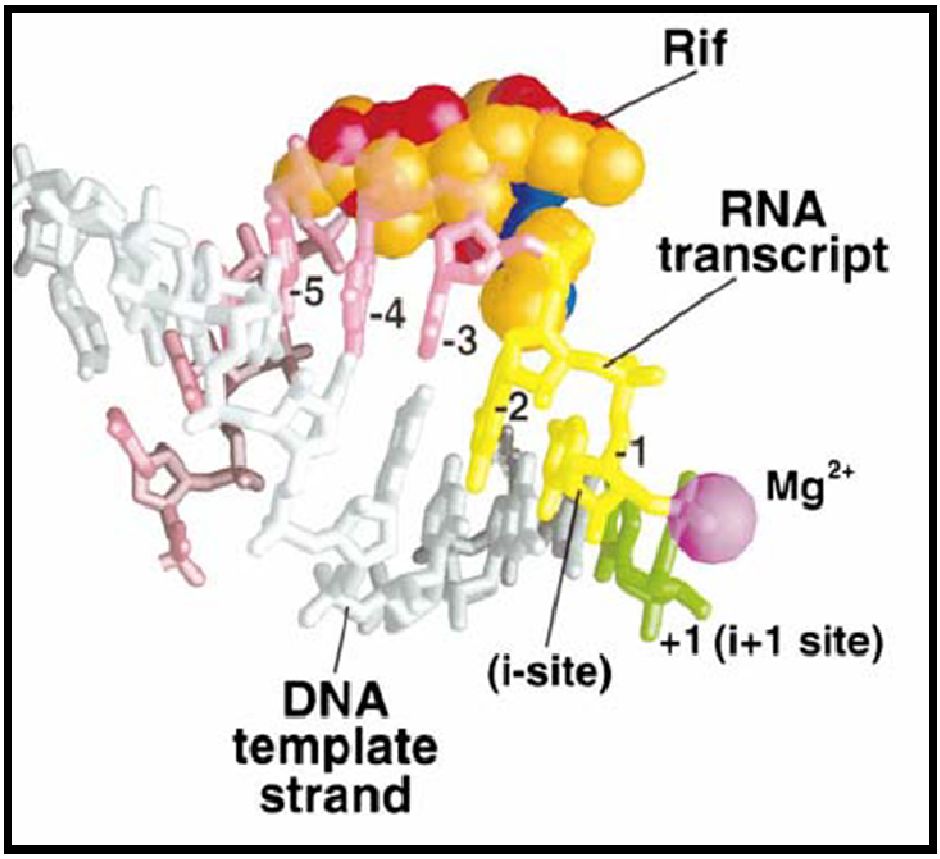


Figure 1.5: Mechanism of RNA polymerase inhibition by rifampicin (Adapted from Campbell *et al.*, 2001). Mg2+ is represented by the **magenta** sphere. The incoming nucleotide substrate at the -1 position is shown in **green** and the -1 and -2 positions that can be accommodated in the presence of rifampicin are coloured in **yellow**. The RNA further upstream (-3 to -8) is coloured **pink.** The DNA template strand is **grey**. Rifampicin is shown positioned in its binding site on the β subunit. The carbon 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.

1.7. Resistance to rifampicin

Despite the highly efficient mechanism of inhibiting bacteria, rifampicin is by no means the perfect antibiotic. The biggest challenge arises from the fact that bacteria can acquire high level resistance to this drug through a variety of mutations. This is surprising, since the reason rifampicin works so well is that it is a rigid molecule, and sits tightly in the pocket where it binds, allowing the bonds to be very strong. However, this also means that if an

amino acid on the edge of the channel with a small side chain is replaced with an amino acid with a large side chain, rifampicin may not be able to bind, simply because it cannot fit in the space. Resistance to rifampicin by mutation of the target is widespread but not unique. Other mechanisms of resistance have been reported, such as duplication of the target, action of RNA polymerase-binding proteins, modification of rifampicin and modification of cell permeability (Tupin *et al*., 2010).

1.7.1. Resistance by mutation of target

Rifampicin interacts with the β-subunit of RNA polymerase encoded by *rpoB* gene. Binding of the molecule to RNA polymerase involves 12 amino acids residues and mutagenesis of at least one of the residues results in a resistant phenotype (Campbell *et al*., 2001). In *E. coli*, rifampicin resistance mutations are usually located in the central region of the polypeptide, within cluster I (amino acids 507-533), cluster II (amino acids 563-572) and cluster III (Floss and Yu, 2005), and they can also occur near the N-terminus of the β-subunit (Severinov *et al*., 1994).

1.8. The Δ7 system

Isolation of rRNA mutants was difficult because the rRNA genes are present as multiple copies in nearly all bacteria. This has become an obstacle to many studies of rRNA because the mutation in one rRNA gene would be masked by the rRNA originating from the other unaltered genes. For example, the *E. coli* (Asai *et al*., 1999) and *Bacillus subtilis* (Javis *et al*., 2000) genomes contain seven and ten rRNA operons respectively. This is contrast to different *Mycobacterium* strains; *Mycobacterium smegmatis* has two rRNA operons while *Mycobacterium leprae* and *Mycobacterium tuberculosis* both have a single rRNA operon (Kempsell *et al*., 1992; Gonzales-y-Merchand, 1996).

Several hypotheses have been proposed to explain the wide variation observed in rRNA copy number. It is generally assumed that the multiple copies of rRNA operons in prokaryotic organisms are required to achieve high growth rates. However, the marginal impact of rRNA

operons inactivation on the maximal growth rate (Condon *et al*., 1997) suggests that the capacity for rapid growth is not the sole determination of rRNA operon copy number. The number of transcripts that can be initiated at an rRNA operon promoter and the transcriptional rate of RNA polymerase set a maximum rate on the number of ribosomes that can be produced from a single rRNA operon. Given the high demand for rRNA transcription and the central role of rRNAs in the regulation of ribosome synthesis, it is conceivable that the number of rRNA operons may dictate the rapidity with which microbes can synthesize ribosomes and respond to favourable changes in growth conditions (Stevenson and Schmidt, 2001).

Despite the obstacle to rRNA studies due to multiple *rrn* operons in experimental organism; this can be overcome by the construction of an *E. coli* strain in which all seven chromosomal rRNA operons are inactivated by deletions spanning the 16S and 23S coding regions. A single *E. coli* rRNA operon carried by a multicopy plasmid supplies 16S and 23S rRNA to the cell. The Δ7 system has been used to successfully isolate rRNA mutants conferring resistance to antibiotics such as streptomycin (Finken *et al*., 2005), chloramphenicol (Kehrenberg *et al*., 2005), and tetracycline (Trieber and Taylor, 2002; Connell *et al*., 2003).

1.9. Aim of the study

The aim of this study was to investigate one possible explanation for coupled transcription-translation coordination that occurs in prokaryotes. Since rifampicin is known to target RNA polymerase and rRNA is a central component of the ribosomal translation process, the focus of my work was to attempt to isolate rRNA mutants conferring resistance to rifampicin. Ability to isolate rRNA mutants on the ribosome that conferred resistance to rifampicin would suggest that there is indeed an intimate transient contact between the two moieties. This hypothesis of a possible intimate transient interaction between these two moieties is supported by the sigma operon observed in *E. coli* K12. This operon encodes DNA primase, the σ-subunit of RNA polymerase and 30S r-protein S21, which are essential for replication, transcription and translation respectively. Another example is the interaction of 50S r-protein L2 with the α-subunit of RNA polymerase (Rippa *et al*., 2010). These studies support the

hypothesis that there is indeed interaction of individual ribosomal proteins with RNA polymerase.

Isolation of rRNA mutants was done by using an *E. coli* strain in which all seven chromosomal rRNA are deleted, and only one rRNA operon borne on a plasmid is present. Mutants were generated and screened using spontaneous or chemical mutagenesis and marker rescue to identify rRNA mutants.

Specific experimental objectives:

1. To obtain rRNA mutants with altered resistance to rifampicin

2. To optimise the phenotypic expression of the mutant(s)

3. Study the effects of mutations on other selected antibiotics

4. Determine the nature and position of mutations by DNA sequencing

5. Perform site-directed mutagenesis to confirm that the mutations detected were responsible for the change in phenotype

CHAPTER 2

MATERIALS AND METHODS

2.1: Bacterial strains and plasmids used in this study

Table 2.1: Bacterial strains used in this study

Strains	Characteristics	Source
Escherichia coli		
MM294-4	*endA1*, *hsdR17*, *gyrA*	E. Dabbs
SQZ10	Δ 7 rRNA, pHK-*rrnC*$^{+}$ *SacB*-KanR, ptRNA67-SpcR	S. Quan
SQ170	Δ7 rRNA, pKK3535, ptRNA67-SpcR	S. Quan

Table 2.2: Plasmids used in this study

Plasmids	Characteristics	Source/reference
pDA71*	*E. coli-Rhodococcus* shuttle vector with the *Eco*RI suicide gene inactivated	E. Dabbs
pKK3535	pBR322 derived vector carrying an *rrnB* operon and Amp-R marker	Brosius *et al*., 1981
pGM1	Δ7 *rRNA*, pKK3535, ptRNA67, rif-R	This work
pGM2	Δ7 *rRNA*, pKK3535, ptRNA67, rif-R	This work

pGEM-T-Easy	*E. coli* cloning vector with lacZ′ gene, Amp-R, single 3′ thymidine overhangs, SP6 and T7 promoters	Promega
pKGEM	*Eco*RI 2051bp fragment from pKK3535 ligated into pGEM-T-Easy	This work
pGM1-pKK7	pGM1 4293bp *Bam*HI fragment ligated to the 7503bp *Bam*HI fragment of pKK3535	This work
pGM2-pKK7	pGM2 4293bp *Bam*HI fragment ligated to the 7503bp *Bam*HI fragment of pKK3535	This work

pGM1-pKK4	pGM17503bp *Bam*HI fragment ligated to the 4293bp *Bam*HI fragment of pKK3535	This work
pGM2-pKK4	pGM2 7503bp *Bam*HI fragment ligated to the 4293bp *Bam*HI fragment of pKK3535	This work

2.2. Media and growth conditions

Luria-Bertani (LB) (Appendix) media was used for growing *E. coli* strains. Liquid cultures were obtained by inoculating a single bacterial colony into 5ml LB medium and incubated at 37°C overnight. For short-term storage, *E. coli* strains were kept on LB-agar plates (Appendix) at 4^{o}C and for long-term storage the strains were grown in 33% glycerol and kept at -70^{o}C.

2.3. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of antibiotics was determined on LB-agar plates for *E. coli* by agar dilution method. Freshly grown single colonies were inoculated into 200µl of sterile distilled water per well of the replicator plate. This was then transferred to the antibiotic plates with a multipoint inoculator. Antibiotic-free plates were used as positive controls. The spot test results were analyzed after incubation at 37°C for 48- 72 hr.

2.4. Plate patching technique

This technique was employed to detect particular phenotypes of *E. coli* under varying conditions of antibiotic concentration. Individual colonies of *E. coli* were picked up using sterile toothpicks and streaked on LA and LA plates supplemented with an antibiotic. The streaking was done in a manner such that each individual colony could be identified on different LA plates. To achieve this, a numbered pattern was placed under each LA plate, and a streak was made on a particular corresponding number. The LA plates were then incubated for various duration of time (1-2 days) at 37°C. The phenotype was assessed based on the ability of the streaked cells to form confluent growth.

2.5. Marker rescue

The marker rescue technique was used to confirm the mutations observed are plasmid-borne. A pool of resistant clones was grown in LB at 37^{o}C overnight. Plasmid DNA was extracted and transformed into *E. coli* SQZ10. Transformants were selected on LA plates supplemented with 200µg/ml ampicillin and transferred to LA plates supplemented with 200µg/ml ampicillin and 6% sucrose (Appendix) after 24 hr. The presence of sucrose in the selection media makes the growth condition to be more favourable to the incoming plasmid, and thus displaces the resident plasmid.

2.6. DNA preparations

2.6.1. *E. coli* bulk plasmid preparations

A single colony was used to inoculate 100 ml of LB with appropriate selective antibiotic for the maintenance of the plasmid. The culture was grown overnight with gentle agitation at 37°C. Cells were harvested by spinning in a JA-10 rotor (Beckman) at 10 000 revolutions per minutes (rpm) for 10 minutes (min) and then resuspended in 5 ml of solution I (Appendix). A 10 ml of solution II (Appendix) was then added to the cell suspension and mixed gently by inversion. The mixture was left to stand at room temperature for 15 min. Then 7.5 ml of solution III (Appendix) was added and shaken vigorously and then left on ice for 15 min.
The cell debris were removed by spinning in a pre-chilled (4°C) JA-20 rotor at 15 000 rpm for 10 min. The supernatant was transferred to a sterile JA-20 centrifuge tube and the DNA was precipitated with 12ml of isopropanol. The precipitation process was allowed to continue at room temperature for 15minutes. This was followed by centrifugation at 15 000 rpm for 15 min at room temperature. The supernatant was decanted off and the DNA pellet washed with 2ml of ethanol. The ethanol was gently poured off and the DNA pellet was vacuum-dried for 20 min. The DNA was re-suspended in 4ml TE buffer (Appendix) for 2 hours with gentle agitation. Thereafter, 4.1 g of cesium chloride (CsCl) was added and dissolved by mixing gently, followed by adding 600µl of 1% ethidium bromide (EtBr) (Appendix). The refractive index was adjusted to be between 1.387 and 1.389 (0.001 units =100mg CsCl if the index was

below or 0.001 units= 100µl TE if the index was above). The mixture was loaded into a Beckman Quick-seal tube using a Pasteur pipette. The tube was sealed, balanced and ultra-centrifuged overnight at 45 000 rpm in a Beckman vertical VTi 65.2 rotor. The plasmid DNA was extracted from the tube using a needle attached to a hypodermic syringe. DNA was purified as described in section **2.7.1.3**.

2.6.2. *E. coli* mini plasmid preparations

Individual bacterial colony was inoculated into 1ml of LB containing the appropriate selective antibiotic. This was incubated at 37°C with agitation overnight. The culture was transferred into sterile Eppendorf tube and the cells harvested by microfuging for 1 min. The supernatant was decanted off and the pellet resuspended in 80µl of solution I by vortexing briefly. Then 160µl of solution II was added to the cell suspension and mixed by gently inversion of the tube. The tube was left to stand at room temperature for 15 min. Thereafter, 120µl of solution III was added and the mixture shaken vigorously and kept on ice for 5 min. Cellular debris was removed by microfuging at 4°C for 10 min. The supernatant was collected into a new sterile Eppendorf tube and placed in a water bath (42°C) for 2 min to warm the supernatant. Isopropanol (220µl) was added and the precipitation process allowed continuing at room temperature for 5 min followed by centrifuging at room temperature for 5 min. The pellet was washed with 150µl ethanol and vacuum-dried for 20 min. The DNA was then resuspended in 100µl of sterile distilled water containing freshly boiled ribonuclease (RNaseA) (1µl of 10mg/ml). A small aliquot was analyzed for presence of plasmid DNA on 0.8% agarose gel.

2.7. DNA manipulations and cloning techniques

2.7.1. DNA precipitation

2.7.1.1. Salt and ethanol precipitation

DNA was precipitated from aqueous solution with 1/3 volume of 1M NaCl and 2 volumes of 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 10-20 min and resuspended in the appropriate volume of sterile distilled water. RNaseA (10mg/ml) was used to remove contaminating RNA.

2.7.1.2. Isopropanol-mediated DNA precipitation

After the addition of isopropanol (220µl), the solution was mixed by inversion and left to stand at room temperature for 5 min. The solution was then centrifuged at room temperature for further 5 min. The pellet was washed with 150µl of 96% ethanol and vacuum-dried for 20 min. The plasmid was resuspended in an appropriate volume of sterile distilled water containing RNaseA.

2.7.1.3. DNA precipitation from CsCl gradient

Ethidium bromide was removed from the DNA by thorough mixing with 0.1 volume of butanol. EtBr suspended in butanol forms a top layer in the tube, which is removed. This procedure was repeated at least 3 times until there were no traces of EtBr. This left the DNA in CsCl solution. The DNA was stored at -20°C until required. The salt was removed by adding 2 volumes of sterile distilled water and 2.5 volumes of 96% ethanol and precipitated

by centrifugation at 4°C for 20 min. The pellet was vacuum-dried and resuspended in appropriate volume of sterile distilled water.

2.7.1.4. Phenol-chloroform extraction

The DNA solution in water was extracted by addition of 1/3 volume TE-saturated phenol (Appendix), mixed by inversion and microfuged at room temperature for 5 min to separate the organic and aqueous phases. The upper aqueous layer was transferred into a new sterile Eppendorf tube and where necessary, as in the case of extracting DNA from agarose gels, a further phenol step was performed. Then 1/3 volumes of chloroform was then added to the aqueous layer and mixed gently by inversion. The organic and aqueous layers were separated by microfuging at room temperature for 2 min. The upper aqueous layer was transferred into a sterile Eppendorf tube and the DNA was precipitated by the addition of 1/3 volume of NaCl as described in **2.7.1.1.**

2.7.2. Restriction enzyme digestions

Enzymes were obtained from Fermentas and used according to manufacturer′s instructions. The total volume of digestion was 15µl (13.5µl DNA and 1.5µl 10× buffer). The mixture was tapped briefly to ensure even buffer distribution and spun down for 5 sec. 0.5-1µl of restriction endonuclease was added and the contents mixed and re-spun briefly. Digestions were incubated at the appropriate temperature for maximal enzyme activity for at least 4 hr. 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 require a lower incubation temperature.

2.7.3. Ligation of DNA

T4 DNA ligase (Fermentas) was used for all ligations procedures. The total volume for ligation was kept minimal at 20µl. Vector DNA and insert DNA were added at a ratio of 1:3 respectively. Ligation buffer and the appropriate volume of sterile distilled water were added to the DNA sample, mixed by tapping and microfuged for 5 sec. Subsequently, 1µl of ligase was added, remixed and re-spun. Ligation was performed in a water bath set at 22°C for 16-22 hr.

2.7.4. DNA extraction kit

Purification of DNA bands excised from digested plasmids loaded on low-gelling agarose was performed by using a NucleoSpin Extraction II kit (Macherey-Nagel) according to manufacturer′s instructions.

2.7.5. The freeze-squeeze method

DNA was digested with appropriate restriction endonuclease and fragments were separated on agarose gel. The fragment of interest was excised from the gel with a scalpel while viewing under long wavelength UV light (366nm). The gel slice was transferred into a sterile Eppendorf tube where it was crushed with a sterile spatula. The crushed slice of gel was kept at -70ºC for 30 min and it was thawed at room temperature. Following thawing it was microfuged for 6 min and the supernatant collected into a sterile tube. A second round of crushing, freezing, thawing and centrifuging was done. The DNA which was suspended in the collected supernatant was purified by phenol-chloroform extraction in **2.7.1.4**. The DNA was re-suspended in appropriate volume of sterile distilled water.

2.7.6. Determination of DNA concentration

In order to measure the concentration of DNA solution, an aliquot was first run on a gel, which was then quantified using UVP LabWorks Image Acquisition and Analysis Software

(Ver.4.5) by comparing band intensity between the DNA and the molecular weight marker, for which the concentration had been predetermined by the manufacturers.

2.7.7. Alkaline phosphatase treatment

Fast thermosensitive alkaline phosphatase (Fermentas) was used to prevent the vector from ligating to itself. The alkaline phosphatase removes the 5′- phosphates that are necessary for ligation by DNA ligase. Without these phosphates the vector cannot ligate on its own but can still ligate to the insert that retains its 5′-phosphates (Weaver, 1999). Following digestion of the vector, 1µl of alkaline phosphatase and 5µl 10x fast alkaline phosphatase buffer was added to the reaction mixture. The reaction mixture was incubated for 10 min at 37°C followed by heat-inactivation at 80°C for 20 min.

2.8. Gel electrophoresis

2.8.1. Agarose gel electrophoresis

Agarose solutions stock solutions were prepared in 0.5× TBE buffer (Appendix) at concentrations of 0.4%, 0.6% or 0.8% (Appendix) depending on a fragment size to be separated. The solutions were sterilized by autoclaving (121°C, 20 minutes). Fragment sizes ≥10kb were analyzed on 0.4% agarose gels and 2-10kb on 0.8% agarose gels. Gels were prepared by melting the agarose stock solution in a microwave oven. A volume of 25ml of the melted agarose was mixed with 2.5µl of a 1% EtBr solution. The mixture was poured in a gel tray with a 12-tooth well comb and allowed to polymerize at 4°C for 20 min. Combs were removed when the gel had completely set.

0.5× TBE electrophoresis buffer mixed with 1% EtBr solution was poured into an electrophoresis tank. DNA samples were loaded with 3µl of bromophenol blue tracking dye. GeneRuler ^{TM} DNA 1KB ladder Plus mix (Fermentas) was used in all electrophoresis runs using a Hoefer PS 500xdc power supply. The process was carried out at room temperature,

80V and a current of 21-28mA, until the dye front reached the bottom of the gel. DNA sizes were quantified from standard curve generated from migrations distances of known molecular weight marker sizes run on the same gel. The concentrations were estimated by comparing the intensity of the bands to bands of similar intensity and known concentration. The DNA was visualized and captured using the UVP BioDoc-It^{TM} system.

2.8.2. Low gelling agarose gel electrophoresis

Prior to usage, 20 ml of 0.6% low gelling agarose in 0.5× TBE buffer was autoclaved. EtBr was added to a final concentration of 1 mg/ml, and the agarose was poured into a tray inserted with a comb and polymerized at 4°C for 30 min. The gel was placed in a pre-cooled electrophoresis unit containing 0.5× TBE. DNA samples were mixed with bromophenol blue tracking dye and loaded into the wells; electrophoresis was conducted at 75 V at 4°C. Using a scalpel under a UV light, a band of interest was excised from the gel, from which the DNA was extracted by either phenol-chloroform extraction or freeze-squeeze method. In case of the phenol-chloroform extraction, the piece of agarose carrying the DNA was melted at 60°C for 30 min, followed by three phenol extractions. Between each extraction, 1/15 volume of 1 M Tris-HCl (Appendix) was added. One chloroform extraction was then conducted, followed by precipitation of the DNA.

2.9. Transformations

2.9.1. *E. coli* $CaCl_2$ transformation

A flask containing 20 ml of pre-warmed LB supplemented with 0.5% glucose (Appendix) was inoculated with 200µl of an overnight culture of *E. coli* SQZ10. The culture was incubated with vigorous shaking at 37ºC until the OD_{600} of 0.2-0.4 had been reached. That OD was generally obtained by incubating for a minimum of 1hour 45 min - 2 hours. The flask was chilled in an ice-water slurry for 5 min and the cells were harvested in a pre-chilled Beckman JA-20 rotor at 10 000 rpm for 5 min at 4ºC. The supernatant was discarded and the

cells re-suspended in 10 ml of ice cold $CaCl_2$ transformation buffer (Appendix). The cell suspension was placed on ice for 15 min and re-centrifuged at 10 000 rpm for 5 min. The supernatant was decanted and the cells re-suspended in 1.3 ml of transformation buffer. The cells were left on ice for 2-24 hours in the cold room (4ºC).

Aliquots 100µl of the cell suspension were placed into pre-chilled sterile Eppendorf tubes and about 5-10µl of plasmid DNA was added and mixed with the cells by bubbling air through. The DNA plus cell suspension was left on ice for 15 min to allow for diffusion. The cells were then heat-shocked at 42ºC for 90 seconds. A volume of 500µl of pre-warmed LB was added to the cells after heat shocking and incubated for 1hr at 37ºC to allow phenotypic expression of the resistance genes. The cells were then spread onto LB-agar containing an appropriate selective agent and further incubated for overnight at 37ºC. Colonies were visible after the overnight incubation for *E. coli* strains.

2.9.2. Electroporation

E. coli SQZ10 cells were prepared for electroporation by inoculating a fresh colony into 5ml of LB broth and incubated overnight at 37ºC. The overnight culture was then diluted 100× on LB broth and grown at 37ºC until the OD_{600} was <0.5. The cells were washed twice in electroporation buffer (Appendix) at 4ºC. An aliquot of 100µl of the *E. coli* cells was transferred into an Eppendorf tube and 5-10µl of DNA was added. This was mixed by bubbling air through and left to stand on ice for 10 min to allow diffusion. The mixture was then transferred into a pre-chilled electroporation cuvette and electroporated (25µF, 2.0kV and 200Ω). LB broth was added immediately to the electroporated cells and incubated for 1 hour at 37ºC for the expression of the antibiotic resistance markers. After incubation the cells were plated on LB agar plates supplemented with an appropriate selectable marker and incubated overnight at 37 ºC.

2.10. Mutagenesis

2.10.1. Ethyl methyl sulfonate (EMS) mutagenesis

A clone in *E. coli* SQ170 was grown overnight at 37ºC in 5 ml of LB to an optical density of between 0, 2 and 0, 4. A 100 fold dilution of the culture was then grown in 5 ml of LB in a 100ml flask to increase aeration and incubated at 37ºC for 4 hr. 1 ml of this culture was spun at room temperature at 15 000 rpm for 30 seconds. The cell pellet was then washed once using A-N buffer (Appendix) and the cells resupended in 500µl of the same buffer, and 500µl of a 2 % solution of EMS was added to the mixture. The culture was incubated at 37ºC for an hour. The cells were pelleted by microfuging for 30 sec at 13 000rpm and then washed using A-N buffer to stop the reaction. The cells were added to 5 ml of LB and incubated overnight at 37ºC. 100µl of the cells was spread on the appropriate plates and incubated overnight at 37ºC. The EMS was detoxified by adding to a strong solution of sodium hydroxide before discarding.

2.10.2. Polymerase chain reaction (PCR)-mediated site-directed mutagenesis

(Adapted from www.finnzymes.com)

A Phusion™ Site-Directed Mutagenesis Kit was used in this study. This is a PCR-base site-directed mutagenesis which uses a highly processive Phusion™ hot start high-fidelity DNA polymerase for exponential PCR amplication of dsDNA plasmid that is mutated. PCR was performed in an MJ MINI™ thermal cycler (Bio-Rad). The wild-type pKK3535 plasmid was used as a template. A control reaction utilised phosphorylated 5′ GTCGACTCTAGATCCCCGGGT 3′ and 5′ CTGCAGGACTGT**A**AGCTTGGCGTA 3′ as the forward and reverse primers respectively. PCR reactions were carried out in a total volume of 50µl as follows:

Table 2.3: Components of mutagenesis PCR

Component	Volume (µl)	Final concentration
5x Phusion buffer	10	1×
10mM dNTP mix	1.0	200µM each
Forward primer	1.0	0.5µM
Reverse primer	1.0	0.5µM
DNA template	1.0	2ng
Sterile water	35.5	-
Phusion DNA polymerase	0.5	0.02U/µl

Table 2.4: Components of control PCR

Components	Volume (µl)	Final concentration
5x Phusion buffer	10	1×
10mM dNTP mix	1	200µM each
Control primer mix	1	0.5µM each
Control plasmid	1	10pg
Sterile water	36.5	-
Phusion DNA polymerase	0.5	0.02U/µl

Finnzyme site-directed mutagenic PCR was used to introduce point mutations at specific positions of the *rrn*B operon of pKK3535. It exploits the high fidelity of Phusion hot start

DNA polymerase and the use of two phosphorylated primers. The, primers, one with the desired mutation, are designed so that they anneal back to back to the plasmid.

The PCR reaction was performed under the conditions listed in **Table 2.5.** Twenty amplication cycles were carried out. Following PCR, the product was subjected to agarose gel electrophoresis to verify the amplication of the fragment of interest. The PCR product was then circularized by ligation with Quick T4 DNA ligase at room temperature for 5 min and transformed into *E. coli*. PCR conditions for the control plasmid are the same as the experimental plasmid, with the annealing cycle omitted.

Table 2.5: PCR conditions used to amplify the *rrnB* gene

Cycle	Temperature (^{o}C)	Time (sec)	Number of cycles
Initial denaturation	98	30	1
Denaturation	98	10	
Annealing	67.32	30	25
Extension	72	3min, 36sec	
Final extension	72	10	1
	4	hold	∞

2.11. Preparation of DNA for sequencing

DNA was prepared using GeneJet Plasmid Extraction Kit (Fermentas) according to manufactures instructions. This plasmid purification protocol is base on a modified alkaline-lysis procedure, followed by the binding of plasmid DNA to a glass fibre matrix under the appropriate pH conditions. Plasmid DNA was eluted in a low ionic strength buffer. After

DNA had been prepared the concentration and purity of the DNA was analyzed on 0.8% agarose gel. Sequencing was performed by Inqaba Biotechnology Industries (Pty) Ltd.

Table 2.6: Internet addresses used for sequence analysis and database access

Program	**World wide web site**
Sequence analysis	
BLASTN	http://www.ncbi.nlm.nih.gov/BLAST/
FASTA	http://www.ebi.ac.uk
EMBOSS	http://www.ebi.ac.uk/Tools/emboss/align/
NEBcutter	http://tools.neb.com/NEBcutter2/
Databases	
GENEBANK	http://www.ncbi.nlm.nih.gov/Genbank/
EMBOSS Explorer	http://embossgui.sourceforge.net/
Reverse complement	http://www.bioinformatics.org/sms/rev_comp.html

CHAPTER 3

RESULTS

3.1. Generation of an *E. coli* strain with a single *rrnB* operon

E. coli SQZ10 is deficient of all seven chromosomal rRNA operons and carries plasmid pHK-*rrnC*$^+$, which contains both the *sacB* and kanamycin resistance genes. To generate rRNA mutants, an *E. coli* strain carrying a single copy of the *rrnB* operon is desirable. This strain was generated by replacing plasmid pHK-*rrnC*$^+$ in SQZ10 with pKK3535 (with the *rrnB* operon). This was done by transformation of SQZ10 with pKK3535 using both the $CaCl_2$-mediated and electroporation methods. Selection of transformants was done overnight on LA plates supplemented with 200µg/ml ampicillin. The results are indicated in **Table 3.1** below.

Table 3.1: Transformation of *E. coli* SQZ10 with pKK3535 using optimized $CaCl_2$-mediated transformation and electroporation techniques.

Sample	**Number of transformants (10^6)/µg DNA**	
	$CaCl_2$	**Electroporation**
No DNA	0	0
pDA71(vector control)	1.02	11.59
pKK3535	0.89	7.93

Two transformation techniques, $CaCl_2$ transformation and electroporation, were compared to determine the technique with a high transformation efficiency. A few transformants, in the case of the pKK3535 were anticipated when using the both the transformation techniques, because pKK3535 is a low-copy number plasmid as compared to pDA71. It was evident from **Table 3.1** that the transformation efficiency of $CaCl_2$ treated cells was at least ten times lower when compared to electroporation. Electroporation was chosen as the technique to be used in subsequent transformation experiments.

To ensure that replacement of pHK-*rrnC*$^+$ with pKK3535 was successful, five SQZ10 transformants were screened for kanamycin-sensitivity and ampicillin-resistance. This was done by streaking single colonies on LA-Kan (100µg/ml) and LA-Amp (100µg/ml). Growth was only observed on LA-Amp plates, and no growth was seen on LA-Kan plates (Data not shown). This observation indicated that pHK-*rrnC*$^+$ (confers kanamycin resistance) was replaced with pKK3535 (confers ampicillin resistance).This strain was designated *E. coli* SQ170 and used for subsequent selection of rifampicin-resistant mutants.

3.2. Determination of sucrose concentrations lethal to *E. coli* SQZ10

The *sacB* gene in *E. coli* SQZ10 codes for levansucrase, an extracellular enzyme that is responsible for the hydrolysis of sucrose and synthesis of levans, which are high molecular weight fructose polymers. Expression of *sacB* in the presence of sucrose is lethal to *E. coli* cells. An accumulation of levans which hinder the periplasm because of their high molecular weight or the transfer of fructose residues to inappropriate acceptor molecules promotes the toxic effects on the bacterial cells (Pelicic *et al*., 1996). It became obvious that prior to any mutagenesis and selection of rifampicin-resistant mutants, the concentration of sucrose sufficient to counter-select pHK-*rrnC*$^+$ had to be determined.

Literature indicates that when transforming mutated plasmid into the original host, a selection condition has to be in favour of the incoming plasmid in order to displace the resident

plasmid. This was done by streaking SQZ10 on LA-plates supplemented with increasing concentrations of sucrose (0%, 3%, 6%, 9% and 12%) and incubated overnight at 37^{o}C.

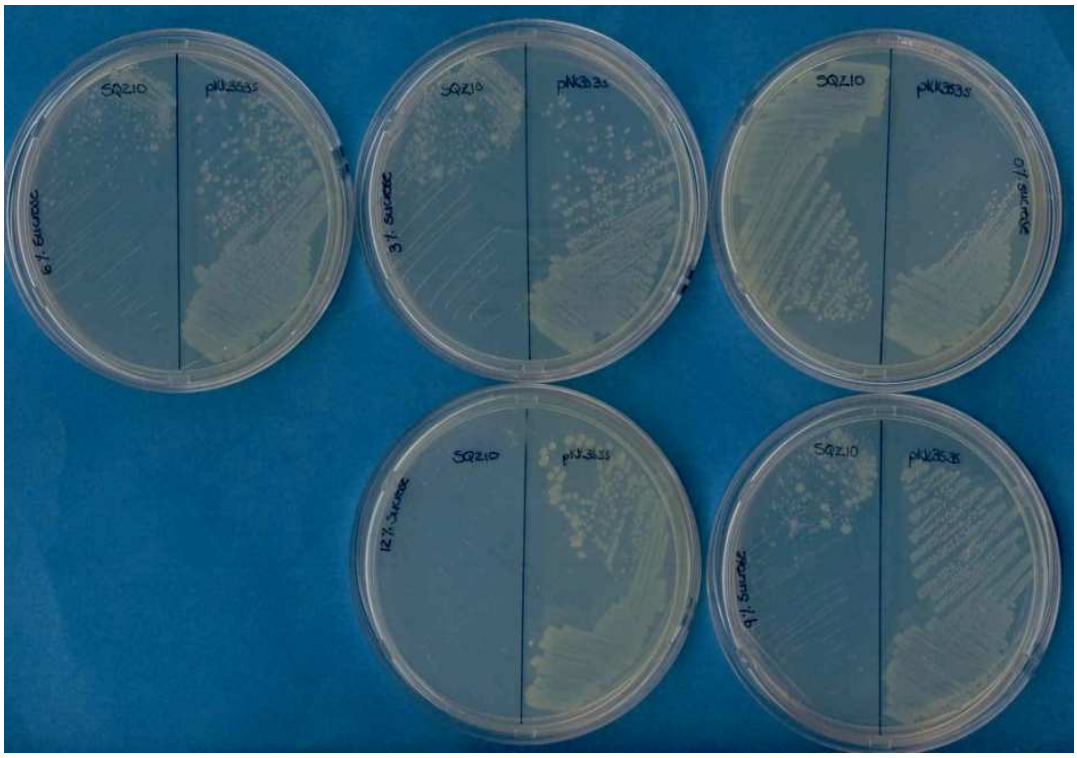


Figure 3.1: Inhibition of *E. coli* SQZ10 growth by different concentations of sucrose. pKK3535 was used as a positive control.

It was confirmed that the growth of *E. coli* SQZ10 was inhibited by the presence of sucrose in the growth media. Significant amount of growth was inhibited at 6% sucrose, and this was indicated by fewer colonies when compared with growth in a plate that was not supplemented with sucrose. 6% sucrose was chosen as the cut-off concentration to counter-select for the plasmid conferring resistance to kanamycin.

3.3. Selection of rifampicin-resistant SQ170 mutants

3.3.1. Selection by spontaneous mutagenesis

The first approach used to select SQ170 mutants with an increased MIC towards rifampicin was by spontaneous selection. Aliquots (100µl) of a culture of SQ170 were spread on broth plates supplemented with 25µg/ml, 50µg/ml, 75µg/ml or 100µg/ml rifampicin. The plates were incubated at 37^{o}C until growth of resistant colonies was observed. The results are indicated in **Table 3.2** below.

Table 3.2: Rifampicin resistant clones obtained from spontaneous selection at 48 hr.

Strain	**Rifampicin (µg/ml)**	**Number of rif-R clones**	
		24hr	48hr
SQ170	0	+++	+++
	25	275	279
	50	246	248
	75	185	185
	100	115	115

+++ indicates confluent growth.

3.3.2. Selection by EMS mutagenesis

SQ170 was subjected to EMS mutagenesis and after overnight growth plated on LA plates supplemented with 50, 100,150 and 200µg/ml of rifampicin. The number of resistant clones

obtained was 10-fold more when compared to those obtained by spontaneous selection and this is shown in **Table 3.3** below.

Table 3.3: Rifampicin resistant clones obtained from EMS mutagenesis at 48hr.

Strain	**Rifampicin (µg/ml)**	**Number of rif-R clones**	
		24hr	48hr
SQ170	0	+++	+++
	50	2200	2320
	100	1500	1500
	150	1100	1100
	200	789	800

+++ indicates confluent growth

The number of rifampicin resistant clones obtained by EMS mutagenesis was 10-fold more when compared to those obtained by spontaneous selection. This was expected, since EMS is a highly potent mutagen and has a mutagenesis rate that is considered to be higher than that of spontaneous mutagenesis.

3.4. Identification of plasmid-borne mutations by marker rescue

Using both spontaneous and EMS mutagenesis, rifampicin resistant mutants of SQ170 were generated. The purpose of the work was to obtain SQ170 mutants that carried rifampicin resistant mutations in the ribosomal RNA (i.e. plasmid-borne). To test if any of the rifampicin mutants carried such mutations; the marker rescue technique was employed. This pool of Rif-R clones was washed off plates and grown overnight at 37^{o}C in LB supplemented with 100µg/ml ampicillin. A mini plasmid preparation of this pool was made. The DNA obtained

was utilized to transform *E. coli* MM294-4 cells via the $CaCl_2$ method. A negative control of un-mutated SQ170 was included. Selection of transformed cells was done LA supplemented with Amp+6% sucrose only, followed by selection on LA-Amp/Rif plates.

Transformation of SQZ10 failed to produce significant number of colonies when using electroporation. Initially it was thought that poor yield of transformants in SQZ10 was due to low yield of plasmid DNA. This proved not to be the explanation since when the same batch of plasmid DNA was transformed in *E. coli* MM294-4, a sufficient amount of transformants (±400) were obtained. To investigate this problem, a number of variables were changed. These included:

1. Isolation of plasmid DNA using a Fermentas GenJet plasmid extraction kit to increase purity and quality of plasmid DNA

2. Comparison of transformation efficiency of *E. coli* MM294-4 and SQZ10. MM294-4 has 20-fold more transformation efficiency than SQZ10.

In spite of changing the above-mentioned parameters, the problem of low transformation efficiency still occurred. To settle the question of what caused a reduction in transformation efficiency even after optimization of electroporation conditions, transformation of SQZ10 with a pool of Rif-R clones was repeated and selection of transformants was done in a three-step process. Firstly, selection was done on LA-Amp only, followed by LA-Amp+6% sucrose and finally LA-Amp+Rif. Selection on LA-Amp only produced more than 4-times transformants as compared to when selection was done on Amp+sucrose. Results are recorded in **Tables 3.4** and **3.5** below. The 3-step selection method was employed for further experiments of marker rescue.

Table 3.4: Transformation of SQZ10 with plasmid DNA (derived from a pool of Rif-R clones generated by spontaneous selection) from a Fermentas GeneJet Miniprep Kit using optimized electroporation conditions.

SQZ10 transformed with DNA:	**No. of transformants on LA-supplement (µg/ml)**	
	Amp200	Amp100+6%sucrose
No DNA	0	0
SQ170	440	88
SQ170-25	550	73
SQ170-50	400	36
SQ170-75	160	42
SQ170-100	275	53

Table 3.5: Transformation of SQZ10 with plasmid DNA (derived from a pool of Rif-R clones generated by EMS mutagenesis) from a Fermentas GeneJet Miniprep Kit using optimized electroporation conditions.

SQZ10 transformed with DNA:	**No. of transformants on LA-supplement (µg/ml)**	
	Amp200	Amp200+6%sucrose
No DNA	0	0
SQ170	423	76
SQ170-50	580	83
SQ170-100	501	67
SQ170-150	300	46
SQ170-200	150	42

Marker rescue results revealed that Rif-R *E. coli* SQZ10 colonies were recovered with plasmid DNA derived from a pool of Rif-R clones, suggesting that the Rif-R phenotype observed was due to mutations in the plasmid DNA.

3.5. Phenotypic characteristics of rRNA mutants

3.5.1. Rifampicin phenotype

The MIC of each mutant was determined by spot tests with concentrations of rifampicin ranging from 0 to 200µg/ml. Plates were incubated at 37^{o}C for 24hrs and the results are shown in **Figure 3.2** below. Six out of the 8 mutants tested displayed very slight growth at 25µg/ml rifampicin. These clones were not studied further as it was believed that the colonies observed were low level resistant rRNA mutants. Only the MIC of the selected clones (pGM1

and pGM2) was studied further because they showed confluent growth on antibiotic plates. In *E. coli* SQ170, pGM1 and pGM2 conferred resistance up to 25µg/ml and 30µg/ml rifampicin as opposed to 5µg/ml for the un-mutated SQ170 control. This indicated an approximately 5-fold increase in rifampicin resistance.

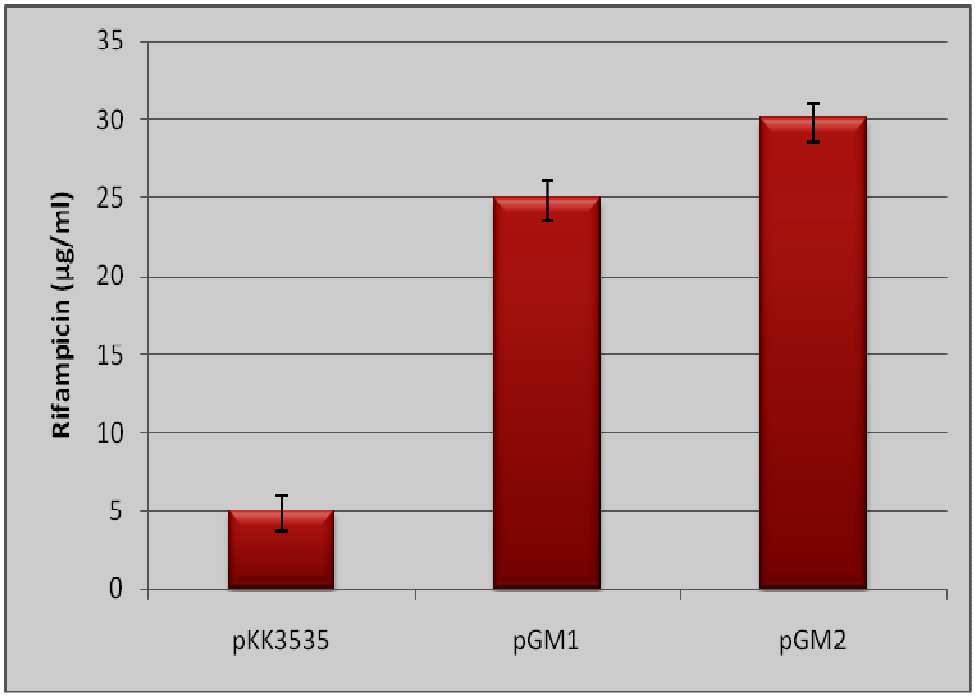


Figure 3.2: Rifampicin MIC of rRNA mutants obtained from spontaneous selection and EMS mutagenesis in *E. coli* SQ170. Each set of experiments was repeated three times. The columns represent the average of results from all three experiments. Standard deviations of the means are indicated by error bars.

3.5.2. Effects of temperature on rifampicin resistance

E. coli SQZ10 cells, transformed with the rRNA mutants, were spotted onto increasing concentrations of rifampicin ranging from 0 to 200µg/ml. The plates were incubated at 18^{o}C, 25^{o}C, 37^{o}C, and 42^{o}C for 24hrs. The MIC of each mutant was determined at varying temperatures in the presence (**Figure 3.3.)** and absence of ampicillin. Rifampicin resistance was higher in the absence of ampicillin. The results indicated that at low temperature, the resistance was reduced. Thus, the rifampicin resistance phenotype in SQ170 was determined to be cold-sensitive. At 42^{o}C, resistance in pGM2 increased from 25µg/ml to 30µg/ml rifampicin. From the results observed, it was clear that pGM1 and pGM2 have different

temperature conditions that allow the phenotype of each mutant to be clearly expressed.pGM1, is expressed better at 37°C, whereas pGM2 phenotype is expressed better at 42°C.

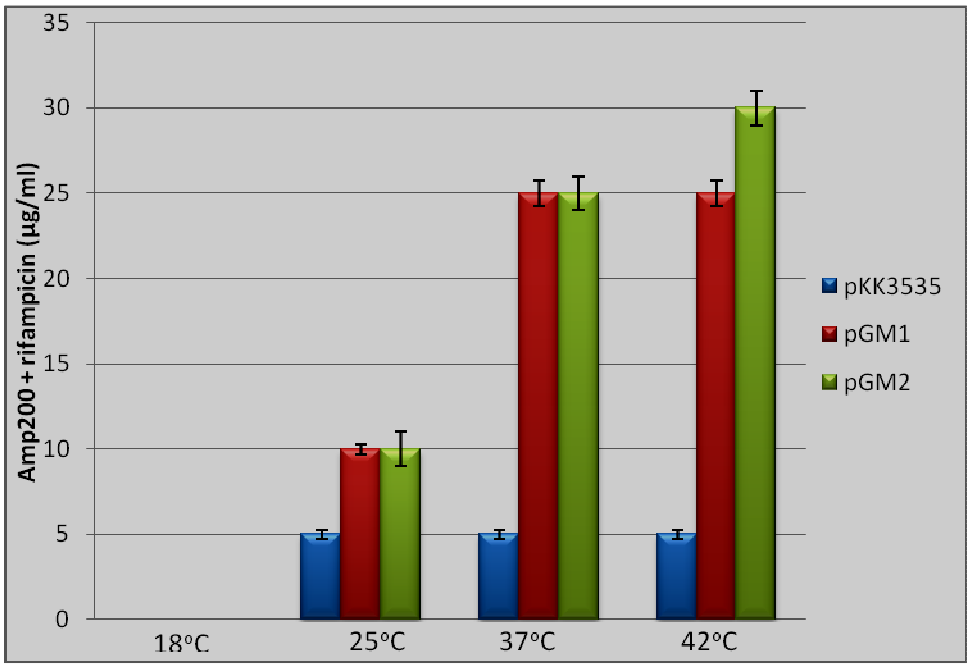


Figure 3.3: Rifampicin MIC of rRNA mutants at varying temperatures of 18°C, 25°C, 37°C, and 42°C in *E. coli* SQ170. Each set of experiments was repeated three times. The columns represent the means of results from all three experiments. Standard deviations of the means are indicated by error bars.

3.5.3. Effects of pH on rifampicin resistance

The MIC of the two mutants (pGM1 and pGM2) was determined by spot tests with concentrations of rifampicin ranging from 0 to 200µg/ml. Plates used for these tests were made from LA whose pH had been adjusted to pH 5.5 using HCl, pH 7.0, and pH 8.5 adjusted by using NaOH. The results indicated that resistance was the highest at neutral pH and alkaline pH. There was a decrease in resistance at acidic pH. Both pGM1 and pGM2 displayed an increase in resistance from 20 to 30µg/ml. The resistance of the SQ170 control was not affected by change in pH conditions as it remained the same in all the tested pH conditions. Results are shown in **Figure 3.4** below.

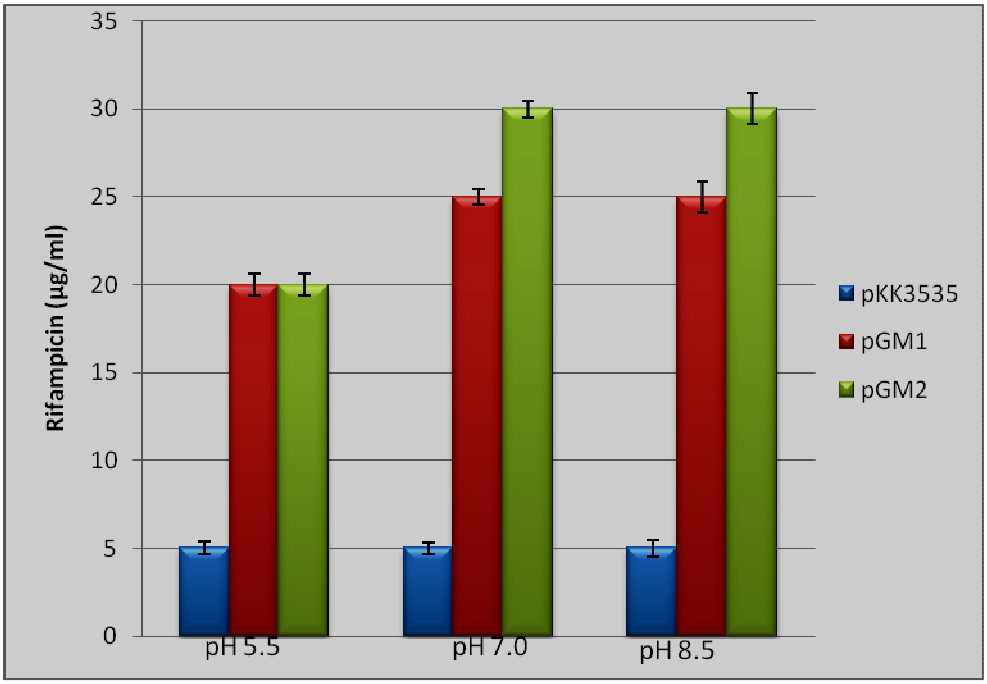


Figure 3.4: Rifampicin MIC of rRNA mutants at varying pH conditions ranging from pH 5.5, 7.0 and 8.5 in *E. coli* SQ170. Each set of experiments was repeated three times. The columns represent the means of results from all three experiments. Standard deviations of the means are indicated by error bars.

3.5.4. Effects of phosphate buffered media on rifampicin resistance

The MIC of pGM1 and pGM2 was determined by spot tests on buffered L (A-N) media with varying pH values. The buffered LA-N was prepared by dissolving the required amount of bacteriological agar in water followed by adjusting the pH to the required values. This was followed by preparation of LB with the required pH. Both the LB and the agar were autoclaved separately and LB was supplemented with glucose and 1x A-N buffer. The two components then used to make rifampicin plates with varying concentrations ranging from 0 to 50µg/ml.

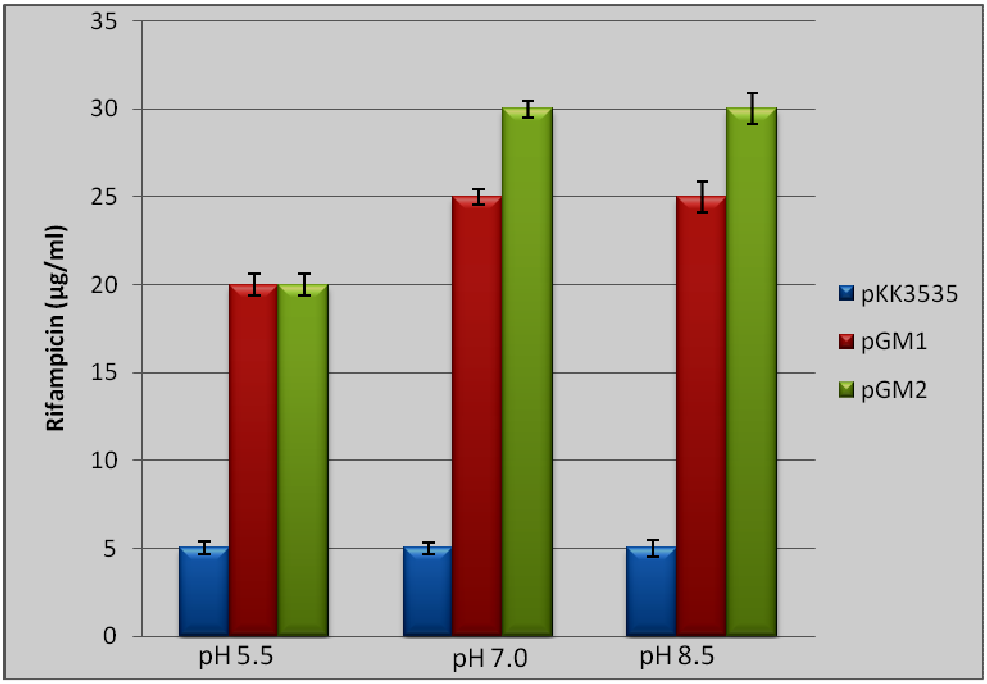


Figure 3.5: Rifampicin MIC of rRNA mutants at varying pH conditions ranging from pH 5.5, 7.0 and 8.5 in buffered L (A-N) media. Each set of experiments was repeated three times. The columns represent the means of results from all three experiments. Standard deviations of the means are indicated by error bars.

The growth patterns observed in **Figures 3.4** and **3.5** suggests that the presence of phosphate buffer in the growth media did not have a significant effect on the resistance phenotype. This was most apparent at neutral pH 7.0 and alkaline pH 8.5 which showed a 4-fold and 5-fold increase in resistance of pGM1 and pGM2 respectively.

3.5.5. Effects of plasmid-borne mutations on other antibiotics MICs

Mutant pGM1 and pGM2 were tested for any increase or decrease in resistance to other antibiotics. For this purpose, antibiotics that were known to inhibit the translation process in different ways were chosen. The spot tests of pGM1 and pGM2 were done on varying concentrations of streptomycin, chloramphenicol, tetracycline, kasugamycin and erythromycin. The results are shown in **Figures 3.6** to **3.10**. *E. coli* SQ170 was used as a control strain.

The results indicated that the resistance/susceptibility of the strains was changed because of the presence of the mutation. pGM2 displayed a 2-fold increase in resistance to streptomycin when compared with *E. coli* SQ170. The results for pGM1 and pGM2 were comparable at a 1-fold and 2-fold increase in resistance to chloramphenicol. Both pGM1 and pGM2 were susceptible to tetracycline whilst no change in MIC was recorded for erythromycin on both mutants. pGM1 displayed no change in MIC and an increased susceptibility on pGM2 was observed.

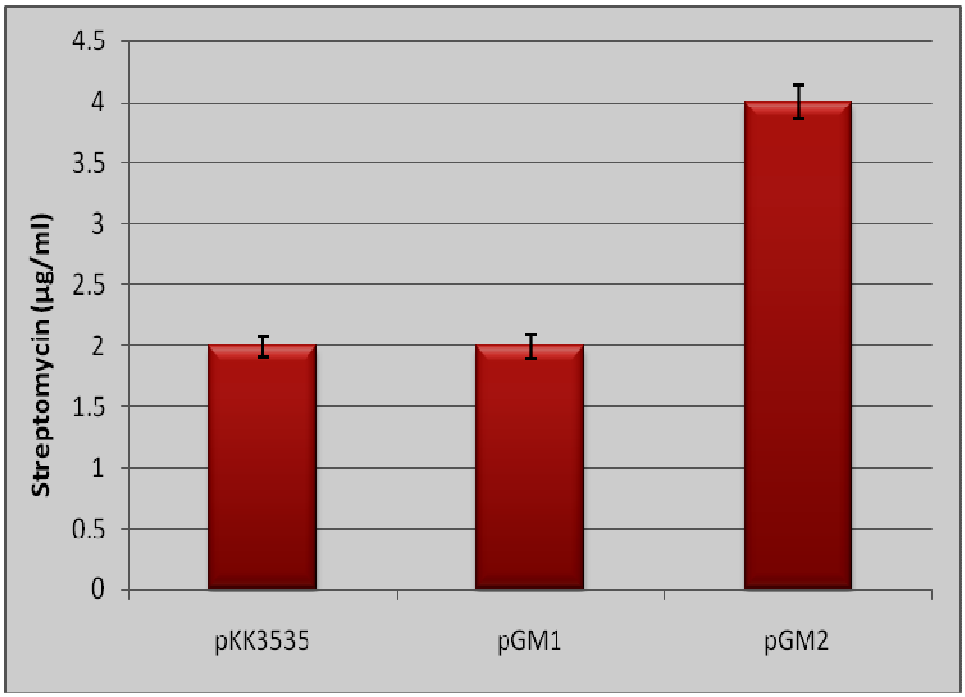


Figure 3.6: Streptomycin MIC of rRNA mutants in *E. coli* SQ170. Each set of experiments was repeated three times. The columns represent the means of results from all three experiments. Standard deviations of the means are indicated by error bars.

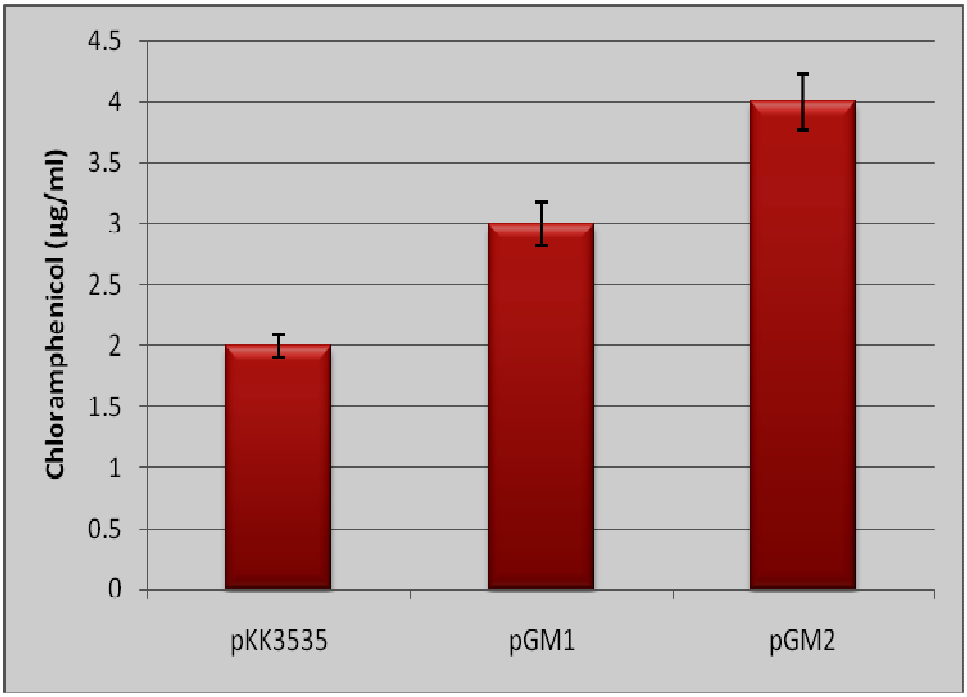


Figure 3.7: Chloramphenicol MIC of rRNA mutants in *E. coli* SQ170. Each set of experiments was repeated three times. The columns represent the means of results from all three experiments. Standard deviations of the means are indicated by error bars.

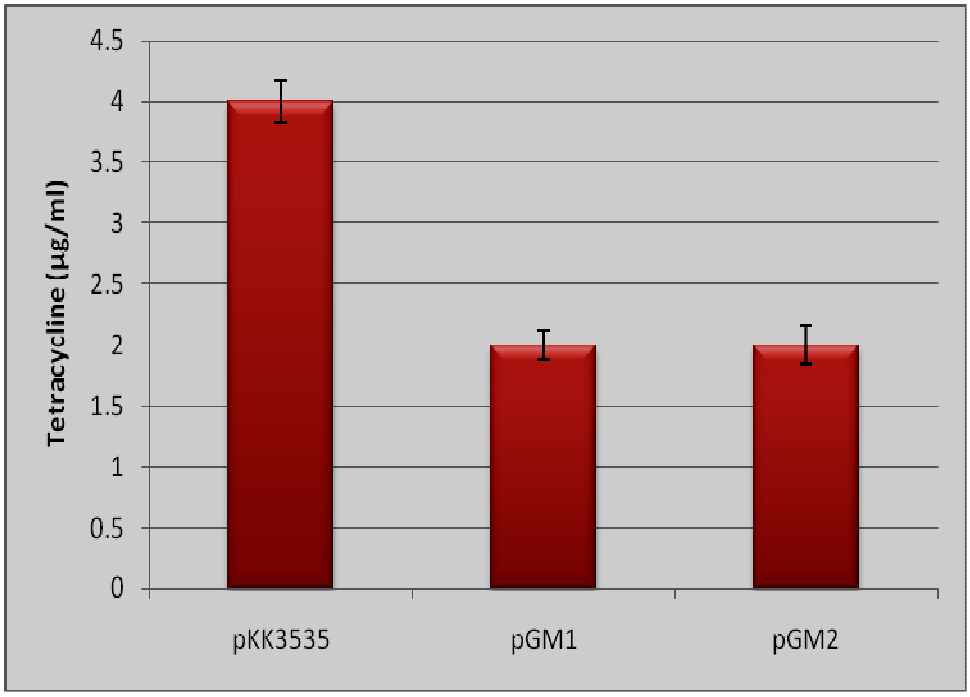


Figure 3.8: Tetracycline MIC of rRNA mutants in *E. coli* SQ170. Each set of experiments was repeated three times. The columns represent the means of results from all three experiments. Standard deviations of the means are indicated by error bars.

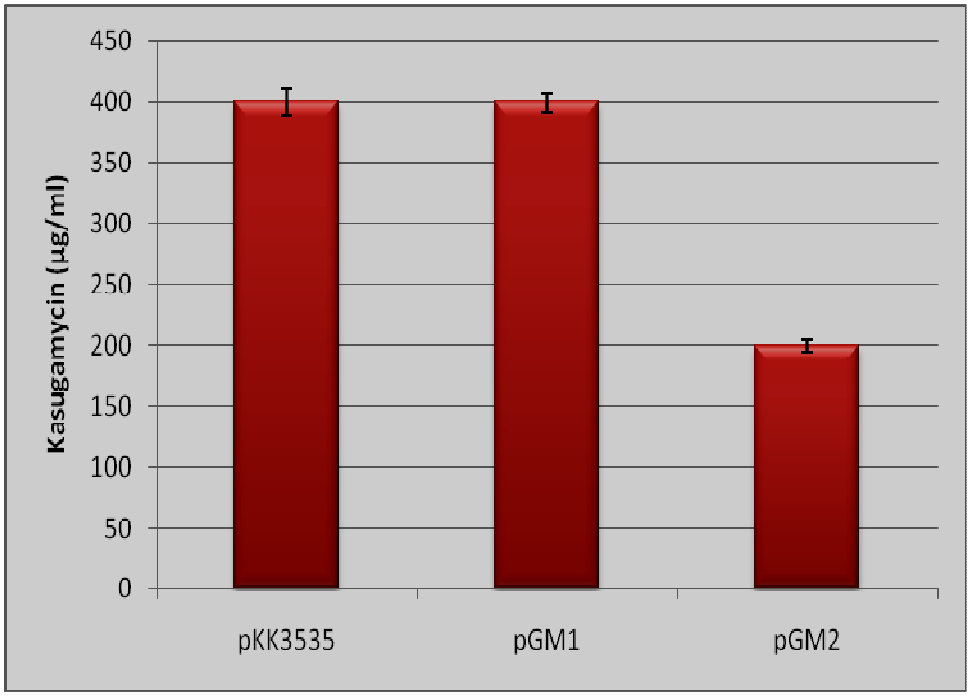


Figure 3.9: Kasugamycin MIC of rRNA mutants in *E. coli* SQ170. Each set of experiments was repeated three times. The columns represent the means of results from all three experiments. Standard deviations of the means are indicated by error bars.

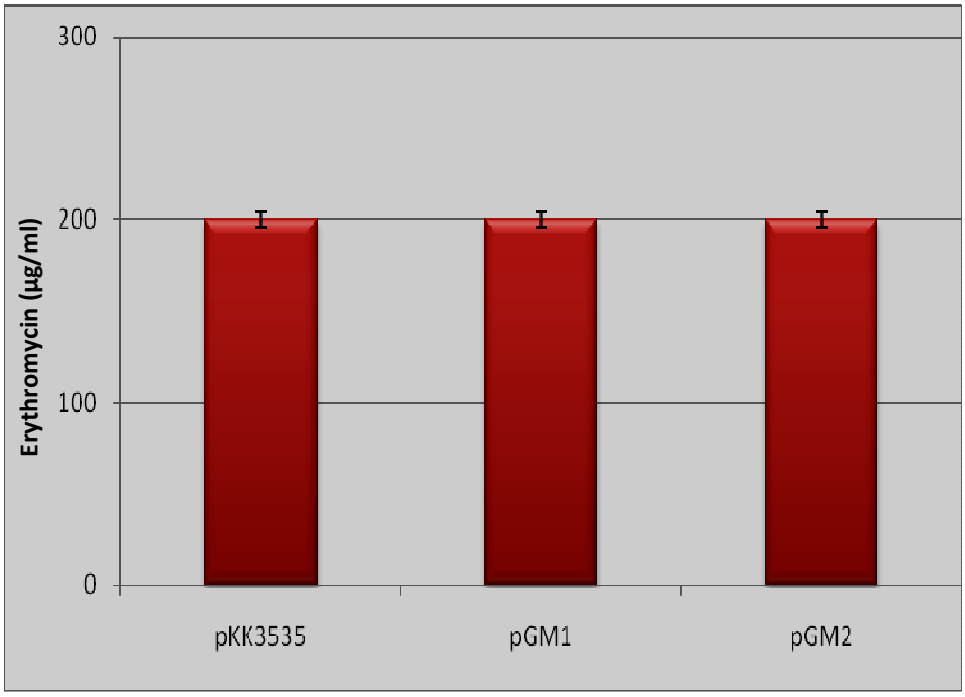


Figure 3.10: Erythromycin MIC of rRNA mutants in *E. coli* SQ170. Each set of experiments was repeated three times.

Table 3.6: Summary of the effects of mutations on tested antibiotics MICs.

Antibiotic	MIC (µg/ml)		
	pKK3535	**pGM1**	**pGM2**
Rifampicin	5	25	30
Streptomycin	2	2	4
Chloramphenicol	2	3	4
Tetracycline	4	2	2
Kasugamycin	400	400	200
Erythromycin	200	200	200

These MIC findings were surprising, in particular the MICs of erythromycin and chloramphenicol. The mutation was expected to be in the 16S rRNA as supported by the MIC changes observed above.

3.6. Restriction fragment exchange

The mutants obtained by spontaneous selection and EMS mutagenesis were further characterized to differentiate between two types of plasmid mutations. Restriction fragment exchange was performed to determine the type of mutation that was responsible for the changed phenotypes observed. Of particular interest were mutations that occur in the *rrn* RNA along the plasmid.

Large scale plasmid preparations of the pool of rifampicin resistant clones and that of pKK3535 were done as described in **2.6.1**. Plasmid DNA of the wild-type and the mutants were digested overnight with *Bam*HI at 37^{o}C. Digestion by *Bam*HI generated two fragments of 7503bp and 4293bp.The larger fragments, containing the *rrnB* operon, were separated

from the smaller fragments by extracting the bands from a 1% low-gelling agarose gel. The purified DNA fragments are indicated in **Figure 3.11** below.

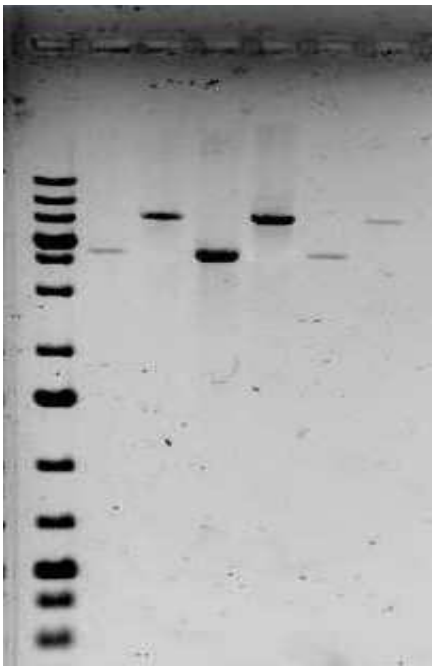


Figure 3.11: Purified *Bam*HI DNA fragments on 0.8% agarose. Lanes 1, 3, and 5 represent the 4293bp fragments of pKK3535, pGM1 and pGM2 respectively. Lanes 2, 4, and 6 represents the 7503bp fragments of pKK3535, pGM1 and pGM2 respectively.

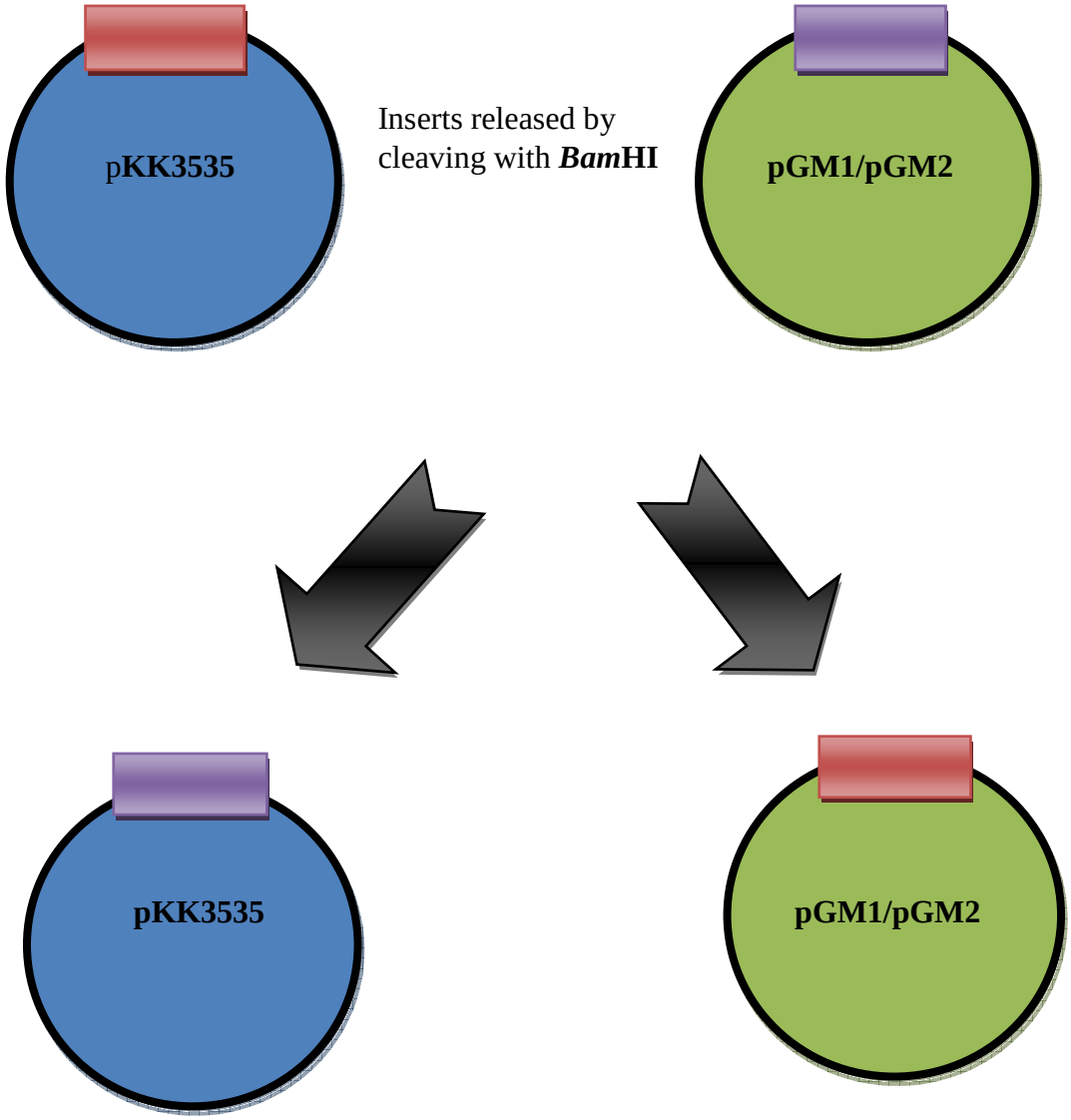


Figure 3.12: Restriction fragment exchange between the wild-type and the mutant recombinant plasmids.

All the 7503bp fragments were regarded as vectors and the 4293bp fragments as inserts. The vectors were treated with alkaline phosphate to prevent re-circularization. Each 4293bp fragments of pGM1 and pGM2 was ligated to the 7503bp fragment of pKK3535. Each of the 7503bp fragment of pGM1 and pGM2 was ligated to the 4293bp fragment of pKK3535 and thus the fragments were exchanged (**Figure 3.12**). The DNA obtained was used to transform *E. coli* SQZ10 using the calcium-chloride method and selection of transformants was done on LA plates supplemented with 100µg/ml. The transformants were analysed for the presence of inserts to ensure that a confident representation of results had been attained. Transformants containing the vector ligated to the insert were observed and these were studied further.

To ensure that ligation was successful, the transformants were grown overnight and plasmid DNA extracted. This was followed by digestion of plasmid DNA with *Bam*HI to separate inserts from vectors.



Figure 3.13: Plasmid DNA of pKK3535 digested with *Bam*HI to release cloned inserts. Lane 1 represents the molecular weight marker and lanes 2-4 represents the plasmid DNA with inserts released.

The transformants that contained the inserts ligated to the vector were streaked on LA-Amp + 6% sucrose plates and then transferred to LA-Rif plates of 0µg/ml to 50µg/ml. Colonies of pGM1 and pGM2 that contained the mutated *rrn*B operon showed resistance growth on LA-Rif plates whilst those with no mutations on the *rrn*B operon showed sensitivity to rifampicin (Data not shown). From the findings of the restriction fragment exchange, it was concluded that the mutations observed on pGM1 and pGM2 were rRNA mutations.

3.7. DNA sequence analysis of rRNA mutants

3.7.1. Nucleotide sequence analysis

The *rrn*B sequences of pGM1 (obtained from spontaneous mutagenesis) and pGM2 (obtained from EMS mutagenesis) were analysed by internet databases. All two sequences were compared to the wild-type *rrn*B sequence using the **B**asic **L**ocal **A**lignment **S**earch **T**ools for nucleotide alignments (BLASTn).

The BLASTn search results detected base substitution for mutant pGM1 (**Figure 3.12**). The *rrn*B operon in pGM1 had a single base change from thymine to adenine at position 3681 of the wild-type sequence. No mutation was detected in the *rrn* region of pGM2 when its sequence was analysed with BLASTn. The observed phenotype of rifampicin resistance might be due to mutation(s) on the plasmid and not on the rRNA. To ensure the accuracy of the BLASTn database, the mutations were analysed using EMBOSS pairwise alignment tool. Using this tool, the same results were recorded for both the mutants. This suggested that the mutations detection of presence or absence of mutations was not because there is no change in the *rrn* region of the plasmid.

The pGM1 mutation was on position 3681 of pKK3535 which places it in the 23S rRNA. This finding is surprising since the observation of the MICs of erythromycin and chloramphenicol, which targets the 23S rRNA, does not show much change in MIC and it was expected that the mutation was likely to be in the 16S rRNA.

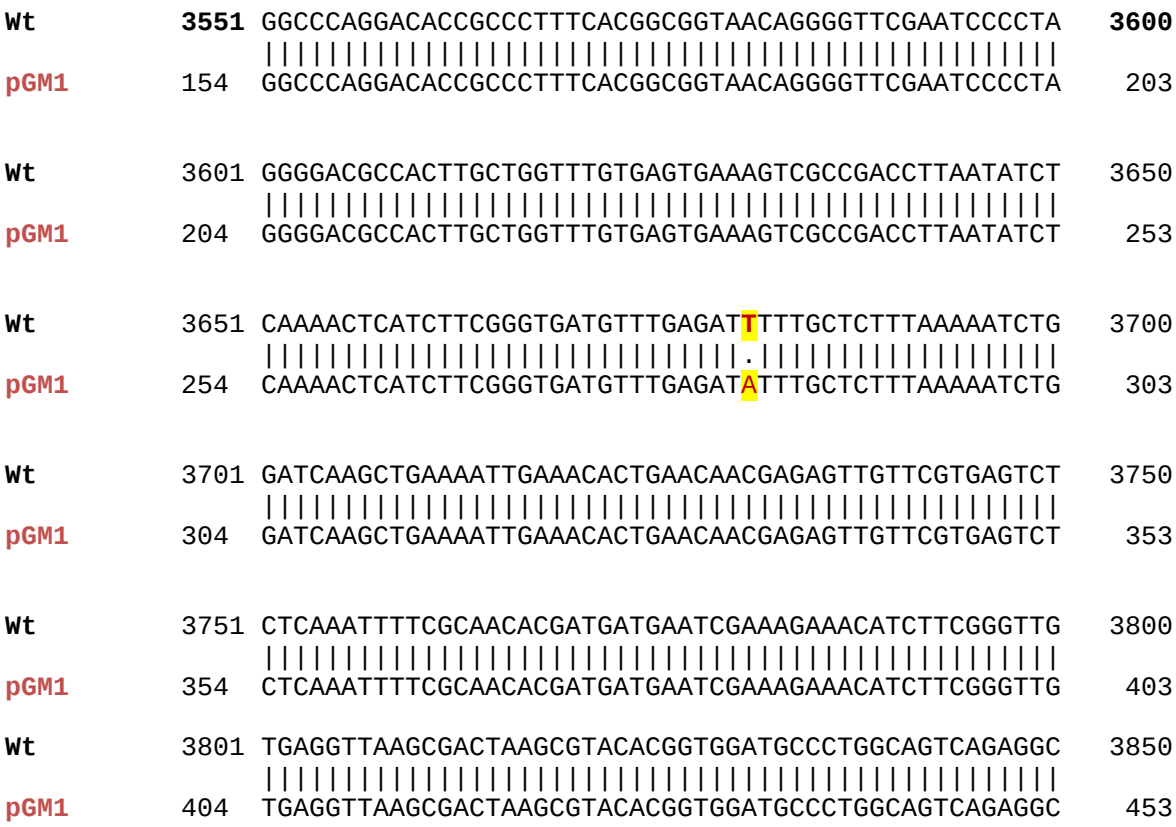


Figure 3.14: Nucleotide alignment of the known *rrnB* operon sequence (wt) compared to its mutated pGM1 counter-part using BLASTN search programme.

3.8. Site-directed mutagenesis of pKK3535

As an alternative strategy, site-directed mutagenesis was used to confirm that the observed phenotype of pGM1 was due to the mutations detected in the *rrn*B operon. To do this, three single base mutations (T to A), (T to C), and (T to G) were introduced into the wild-type pKK3535 with the aid of a Finnzyme Phusion Site-Directed Mutagenesis Kit. This mutagenesis uses a high fidelity Hot Start DNA polymerase which only allows mutations to be introduced at positions of interest. Mutagenic primers were designed according to Finnzyme recommendations, with modifications as desired.

Table 3.7: Mutagenic primers used to construct 23S rRNA mutants

Primer (5'-3')	**Tm (^{o}C)**	**%GC**
Forward:GTGATGTTTGAGATATTTGCTCTTTAAAA	64.32	37%
Reverse:CCGAAGATGAGTTTTGAGATATTAAGGTC	66.56	27%
Forward:GTGATGTTTGAGATCTTTGCTCTTTAAAA	65.53	39%
Reverse:CCGAAGATGAGTTTTGAGATATTAAGGTC	66.56	27%
Forward:GTGATGTTTGAGATGTTTGCTCTTTAAAA	67.24	39%
Reverse:CCGAAGATGAGTTTTGAGATATTAAGGTC	66.56	27%

The site of adenine, cytosine and guanine insertion(s) are highlited in red.
$\mathbf{T_m}$ was calculated as per formular supplied by the Finnzyme mutagenesis kit (www.finnzymes.com).

Amplification was carried out in a PC-960G Gradient Thermal Cycler. The reaction parameters were set according to the Finnzyme specifications with the extension time of 5min, 51sec. The PCR product was a linear plasmid molecule therefore it was circularized with the Quick T4 DNA ligase. The circularized plasmid was then transformed into *E. coli* MM294-4. DNA minipreparations of 5 transformants were performed and digested with to release the inserts.

The purified PCR products were sent to inqaba Biotec for sequencing . Sequencing the PCR products was unseccessful due to a number of reasons. The sequences were analysed by bioinformatics tools and unfortunately no desired mutations were observed. It was speculated that this was due to that the DNA template (pKK3535) was large and thus the site-directed mutagenesis did not work. This was supported by the fact that the PCR product was a faint band. To improve the results, the DNA fragment with the position where the mutation was to be introduced was cloned into pGEM and thus the mutagenesis carried out. It was believed

that this would highly increase the efficiency and accuracy of the DNA polymerase during mutagenesis PCR because the DNA fragment to be mutated is shorter in length.

To reduce the size of the template for optimum amplification, pKK3535 was digested with *Eco*RI to release a DNA fragment of 2051bp. The DNA fragment was cloned into pGEM and transformed into *E. coli* MM294-4. Transformants were screened for the presence of inserts by linearizing the DNA obtained from mini preparations with *Pst*I. Successful amplification was carried as described above (**Figure 3.15**) and the PCR products were sent to Inqaba Biotec for sequencing.

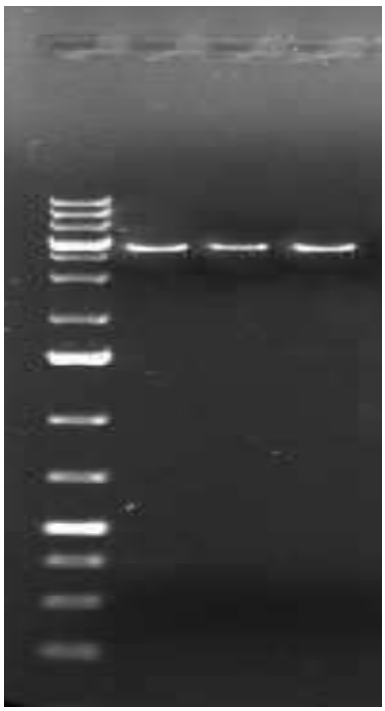


Figure 3.15: Amplified *rrnB* with desired mutations.

After three attempts of site-directed mutagenesis and again no desired mutations were detected after DNA sequencing .

CHAPTER 4

DISCUSSION

4.1. Isolation of rifampicin rRNA mutations

The *E. coli* genome carries seven rRNA (*rrn*) operons each containing a 16S, 23S and 5S rRNA gene. The presence of multiple operons has been an obstacle to many studies of rRNA genes because the effect of mutation(s) in one operon is diluted by the remaining six wild-type copies. In light of the above statement, genetic efforts to study the interaction of the ribosome and RNA polymerase have been difficult. The development of an *E. coli* system that carries a single rRNA operon located on a plasmid (Asai *et al*., 1999) has helped overcome a large gap in the understanding of the function of rRNA; of particular interest being is there a physical but perhaps transient interaction of ribosomes and RNA polymerase during coupled transcription-translation in bacteria. In this context the contribution of research into antibiotics that target transcription and translation machinery has been fruitful for furthering the understanding of functional interactions between these cellular structures. Thus, it became possible to isolate rRNA mutants that confer antibiotic resistance to *E. coli* and to locate the positions where the mutations occur on the rRNA.

Rifampicin is a chemotherapeutic agent used to combat infections by pathogenic nocardioforms and *Mycobacterium tuberculosis*. Soon after its introduction to clinical practise in 1972, resistant mutants were identified (Gillespie, 2002). Resistance has been primarily through target site alteration but in addition four inactivation mechanisms has been identified, but only mutation by alteration of target site will be discussed as it is the main focus in this study.

A strategy was developed for the generation of rifampicin resistance mutants in the rRNA of *E. coli*. This involved the use of a previously described SQ170 strain that is deficient of all seven chromosomal *rrn* operons. Mutants of this strain would be generated by spontaneous and chemical mutagenesis, and selected directly for the desired phenotype of rifampicin resistance. Furthermore, it should be possible to select into the host strain, since only one plasmid-borne rRNA operon was present in this background.

4.2. Mutational analysis of rRNA

Two rifampicin resistant rRNA mutants were characterised in this work. These were pGM1 (obtained from spontaneous selection), and pGM2 (obtained from EMS mutagenesis). The nature of the mutations in each of these mutants was examined in terms of nucleotide substitutions. The implication of these changes on the functioning of the rRNA and how ribosomal proteins (r-proteins) will interact with the region of rRNA will then be considered. These are important as it is known that there is a high cooperative nature of the interaction between rRNA and r-proteins that is essential for functional translational activity. r-proteins do affect the function of ribosomes, as is mostly clearly evidenced by mutations in r-protein genes that change the rRNA structure (Gregory and Dahlberg, 1999), ribosome sensitivity to antibiotics and the accuracy at which the ribosome translates the genetic code (Gao *et al*., 2003).

The ability to successfully isolate rRNA mutants conferring resistance to rifampicin in this study suggests that there either is a hitherto unknown but weak rifampicin binding site on the ribosome or that there is an intimate transient contact between RNA polymerase and the ribosome during gene expression. The detailed molecular mechanism of this contact would be the subject of future work.

4.2.1. Mutational analysis of pGM1

The rRNA gene in pGM1 had a single base substitution from thymine to adenine at position 355 of domain I in the 23S rRNA. **Figure 4.1** shows a 3D-structure of the large ribosomal subunit, indicating different domains of the subunit, which gives insight on where the mutation is situated**.** This transversion might lead to structural changes in the large subunit either in terms of tertiary structure of the large subunit RNA or it may affect the way ribosomal proteins interact with this subunit.

4.3.2. Mutational analysis of pGM2

No base substitution mutation was detected in the nucleic sequence analysis of the *rrn* region of pGM2. Sequencing errors were ruled out as the cause after sequencing was repeated twice and the same results were observed. Since this was the case for this mutant, we reasoned that the observed phenotype of rifampicin resistance could have resulted from a mutation elsewhere on the plasmid rather than the 23S RNA gene.

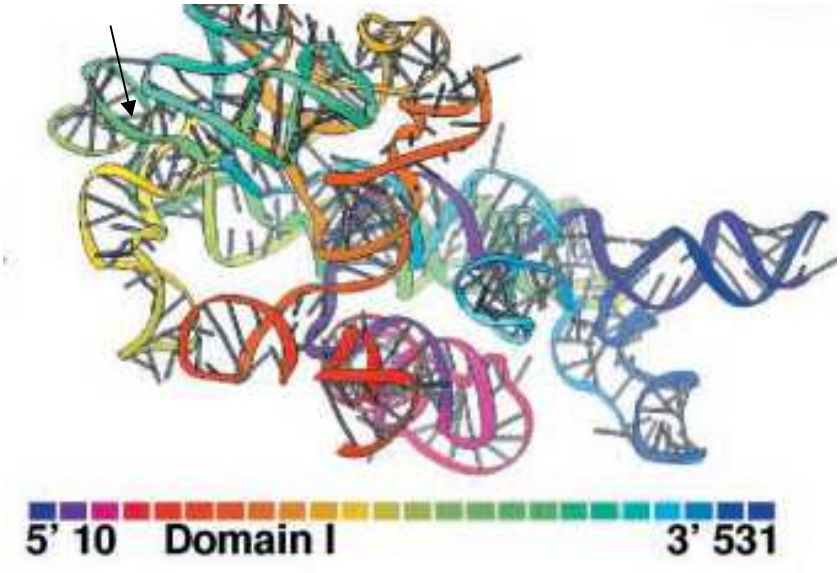


Figure 4.1: Tertiary structure of the large ribosomal subunit domain I in *Haloarcula marismottui* (Adapted from Ban *et al*., 2000). An arrow indicates the position of the mutation.

In terms of the MIC changes observed in chloramphenicol and erythromycin, the mutation is expected to be in the small rRNA but this was not the case when the DNA sequences were analysed. This finding is surprising since the observation of the increased MICs of streptomycin and kasugamycin while erythromycin and chloramphenicol, which target the 23S rRNA, did not show much change in MIC and this strongly indicates that the mutation is likely to be in the 16S rRNA.

4.3. Identification of r-proteins associated with RNA polymerase

The assembly and mechanism of the ribosome involve the cooperative interaction of r-proteins and RNA components. This is observed by the many r-proteins that permeate the large subunit extensively by making van der Waals forces with the nucleotides. Considerable evidence indicates the participation of r-proteins both in the stabilization of the proper rRNA tertiary structure and in facilitating dynamic conformational changes of rRNA during protein synthesis (Gregory and Dahlberg, 1999). Numerous proteins have been recorded to interact with Domain I of the large subunit, where the mutation of pGM1 is. These proteins include L4, L15, L22, L24, L29, L37e, L39e, and L44e **(Figure 4.2).**

A **B**

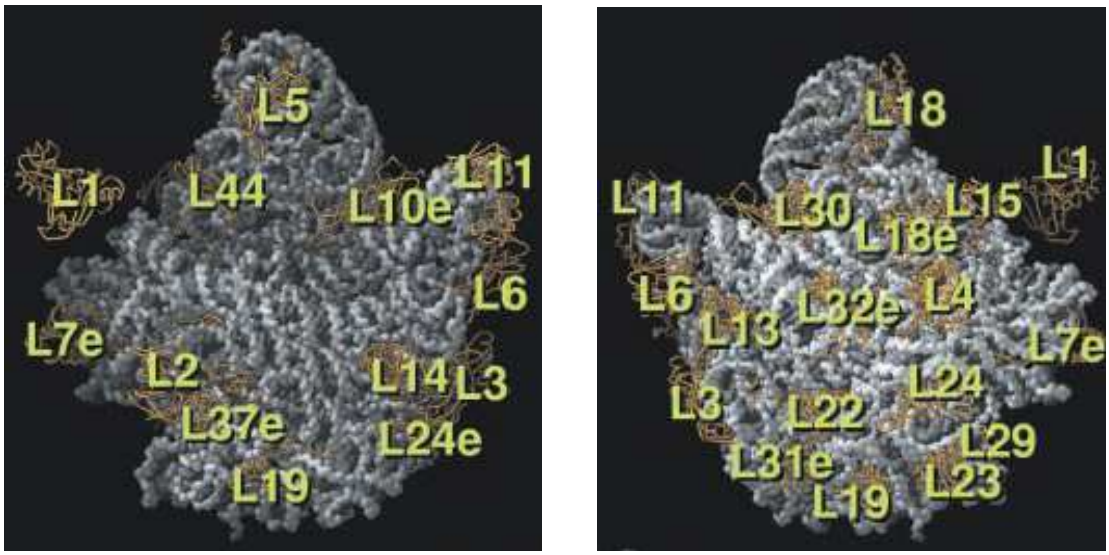


Figure 4.2: Proteins that appear on the surface of the large ribosomal subunit. (**A**) The crown view of the subunit. (**B**) The back side of the subunit in the 180oC rotated crown view orientation (Adapted from Ban *et al*., 2000).

A study by Dabbs (Dabbs, 1979) reported that r-proteins L15, L24 or L29 are not essential for the translational function of the ribosome, since mutations lacking these proteins are viable although some of them show severe defects indicated by conditional lethality (temperature sensitivity). But recently, studies have contradicted this statement. Stelzl *et al*., 2000 reported that L15 is essential for the late assembly step that is important for an active ribosomal conformation and binds erythromycin in solution whereas L24 plays an important role for assembly initiation by forming an assembly starting point at the 5'end of 23S rRNA. Mutations in L4 and L22 have been reported to confer resistance against erythromycin, by interfering with the binding site of the drug. Erythromycin blocks the insertion of the growing peptide chain into the tunnel of the large ribosomal subunit (Stelzl, *et al*., 2000). A recent study by Rippa *et al*., 2010; reported interaction of a 50S r-protein L2 with the α-subunit of RNA polymerase.

4.5. Conclusions

In summary, successful isolation of Rif-R rRNA mutants is consistent with the hypothesis that there is intimate transient contact between the ribosome and RNA polymerase in bacteria. Furthermore, based on the position of the mutation we determined that RNA polymerase might contact the ribosome in the region of proteins L4, L15, and L22. Screening of a large number of ribosomal protein mutants (Dabbs and Wittmann, 1976) revealed several with altered rifampicin MIC (Dabbs, personal communication). The applications of these findings in molecular biology will enhance our understanding of the nature how the interacting proteins in stable complexes are essential to understanding the mechanisms that regulate cellular processes at molecular level.

4.6. Future work

Additional experiments could in future distinguish between the alternatives. These include rRNA mutations suppressing a conditional lethal (Ts or cs) *rpoB* rifampicin resistance or by doing cross-linking experiments involving the ribosome and RNA polymerase. Positive

results for either of these would support the contact model since if the 23SRNA mutation alters a hitherto unknown weak rifampicin binding site such outcomes would not be expected.

Rifampicin mutations in the *rpoB* gene may be conditionally lethal. Conditions such as temperature can be used to suppress the Rif-R phenotype and intergenic suppressors can be determined by marker rescue. The presence of suppressor mutations that have Rif-R rRNA mutants at a different site than the original site would support that RNA polymerase or rifampicin has a binding site in the ribosome.

The interaction of RNA polymerase and r-proteins in a cell can be investigated by crosslinking experiments. A chemical crosslinker, which is sensitive to the desired proteins, can be used to probe these proteins and link them together. Protein interactions are often too weak or transient to be easily detected, but by crosslinking, the interactions can be captured and analyzed.

CHAPTER 5

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CHAPTER 6

APPENDICES

APPENDIX A: MEDIA

Luria Bertani (LB) broth

Tryptone	3g
Yeast extracts	1.5g
NaCl	1.5g
Distilled water	300ml

LA (Luria Bertani agar)

Tryptone	3g
Yeast extracts	1.5g
NaCl	1.5g
Agar	4.5g
Distilled water	300ml

LAS (LA supplemented with 8% sucrose)

Tryptone	3g
Yeast extracts	1.5g
NaCl	1.5g
Agar	4.5g
Sucrose	18g
Distilled water	300ml

L (A-N)

Tryptone	3g
Yeast extracts	1.5g
NaCl	1.5g
Agar	4.5g

10× A-N buffer	10ml
Distilled water	300ml

pH was adjusted to pH 5.5, pH 7.0, and pH 8.5 as required for experiments.

APPENDIX B: SOLUTIONS

A6.1: Solutions used for plasmid preparations from *E. coli*

Solution I

Glucose	0.9g
1M Tris-HCl (pH 8.0)	2.5ml
EDTA (pH 8.0)	2ml
Distilled water	95.5ml

Solution II

NaOH	0.8g
SDS	1g
Distilled water	100ml

Solution III

5M CH_3COOK solution	60ml
CH_3COOH	11.5ml
Distilled water	28.5ml

RibonucleaseA

10mg/ml solution in sterile distilled water, heated to 96ºC immediately before use

1M Tris-HCl pH 8.0

Tris-HCl	15.8g
Distilled water	100ml
Autoclave	

0.5M EDTA pH 8.0

EDTA	18.6g
Distilled water	100ml
Adjust to pH 8.0 with NaOH	

5M CH_3COOK (pH 6.0)

CH_3COOK	4.9g
Distilled water	100ml
Adjust to pH 6.0 with CH_3COOH	

A6.2: Solutions used for transformations

$CaCl_2$ Transformation buffer

Tris-HCl (pH 8-0)	20mM
$CaCl_2$	100mM

pH adjusted to pH 8.0 and the solution autoclaved.

20% Glucose

Glucose	4g
Distilled water	20ml

A6.3: Solution for DNA analysis

Agarose gels

Agarose	0.8g (0.4%), 1.2g (0.6%) or1.6g (0.8%)
0.5× TBE	50ml

5 × TBE buffer

Tris base	54.0g
H_3BO_3	27.5g
0.5M EDTA	20ml, pH 8.0
Distilled water	1000ml
Autoclave	

Bromophenol blue tracking dye

30% glycerol (w/v) in TE and 0.0025% bromophenol blue

Running buffer (0.5x TBE)

5× TBE	50ml
Distilled water	450ml

Molecular weight markers

6× loading dye	100µl
Sterile distilled water	400µl
Molecular weight marker	100µl

TE buffer

1M Tris-HCl (pH 7.5)	10ml
500mM EDTA (pH 8.0)	2ml
Distilled water	88ml

TE-saturated phenol

Phenol	14g
TE buffer	10ml

6.4: Solutions for mutagenesis

10× A-N buffer

K_2HPO_4	70.0g
KH_2PO_4	26.8g

$Na_3C_6H_5O_7$	5.0g
$MgSO_4$	1.0g
Distilled water	1000ml

Table A6.1: Antimicrobial agents

Agent	Stock (mg/ml)	Solvent	Supplier
Ampicillin	100	70% ethanol	Sigma-Aldrich
Chloramphenicol	20	ethanol	Boehringer Mannheim
Erythromycin	20	ethanol	Boehringer Mannheim
Kanamycin	10	sdH_2O	Melford
Kasugamycin	100	sdH_2O	Boehringer Mannheim
Nalidixic Acid	10	sdH_2O	Sigma-Aldrich
Rifampicin	10	methanol	Sigma-Aldrich
Streptomycin	20	ethanol	Boehringer Mannheim

Table A6.2: Chemical suppliers

Name of the chemical	Supplier
Agarose	Bio-Rad
Acetic acid	Merck
Bacteriological agar	Biolab
Boric acid	Saarchem
Bromophenol blue	Sigma-Aldrich
Cesium chloride	Sigma-Aldrich
Chloroform	Saarchem
Dipotassium hydrogen Phosphate	Merck
EDTA	Sigma-Aldrich
EMS	Sigma-Aldrich
Ethanol	Saarchem
Ethidium bromide	Sigma-Aldrich
Glucose	Fluka Biochemika
Glycerol	Merck
Hydrochloric acid	Merck
Isopropanol	uniLAB
Magnesium sulphate	Sigma-Aldrich
Potassium acetate	Sigma-Aldrich
Potassium dihydrogen phosphate	AnalaR-analytical reagents
Phenol	Saarchem
RNaseA	Sigma-Aldrich
SDS	Boehringer Mannheim

Sodium chloride	Saarchem
Sodium hydroxide	Saarchem
Sucrose	Supermarket
Tri-sodium citrate	Sigma-Aldrich
Tris-HCl base	Saarchem
Tryptone	Biolab
Yeast extracts	Biolab

APPENDIX C: PLASMIDS AND MOLECULAR WEIGHT MARKERS

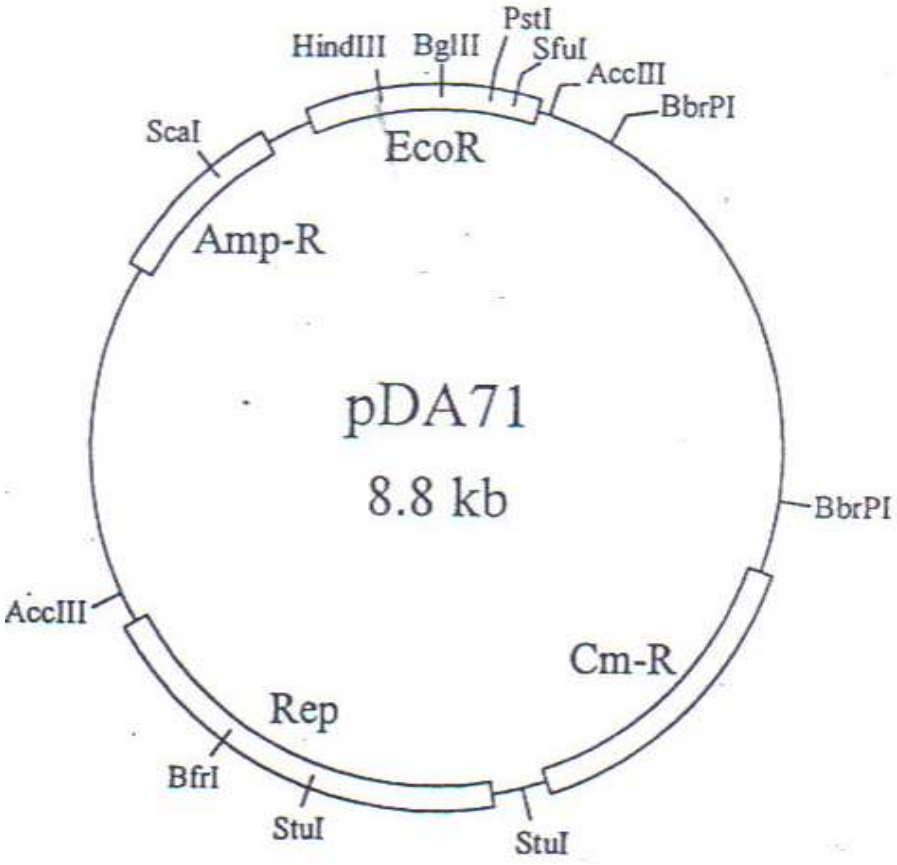


Figure A6.1: Restriction map of pDA71

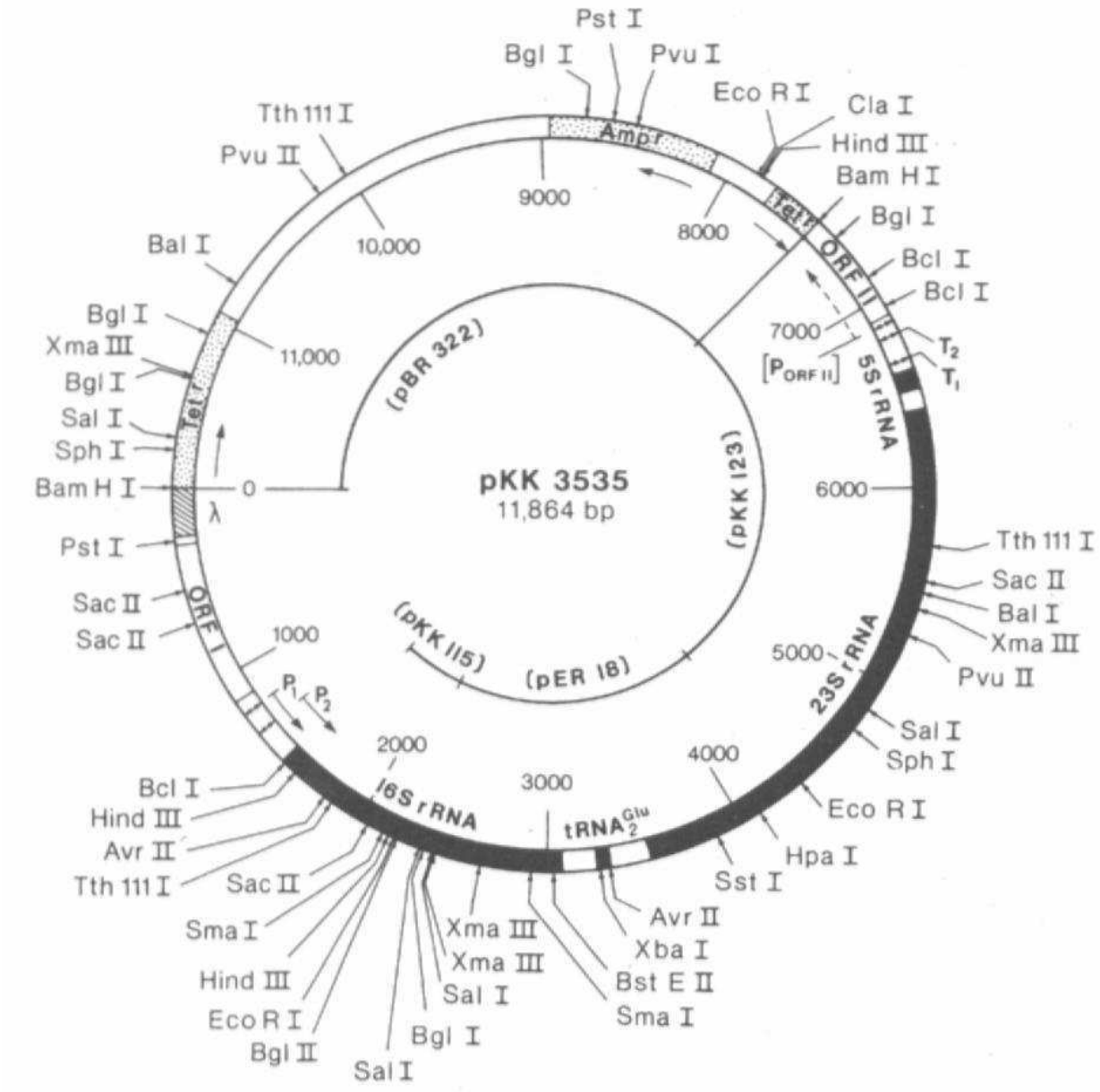
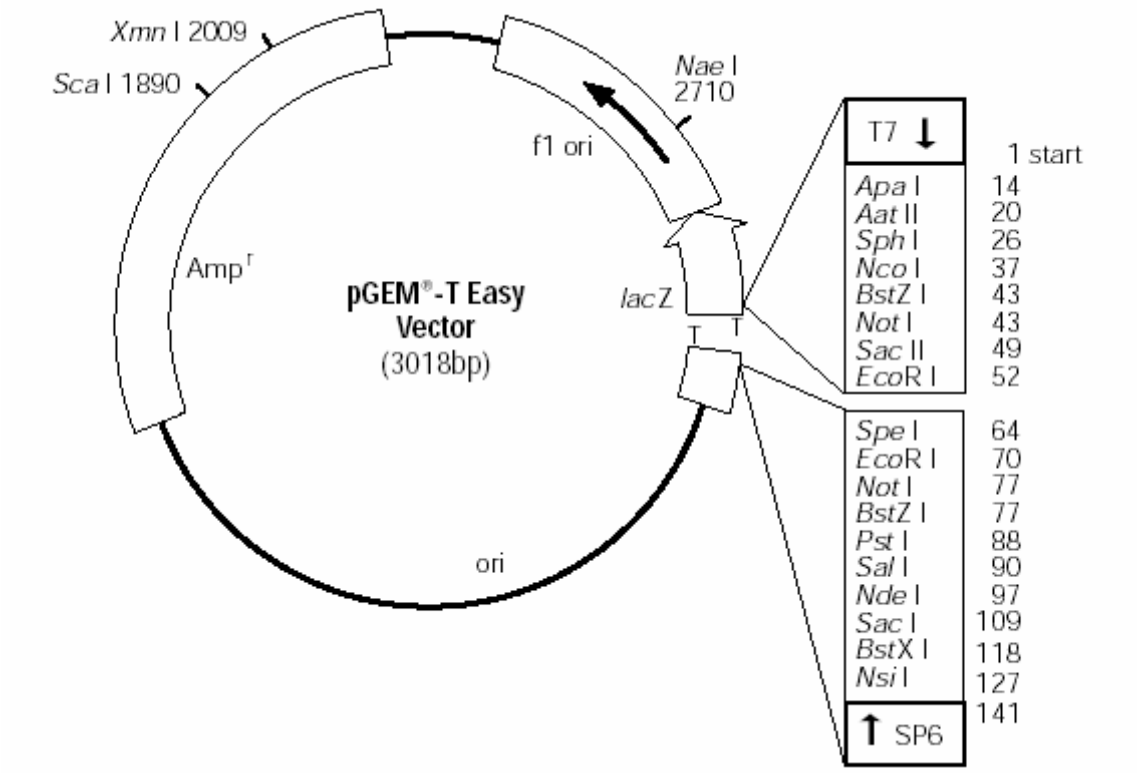


Figure A6.2: Restriction map of pKK3535 (Brosius *et al.*, 1981)



pGEM®-T Easy Vector Sequence reference points:

T7 RNA Polymerase transcription initiation site	1
SP6 RNA Polymerase transcription initiation site	141
T7 RNA Polymerase promoter	3002-6
SP6 RNA Polymerase promoter	136-158
multiple cloning site	10-128
*lac*Z start codon	180
lac operon sequences	2839-2999, 166-395
lac operator	100-216
β-lactamase coding region	1337-2197
phage f1 region	2383-2838
binding site of pUC/M13 Forward Sequencing Primer	2959-2975
binding site of pUC/M13 Reverse Sequencing Primer	176-192

Figure A6.3: Restriction map of pGEM-T-Easy (Adapted from www.promega.com)

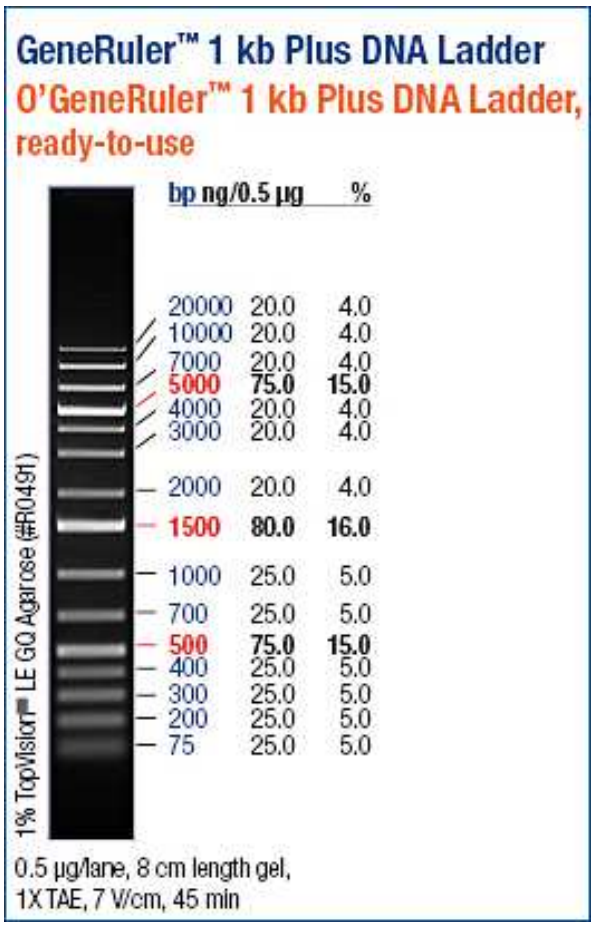


Figure A6.4: 1Kb DNA ladder plus, Fermentas (http://www.fermentas.com/catalog/electrophoresis/generulers.htm).