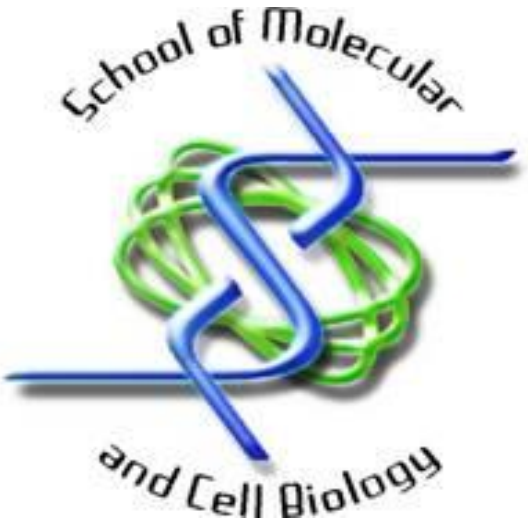
USING ERROR PRONE PCR IN DIRECTED EVOLUTION TO SELECT NOVEL ANTIBIOTIC RESISTANCES



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

AUGUST, 2011

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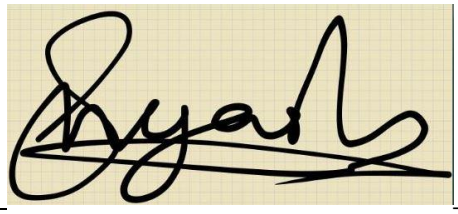
Declaration

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



06 August 2013

Mogashoa, Phokela Apollonarius Comet

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Dedications

This is dedicated to my grandparents JVM (I miss your blessing cross on my forehead grandpa, may your soul rest in peace) and MC Mogashoa, BJ and MW Sepota.

Abstract

The evolution of antibiotic resistance presents an escalating problem in the treatment of various infectious diseases worldwide. Although the origin of antibiotic resistance genes is not generally clearly documented, it has been thought that they evolved from specific genetic elements which eventually managed to spread to other microorganism of different strains and species through mobile genetics elements, transposons and plasmids. Extensively studying all aspects of these genes and their impact on the development of new treatments and drugs is of extreme importance. This study focuses on evolving and understanding how novel antibiotic resistance develops. Error prone PCR (EP-PCR) was used to introduce random mutation in an *arr* gene which confers high level resistance to rifampicin in *E. coli*. The clones obtained from EP-PCR were screened on different antibiotics with varying concentration in an attempt to isolate a clone with an increased minimum inhibitory concentration (MIC) as compared to the wild type parent strain (pBstN49).

Several clones showed decreased levels of resistance against rifampicin but however none showed any significant increase in any of the other antibiotic MICs tested.

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List of abbreviations

ADP:	Adenosine diphosphate
Amp:	Ampicillin
Amp^R:	Ampicillin resistant
bp:	Base pairs
$CaCl_2$:	Calcium chloride
Cam:	Chloramphenicol
Cam^S:	Chloramphenicol sensitive
Ctc:	Chlortetracycline
dH_2O:	Distilled water
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetate
EP-PCR:	Error prone polymerase chain reaction
Ery:	Erythromycin
HCl:	Hydrochloric acid
hr:	Hour(s)
Kan:	Kanamycin
Kb:	Kilo base
Ksg:	Kasugamycin
LA:	Luria agar
LB:	Luria broth
LDR:	Lysozyme, DNAse, RNAse
MAC:	*Mycobacterium avium* complex
MgAc:	Magnesium acetate
µg:	Microgram(s)
µl:	Microliter(s)
µM:	Micromolar
ml:	Milliliter(s)

mM:	Millimolar
MIC:	Minimum inhibitory concentration
min:	Minute(s)
M:	Molar
NaCl:	Sodium chloride
NAD:	Nicotinamide adenine dinucleotide
Nal:	Nalidixic acid
Nal^R:	Nalidixic acid resistant
NaOH:	Sodium hydroxide
ng:	nano gram(s)
OH:	Hydroxyl
PCR:	Polymerase chain reaction
Rif:	Rifampicin
Rif^R:	Rifampicin resistant
RNA:	Ribonucleic acid
Rpm:	Revolutions per minute
sdH_2O:	Sterile distilled water
SDS:	Sodium dodecyl sulphate
sec:	Second(s)
Spc:	Spectinomycin
Spc^R:	Spectinomycin resistant
Str:	Streptomycin
TBE:	Tris base, boric acid, EDTA
TE:	Tris base, EDTA
Tris:	Tris (hydroxymethyl)-aminomethane
tRNA:	Transfer ribonucleic acid
UDPG:	Uridine diphosphate glucose
UV:	Ultra violet
V:	Volts

1. Introduction

1.1. *Mycobacterium smegmatis*

Mycobacterium smegmatis is an acid fast, bacilli shape member of the phylum *Actinobacteria*. It is generally considered non-pathogenic but is known to cause certain infection especially in immunocompromised animals. *M. smegmatis* is considered to be a "fast-growing" microorganism in its genus with the ability to produce visible colonies in 3-5 days on solid media. Since *M. smegmatis* shares similar genes with other pathogenic mycobacteria species and grows relatively faster, it is an attractive model organism in molecular biology. It can be readily grown in a number of different media and it has been shown to express genes from pathogenic species such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*. The *arr* gene studied in this work was cloned from *M. smegmatis* (Quan *et al*., 1997).

1.2. **Antibiotics**

The term "antibiotic" was coined by Selman Waksman in 1942 to describe a metabolite that was produced by a microorganism which at low concentrations inhibit or kills other microorganisms. Various groups of antibiotics exist naturally such as β-lactams and aminoglycosides while other groups such as quinolones are created through synthetic means. Antibiotics are divided into various sub groups depending on the structure and/or mode of action: sulfa drugs, folic acid analogs, β-lactams, aminoglycosides, tetracyclines, macrolides, lincosamides, streptogramins, fluoroquinolones, polymixins, rifamycins, mupirocin, cycloserine, aminocyclitols, glycopeptides and other functionally related molecules.

1.2.1. Ansamycin antibiotics

Ansamycins are characterized by the presence of an ansa-bridge connecting two separate positions of an aromatic nucleus (August *et al*., 1998). Two distinct aromatic moieties of ansamycin have been studied and they are the basis on which these compounds are classified and

identified. The first aromatic moiety has a benzene based nucleus while the second group is based on a naphthalene nucleus. The latter group includes agents such as rifampicin and other rifamycin based antibiotics. Although the benzoquinone and the naphthoquinone ring systems are generally regarded as sub classes of the ansamycin antibiotics (Laport *et al.*, 2009) they act in a completely different manner on prokaryotes and eukaryotes (Xu *et al*,. 2003).

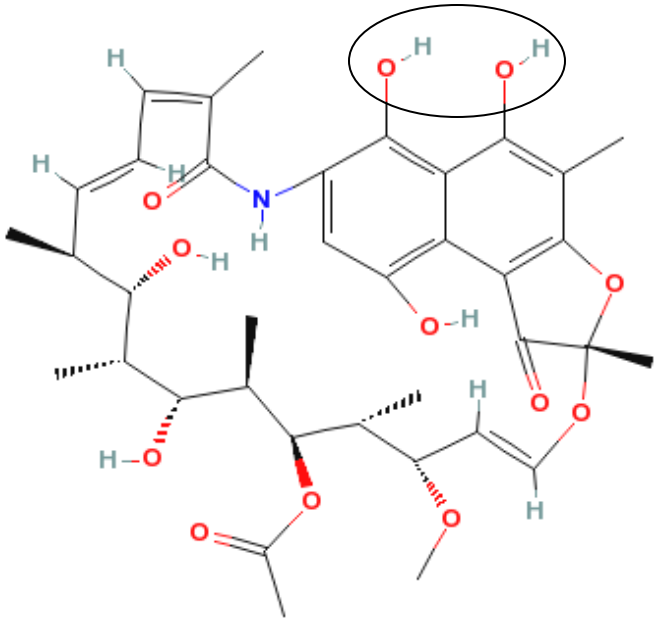

Figure 1: General structure of rifamycin antibiotics (adapted from http://www.lookchem.com/300w/546/5459948.png). The circled region indicates the generalized active site of the antibiotic

Rifamycins are a class of antibiotics which are very effective against bacterial genera such as *Mycobacterium, Nocardia, Gordona,* and *Rhodococcus*. This makes them useful in treating infections associated with these organisms such as tuberculosis and leprosy. This antibiotic class was first isolated in 1957 from the bacterium *Streptomyces mediterranei* (later renamed to *Amycolatopsis mediterranei* in 1960). Several rifamycins were isolated from the culture broth and designated as A, B, C, D and E. Through spontaneous expulsion of glycolic acid and reduction reactions, rifamycin S and SV were produced (Xu *et al.*, 2003).

The introduction of semi synthetic derivatives of rifamycins (S and SV) such as rifabutin and rifapentine has provided a new direction on the treatment of diseases due to *Mycobacterium* and

other related species (Wallace *et al*., 1995). Rifabutin is a semi synthetic derivative of rifamycin S and its mode of action involves blocking the action of DNA dependent RNA-polymerase of gram positive bacteria especially *Mycobacterium* species. It is recommended as a first line treatment drug for pulmonary tuberculosis patients and is widely used to treat *Mycobacterium avium* complex (MAC) disease (Brogden and Fitton, 1994).

Rifapentine is a cyclopentyl-substituted rifamycin whose serum half-life is five times that of rifampin (Vernon *et al.*, 1999). It has been shown to be more effective than rifampicin at treating tuberculosis. However rifampicin resistant organisms usually develop cross resistance to rifapentine (Zhang *et al.*, 2011).

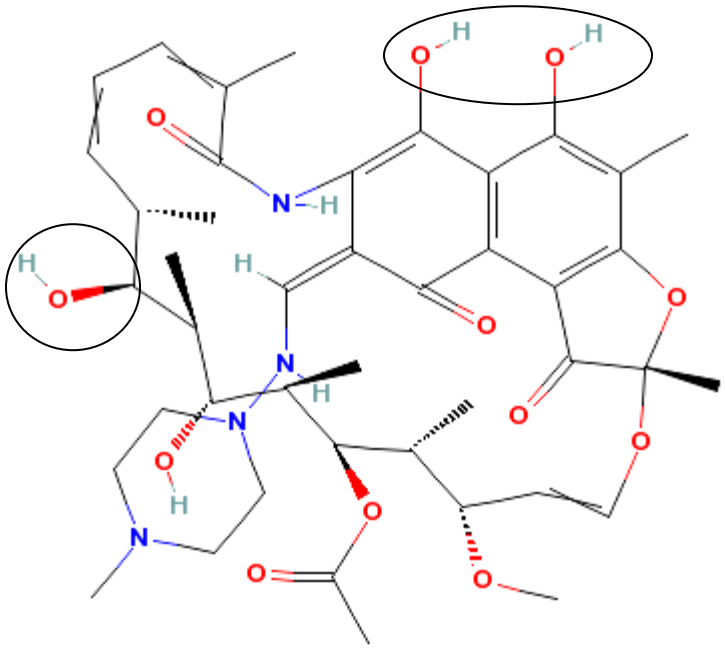

Figure 2: Chemical structure of rifampicin (adapted from http://pathmicro.med.sc.edu/mayer/rifampin.gif). The regions circled indicated the two domains which are responsible for the antimicrobial action of rifampicin (Campbell *et al.*, 2001).

1.2.2. Tetracyclines

Tetracyclines antibiotics inhibit the aminoacyl-tRNA from binding to the ribosome during protein synthesis. This useful class of antibiotics inhibits protein synthesis by binding to the 16S

complex of the 30S ribosomal subunit and thus preventing the charged tRNA from entering the A site (Olson *et al.*, 2006). Three different resistance mechanisms against tetracycline antibiotics have been characterized to date: enzymatic inactivation, active efflux and ribosomal modification. For instance, the plasmid pBR322 has genes that encode a membrane protein which can effectively remove tetracycline from the cell (Allard and Bertrand, 1992). Tetracyclines (derivatives of polycyclic naphthacene carboxamide) possess four hydrocarbon rings. More specifically, they are defined as a subclass of polyketides having an octahydrotetracene-2-carboxamide skeleton.

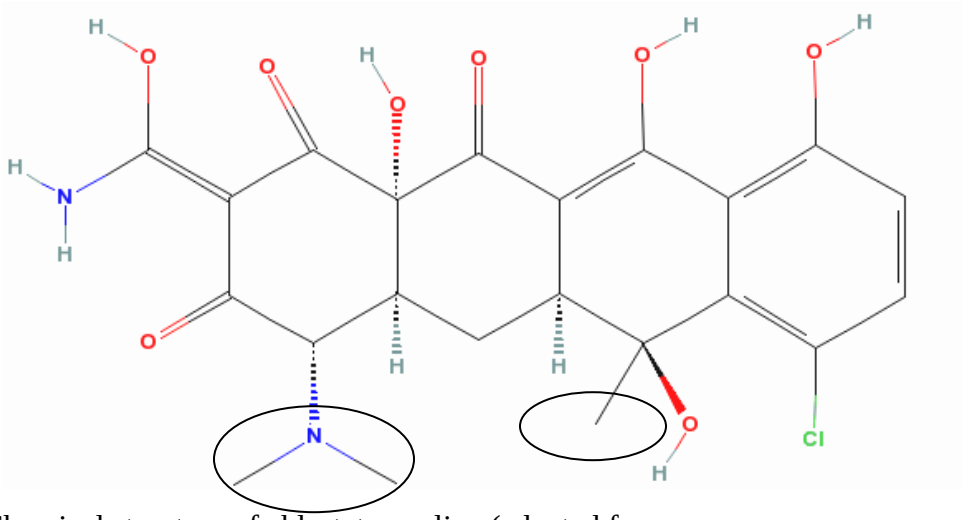

Figure 3: Chemical structure of chlortetracycline (adapted from http://www.lookchem.com/UserFilesUpload/1181-54-0.gif). The circled regions indicate the points were the antibiotic interacts with its substrate to reduce/halt its function (Chi *et al.*, 2011)

1.3. **Antibiotic resistance**

Antibiotic resistance is the innate or acquired ability of a microorganism to survive and thrive in the presence of an antibiotic that should be able to kill it or at least interfere with its cellular growth and reproduction cycles. Antibiotic resistance evolves via natural selection and is often the results of random mutation or lateral gene transfer between two organisms. This phenomenon can also be engineered by applying an evolutionary stress on a population under controlled laboratory conditions. There are four general types of mechanisms that microorganisms use for

antibiotic resistance: Target alteration, drug inactivation, decrease in membrane permeability and active exclusion of the metabolite from the cell.

Table 1: Key features of various antibiotics used in this work

Antibiotic name	**Target site**	**Spectrum**	**Class**	**Resistance mechanism**	**Reference**
Rifampicin	β-subunit of bacterial DNA dependent RNA polymerase	Broad spectrum	Ansamycin	Target alteration	Davies and Davies, 2010
Chlortetracycline	Bacterial 30S ribosomal subunit	Broad spectrum	Tetracycline	Active efflux, ribosome protection (target alteration) and drug inactivation	Davies and Davies, 2010
Erythromycin	Bacterial 50S ribosomal subunit	Gram + cocci	Macrolide	Ribosome (target) modification	Davies and Davies, 2010
Streptomycin	Bacterial 30S ribosomal subunit	Broad spectrum	Aminoglycoside	Target alteration	Davies and Davies, 2010
Kasugamycin	Bacterial 30S ribosomal subunit	Broad spectrum	Aminoglycoside	Target alteration	Davies and Davies, 2010
Chloramphenicol	Bacterial 50S	Gram +,	Amphenicol	Reduced	Davies and

	ribosomal subunit	gram- and anaerobes		membrane permeability, drug inactivation and target alteration	Davies, 2010

1.3.1. Alterations in membrane permeability

Some bacteria have protein receptors on the cell wall which have been found to be involved in the translocation of certain substances across the membrane. Sato and Nakae (1991) reported on the difference in membrane permeability of *Acinetobacter calcoaceticus* and *Escherichia coli* which contributes to the resistance of *A. calcoaceticus* to cephalosporins.

1.3.2. Active efflux

This type of antibiotic resistance involves an energy dependent pump system that actively prevents the intracellular antibiotic from accumulating. Many gram-negative bacteria communicate by *N*-acyl homoserine lactose signals called autoinducers (Pearson *et al.*, 1999). This communication channels also serve as pump centers to actively removed or accumulate a specific substance in the cell. A well-known example of this type of resistance mechanism is found in the plasmid pBR322. This plasmid has a gene that encodes a membrane protein that removes tetracycline from the cell and prevents it from accumulating intracellular. According to a previous study (E Dabbs, personal communication) the expression of *arr* gene (the focus of this work) confers low level resistance to chlortetracycline and spectinomycin in *Escherichia coli*.

1.3.3. Resistance by target alteration

Two novel mechanisms of macrolide resistance of *Pneumococcus* species have been described, which complicate the inference of the genetic mechanism of resistance based on the phenotype.

These two mechanisms involve mutations in the fifth domain (domain V) of the 23s rRNA subunit and mutations of two different ribosomal proteins that constitute part of the assembled 50s rRNA, ribosomal protein L4 and ribosomal protein L22 (Tait-Kamradt *et al.,* 2000). Although such mutations preserve the active site, the protein acquires new biophysical properties that might not be recognized by the antibiotic thus the effects of the antibiotic on the organisms are greatly reduced. This is the principle idea of resistance by target alteration.

1.3.3.1. Rifampicin resistance by target alteration

Several studies indicated there are mutations on the *rpoB* gene in *E. coli* (Jin and Gross, 1988), *M. leprae* (Honore and Cole, 1993) and *M. tuberculosis* (Telenti *et al*, 1993) that results in an altered β-subunit of the RNA polymerase which the antibiotic cannot recognize and bind to (Alexander *et al*., 2003). Such alterations were also observed in rifampicin-resistant *Mycobacterium tuberculosis* and *Mycobacterium leprae* strains (Quan *et al.*, 1999). Some bacterial strains such as *Mycobacterium smegmatis* do not have mutations on the *rpoB* gene that lead to an altered β-subunit but instead they possess an antibiotic inactivation mechanism that involves ADP-ribosylation of rifampicin. The ribosylated antibiotic is reported to have little effect on the growth and proliferation of this particular strain at moderate concentrations (Quan *et al.*, 1999).

1.3.3.2. Spectinomycin resistance by target alteration

Spectinomycin binds to the 30S ribosomal subunit and interferes with the interaction of mRNA and tRNA thus disrupting protein synthesis. Spectinomycin antibiotics have been used to treat sexually transmitted diseases such as gonorrhea with success. Besides inactivation of this drug, a new form of resistance arose from mutations in the 16S ribosomal rRNA and ribosomal protein S5. These mutations lead to an altered 30S ribosomal subunit which can stay active even in the presence of this antibiotic (Kehrenberg and Schwarz, 2007). High level resistance has emerged in the 16S ribosomal RNA in *Pasteurella multocida* (Kehrenberg and Schwarz, 2007).

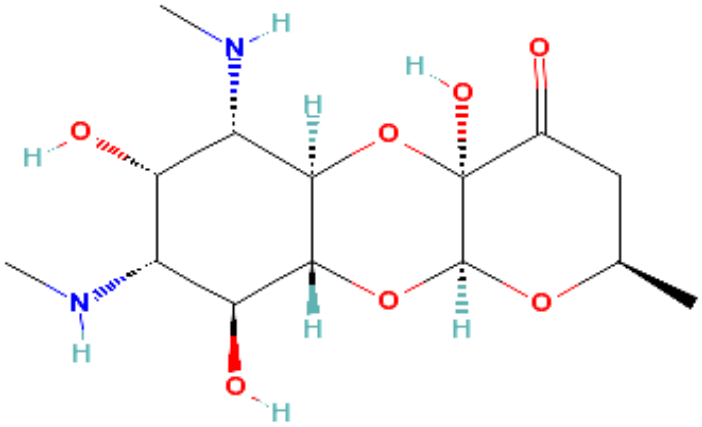

Figure 4: Chemical structure of spectinomycin (adapted from http://pathmicro.med.sc.edu/mayer/spectinomycin.gif).

1.3.4. Drug inactivation

Some organisms produce enzymes that can inactivate the antibiotic by either degrading or changing it to a less or non-effective form. For example, penicillin-resistant strains of bacteria often harbor a plasmid that specifies the gene for β-lactamase that destroys the β-lactam ring based antibiotics (Mathews *et al.*, 2000; Senda *et al.*, 1996).

1.3.4.1. Decomposition of rifampicin

Decomposition is the most widespread of the four rifamycin inactivation mechanisms and has been identified in *Nocardia, Rhodococcus* and *Mycobacterium,* (Tanaka *et al.,* 1996) as well as *Bacillus* species (Andersen *et al.,* 1996). These species produce an oxidoreductase enzyme (monooxygenase) that catalyses the transfer of a hydroxyl group to rifampicin. The subsequent addition of the hydroxyl group is believed to attack the naphthalenyl moiety of rifampicin based on its sequence similarity to many monooxygenases acting on phenolic compounds (Hoshino *et al*., 2009).

1.3.4.2. Glucosylation and phosphorylation of rifampicin

Glucosylation of antibiotics is a resistance mechanism against macrolides and lincomycin antibiotics usually found in *Rhodococcus* spp and *Nocardia* spp (Yawaza *et al*., 1994). It involves the transfer of a β-D-glucose moiety to the C_{23}-OH group of rifampicin acceptor to produces a less toxic derivative of the antibiotic. Phosphorylation of rifampicin occurs at the hydroxyl group at C_{23} and blocks interaction with the RNA polymerase target (Mayers, 2009).

1.3.4.3. ADP-Ribosylation of rifampicin

Reversible ADP-ribosylation is utilized as an enzyme regulatory mechanism in prokaryotes (Baysarowich *et al.*, 2008). The most thoroughly studied example of this type of control mechanism is the ADP-ribosylation of the dinitrogenase reductase from *Rhodospirillum rubrum.* This enzyme is one of the key enzymes in the nitrogen assimilation. Nitrogen fixation is costly to the microbe therefore this process is under tight control. In the presence of fixed forms of nitrogen (ammonia or nitrate salts), the activity of nitrogenase enzyme is rapidly shut off. This is achieved by the addition of an ADP-ribosyl unit to the enzyme (Ludden, 1994). ADP-ribosylation also plays a key role in the mode of action of toxins such as diphtheria and cholera toxins.

In the presence of diphtheria toxin the ADP-ribose portion of NAD is transferred to an aminoacyl transferase II enzyme (Honjo *et al.*, 1968). Following the subsequent ADP-ribosylation of this enzyme, the assembly of amino acids into a polypeptide chain is compromised. Cholera toxin (choleragen) also catalyzes the hydrolysis of NAD to ADP-ribose. Moss and Vaughan (1977) have previously demonstrated that choleragen and its α-promoter can catalyze the hydrolysis of NAD to ADP-ribose and nicotinamide (Morisaki *et al.*, 2000). This NADase activity is analogous to that exhibited by the α-fragment of diphtheria toxin, which has been shown to inhibit protein synthesis through an NAD-dependent ADP-ribosylation of elongation factor II (Moss and Vaughan, 1977). ADP-ribosylation can also be used to inactivate or modify an antibiotic to reduce its toxicity and effectiveness against the host organism.

There are bacterial species that possess the ability to add a functional group to rifampicin which reduces its toxicity and effectiveness towards the organism. Studies (Quan *et al.*, 1999) showed that rifampicin is modified to give a glucosylated or phosphorylated derivative in pathogenic *Nocardia spp*. A variety of other *Mycobacterium* strains such as *M. smegmatis* DSM43756, *M. chelonae* subsp. *abscessus, M. flavescens, M. vaccae,* and *M. parafortuitum* as well as *Gordona* and *Tsukamurella strains* have the ability to inactivate rifampicin by ribosylation (Imai *et al*., 1999). ADP-ribosylation of rifampicin happens on oxygen (O) atom at position C_{23} as opposed to a nitrogen (N) atom in the protein acceptors of various bacterial toxins mentioned above. Mono ADP-ribosyltransferases are predominantly found in prokaryotes while poly ADP-ribosyltransferases in eukaryotes (Hottiger *et al.*, 2010).

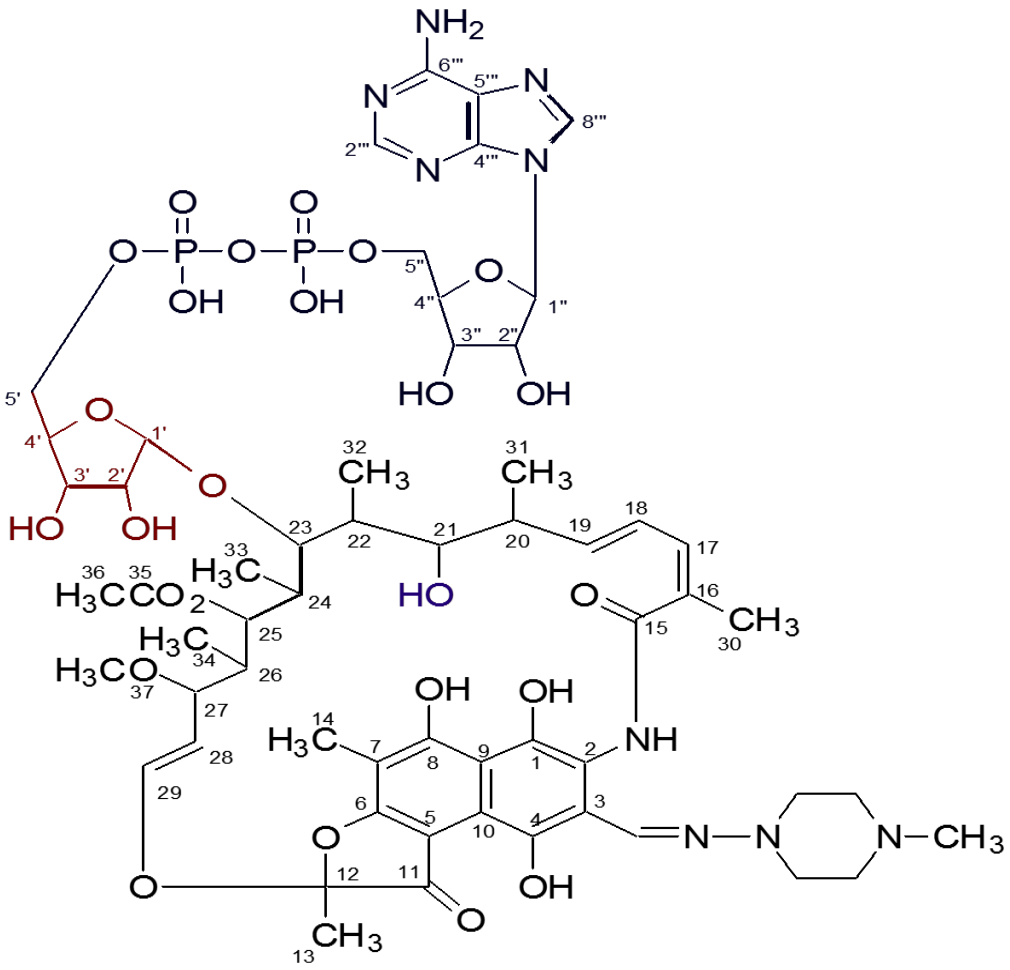

Figure 5: Chemical structure of ADP-ribosylated rifampicin (adapted from Imai *et al.*, 1999).

As rifampicin is a compound that is of semi-synthetic nature (Baysarowich *et al.*, 2008), it is surprising that enzymes that act on this compound in preference to the natural product (rifamycin SV) exist.

Due to the recent emergence of extreme drug resistant pathogenic strains, like *M. tuberculosis* (Daley and Caminero, 2013), it is clear that antibiotic resistant is going to be a huge problem if the appropriate treatment is not implemented soon. To archive this, information regarding the sub-cellular interactions of the pathogenic strains needs to be collected in an attempt to facilitate the development of better and more efficient drugs to treat the infections.

1.4. **The *arr* gene**

ADP-ribosylation is a post-translational modification of primary proteins and/or antibiotic and is involved in various metabolic processes including carcinogenesis (Lakadong *et al.*, 2010). ADP-ribosylation is catalyzed by ADP-ribosyltransferase. These is achieved through the catalysis and transfer of nicotinamide adenine dinucleotide (NAD) and adenine diphosphate ribose (ADPR) respectively, hence the transfer of the ribose moiety to an acceptor molecule which could be either protein or an antibiotic (Dabbs *et al.*, 1995). The ribosylation of proteins in prokaryotes usually leads to the production of potent toxins in species such as *Corynebacterium diphtheriae* (diphtheria toxin), *Pseudomonas aeruginosa* (exotoxin A and exoenzyme S), *Vibrio cholera* (cholera toxin), *Escherichia coli* (labile toxin) and *Bordetella pertussis* (*Bordetella pertussis* toxin).

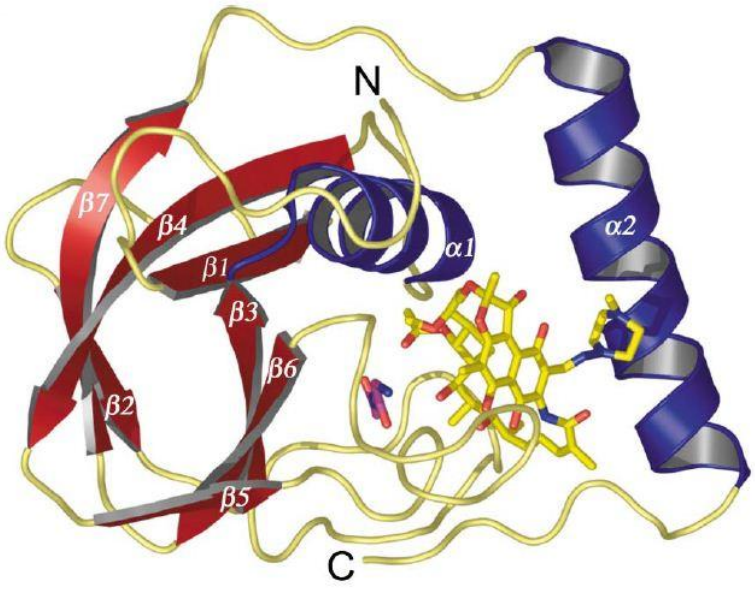

Figure 6: Crystal structure of ADP-Ribosyltransferase (adapted from Baysarowich *et al.*, 2008).

2. Justification

The emergence of antibiotic resistant strains is becoming a huge problem especially in pharmaceutical and medical industries. Antibiotic resistance is mainly driven by the selective pressure imposed on the particular organism. This is usually the result of inadequate exposure to an antibiotic from which a few cells from the bacterial colony managed to survive and recover over time, acquiring resistance towards the antibiotic. *Mycobacterium tuberculosis*, the causative agent for tuberculosis (TB), has been documented to have multiple drug resistant strains. Tuberculosis is now attributed to a number of countries worldwide losing up to 7% of their GDP (World health organization (2009): fact sheet on tuberculosis).

Antibiotic resistance can either be passed on between different bacteria (on a plasmid, transposons or acquiring free/naked DNA from the environment for example) or can result from a mutation in the genetic makeup of the organism. This research aims at the development of such resistance from a mutation in a specific gene (*arr* gene). Understanding how this process happens will assist in the development of new or more effective drugs to treat the infection the particular strain causes

2.1. **Specific objectives**

- *arr* gene expression in *E. coli*
- Random mutagenesis of the *arr* gene
- Screening for increased/decreased resistance on alternative antibiotics
- Select and sequence clones with altered minimum inhibitory concentration (MIC) values

3. Materials and methods

3.1. **Bacterial strains and plasmid vectors used**

Table 2: Bacterial strains and plasmid vectors used in this work

	Phenotype/Markers	**Source**
Bacterial strain		
Escherichia coli MM 294-4	Nal^R , *end A1, hsd*R17, *gyr*A	ER Dabbs
Bacillus subtilis 1 A3-1	Spc^R rifampicin assay organism	ER Dabbs
Rhodococcus erythropolis SQ 1	Cam^S, increased transformability	ER Dabbs
Plasmid vectors		
pGEM	*lac Z'* gene, *Sp6* and T7 RNA polymerase promoters, Amp^R	ER Dabbs
pBstN49	pGEM with *arr* gene insert. Rif^R	Quan *et al*., 1997
pGEM T-Easy	*lac Z'* gene, 3´ thymidine over hangs, *Sp6* and T7 RNA polymerase promoters, Amp^R	Promega

3.2. **Plasmid isolation**

This procedure was carried out by growing a single colony of the bacterial strain in LB (see appendix A) supplemented with 100 µg/ml of ampicillin and 100 µg/ml of nalidixic acid (see appendix A) overnight at 37°C. Aliquots of 1.5 ml were transferred to Eppendorf tubes and microfuged for 2 min at 13000 rpm using a Sigma 1-14 microfuge. The pellet was resuspended in 100 µl of solution I (see appendix A) and left for 2 min after which 200 µl of solution II (see

appendix A) was added and the mixed by gentle inversions. A volume of 200 µl of a pre-chilled solution III (see appendix A) was added and the mixture shaken vigorously then chilled in ice water slurry for 5 min. The solution was microfuged for 5 min at 4°C. The supernatant was transferred to a clean Eppendorf tube and warmed up to 42°C. A volume of 250 µl of isopropanol was added and left at room temperature for 5 min and microfuged for 5 min. The pellet was washed with 150 µl of 96% ethanol and dried at 42°C for 20 min. The dry pellet was then resuspended in 100 µl sterile distilled water (sdH_2O) and 10 µl of RNAse stock solution (see appendix A) was added. The solution was incubated at 65°C for 10 min then chilled on ice.

3.3. **Transformation (*E. coli*)**

A single colony of *E. coli* MM294-4 was grown overnight at 37°C in LB (see appendix A) supplemented with 100 µg/ml of nalidixic acid (see appendix A). 200 µl of the pre-grown culture was added to 20 ml of LB and 200 µl of 20% glucose (see appendix A) and incubated at 37°C for 1 hr 45 min to 2 hr 15 min. The culture was cooled in ice water slurry for 5 min. The culture was transferred to a JA-20 centrifuge tube and centrifuged at a speed of 10000 rpm and temperature of 4°C. The pellet was resuspended in half the volume (10 ml) of $CaCl_2$-Tris buffer and put on ice for 15-30 min. The cells were recovered by centrifugation at 10000 rpm for 5 min at 4°C. The pellet was resuspended in 1/15 volume (1.33 ml) of $CaCl_2$-Tris buffer (see appendix A) and left on ice for 2-24 hr to induce competency. The competent cells were then mixed with the transforming DNA vector in a clean sterile Eppendorf tube at a ratio of 5:1 respectively and put on ice for 15-30 min. The cells were then heat shocked at 42°C for 90 sec. 500 µl of LB was added and the cells incubated at 37°C for 1 hr. The transformants were spread plated on LA (see appendix A) which has been supplemented with the selected screening antibiotic.

3.4. **Gel electrophoresis**

Agarose stock solutions (0.4x, 0.8x and 1.0x) were prepared in 0.5x TBE (see appendix A). After heating the appropriate stock solution so as to liquefy it, a volume of 50 ml of the agarose was poured into a conical flask and 5 µl of 1% ethidium bromide was added and mixed. The agarose was poured into a gel tray with the appropriate comb inserted and left at 4°C for 20-30 min. The

gel was transferred to a gel tank containing 0.5x TBE (see appendix A) and the comb removed to expose the wells. The molecular weight marker and the samples to be analyzed were loaded into the sequential wells with 2-5 µl of tracking dye (see appendix A) added to each sample with exception to the molecular weight marker. The electrophoresis was conducted with a power source at 100 V at room temperature for 1 hr or until adequate separations has been achieved and the gel was viewed with the Biorad Gel-Doc system.

For low melting agarose gel electrophoresis, stock solution of 0.8% low gelling agarose was prepared in 0.5x TBE (see appendix A). A volume of 40 ml was poured into a conical flask and 4 µl of 1% ethidium bromide was added. The gel was polymerized at 4°C for 20-30 min. The gel was transferred to an electrophoresis tank with chilled 0.5x TBE. The samples were loaded with the tracking dye and the electrophoresis run at 80 V at 4°C for 1 hr or until adequate separation has been achieved. The gel was viewed with the Biorad Gel-Doc system

3.5. **DNA manipulation techniques**

3.5.1. Phenol-chloroform isolation

Following the isolation of plasmid DNA a phenol-chloroform extraction would be performed. The volume of the plasmid DNA solution was filled to 300 µl with TE buffer (see appendix A) if it was less. One third volume (100 µl) of phenol solution was added and the tubes were mixed by ten inversions. The mixture was centrifuged at 13000 rpm for 3 min after which the top layer was transferred to a new Eppendorf tube and the procedure repeated two to three more times with the sequential additions of 10 µl of 1 mM Tris-HCl buffer (see appendix A) between the transfers. The solution was centrifuged and the top layer transferred to a new Eppendorf tube. Twice the volume (600 µl) of chloroform was added and the tubes mixed by ten inversions. The solution was centrifuged at 13000 rpm for 5 min and the top layer was treated with salt-ethanol precipitation technique outlined in 3.5.2.

3.5.2. Salt-ethanol precipitation

The plasmid DNA was precipitated by adding half volume of 1 M NaCl (see appendix A) and two fold volume of 96% ethanol. The DNA was left to precipitate out at -20°C overnight and pelleted at 13000 rpm for 30 min at 4°C. The supernatant was discarded and the pellet dried for 20 min at 42°C. The dry pellet was resuspended in 100 µl of sterile distilled water.

3.5.3. Ligations

The ligations were done by adding the appropriate vector and insert at a ratio of 1:5 – 1:10 respectively. Ligase buffer and ligase enzyme (both supplied by Promega) were added to the concentrations outlined by the manufacturer in the instruction protocol.

3.5.4. Cloning of EP-PCR products (TA cloning)

The cloning of EP-PCR (error prone PCR) products was carried out by a TA cloning technique with the Promega pGEM T-Easy cloning vector kit. Prior to cloning, the pGEM T-Easy vector was briefly microfuged and the 2x ligation buffer vigorously vortexed to ensure they are properly mixed. The ligation buffer, vector (pGEM T-Easy), EP-PCR product and T4 DNA ligase (3 Weiss units/µl) were added at volumes of 5, 0.5, 3 and 1 µl respectively. The mixture was filled upto 11 µl with sdH_2O and incubated overnight at 4°C. The ligation mixture was boiled for 10 min at 65°C and placed on ice or stored at -20°C until needed.

3.6. **Gel extraction**

3.6.1. Low melting agarose extraction

Following low melting agarose gel electrophoresis, the required band was excised using a scalpel under UV light and place in an Eppendorf tube. The tube was heated to 65°C in a water bath so as to melt the agarose gel. The liquefied gel was subjected to phenol-chloroform isolation followed by salt-ethanol precipitations as stated in 3.5.1 and 3.5.2 respectively.

3.6.2. Freeze-squeeze gel extraction

Following gel electrophoresis, the required band was cut out using a scalpel under UV light and transferred into an Eppendorf tube. The agarose containing the desired band was crushed and frozen at -70°C for 30 min then thawed at room temperature. The Eppendorf tube was centrifuged for 6 min at 13000 rpm and the supernatant was transferred to a new tube. The pellet was again crushed, frozen, thawed and centrifuged. The supernatant from the second round was pooled together with the one from the first round and subjected to phenol-chloroform and salt-ethanol precipitation as in section 3.5.1 and 3.5.2.

3.7. **Inactivation assays**

The ability of the clones to inactivate rifampicin was assayed using the plate inactivation method. In order to achieve accurate results two approaches were adopted for this purpose: *In vitro* and *In vivo* inactivation assay. The former refers to using sub-cellular components of a bacterial cell to assess the effect/impact of an experiment while the latter refers to using intact bacterial cells to obtain a similar objective. Living organisms contain a complicated system of metabolic pathways, proteins, nucleic acids, other organic and inorganic compounds which all interact to sustain life. Since *in vitro* inactivation assay requires disrupting or even killing the cell to get to the intracellular components, it is easy to come to a wrong conclusion when analyzing the results obtained from this assay. However it offers a simplified module to study and extrapolate data on a specific intracellular component without interference. *In vivo* inactivation assay, however, uses intact cells to observe the overall effect on the whole cellular metabolic pathways. This assay is more preferred in molecular biology since it gives a better picture of how the organism will react to a particular stressor or stimulant. For the purpose of this study both methods were used to study the effect of random mutagenesis of the *arr* gene on rifampicin resistance

3.7.1. In vitro inactivation assay

The clone containing the required plasmid was cultured in LB supplemented with 100 µg/ml of ampicillin (see appendix A) and 100 µg/ml of rifampicin (see appendix A) overnight at 37°C. The cells were harvested at 15000 rpm in a JA20 rotor for 15 min. The pellet was washed twice with sonication buffer and resuspended in 1 ml sonication buffer. The cells were sonicated for 10 sec 4 times over ice in Eppendorf tubes and the homogenate recovered at 15000 rpm for 5 min in a JA20 rotor. The following reaction mixture was setup:

Table 3: Reaction mixture for *In vitro* inactivation assay

Reagent	Volume (µl)
Sonication Buffer	31.5
Antibiotic (10 mg/ml of Rif)	1.5
Co-factor (NAD or UDPG)	15
Co-enzyme A	15
MgAc (2 mM)	15
Glutathione	7.5
LDR	6
Cell homogenate	58.5
Total Volume	150 µl

The reaction mixture was incubated for 14-16 hr at 37°C and boiled for 10 min. A volume of 65 µl was added to the wells of a bioassay plate (half agar plates spread with a confluent growing *Bacillus subtilis* culture) then incubated at 4°C for 4 hr and 42°C for 4-5 hr and finally at 37°C overnight.

3.7.2. In vivo inactivation assay

The clone containing the required plasmid was cultured in LB supplemented with 100 µg/ml of ampicillin and 100 µg/ml of rifampicin overnight at 37°C. The reaction mixture was setup as in table 4 below.

Table 4: Reaction mixture for *In vivo* inactivation assay

Reagent	Volume (µl)
Sonication Buffer	31.5
Antibiotic (10 mg/ml of Rif)	1.5
Co-factor (NAD or UDPG)	15
Co-enzyme A	15
MgAc (2 mM)	15
Glutathione	7.5
LDR	6
Pre-grown culture	58.5
Total Volume	150 µl

The reaction mixture was incubated for 14-16 hr at 37°C and boiled for 10 min. A volume of 65 µl of the reaction mixture was added to the wells of a bioassay plate (half agar plates spread with a confluent growing *Bacillus subtilis* culture) then incubated at 4°C for 4 hr and 42°C for 4-5 hr and finally at 37°C overnight

3.8. **Random mutagenesis**

3.8.1. The polymerase chain reaction (PCR)

The polymerase chain reaction is a molecular biology technique that uses the natural process of DNA replication to amplify copies of a DNA template. The newly synthesized copies serve as templates for the next cycle and these results in an exponential amplification. In theory, a single

copy of DNA will generate over a million copies of DNA in twenty amplification cycles. PCR works in a three step manner: denaturation, annealing and elongation.

During denaturation, the DNA is heated to its melting points so as to separate the two strands to allow for primer annealing. *In vivo*, this process is facilitated by enzymes such as DNA helicase and DNA binding proteins. After primer annealing at cooler temperatures, the *Taq* DNA polymerase is allowed to catalyze the elongation of the two respective strands using the parent strand as the template. PCR employs a heat stable *Taq* polymerase isolated from *Thermus aquaticus* since it uses high temperatures to denature the native DNA.

To achieve high and efficient amplification of the target DNA the conditions within the reaction mixtures and the environment were the PCR reaction proceeds need to be optimized. Usually this involves addition of precise concentrations of Mg^{2+} ions and buffered solutions to the reaction mixture.

3.8.2. Error prone PCR

Often when probing the structure or function of a protein or nucleic acid, a library of mutant strains is generated and the desirable strains selected by a specific screening method (Cadwell and Joyce, 1994). The substitution of Mg^{2+} ions with Mn^{2+} ions is one of the factors that can greatly reduce the fidelity of the DNA polymerase leading to more random base substitutions. The error rate of *Taq* polymerase is the highest of the known thermostable DNA polymerases, in the range of 0.1×10^{-4} to 2×10^{-4} per nucleotide per pass of the polymerase, depending on reaction conditions (Cadwell and Joyce, 1994).

3.9. **Error prone polymerase chain reaction (EP-PCR)**

Error-prone PCR was conducted under the parameters shown below in tables 5, 6 and 7 below.

Table 5: The reaction mixture used for EP-PCR

	Concentration of stock solution	Volume added (µl)	Concentration in final mixture (50 µl)
sdH_2O	-	30.75*	-
Taq buffer	10x	5.0	1x
$MgCl_2$	25 mM	3.0*	1.5 mM*
$MnCl_2$	50 mM	0.0*	0.0*
dNTP's	2 mM	5.0	200 µM each
DMSO	-	2.0	4%
Forward primer	100 µg/µl	1.0	
Reverse primer	100 µg/µl	1.0	
Template DNA	100 ng/µl	2.0	
Taq polymerase	5 units/µl	0.25	1.25 units

*These volumes were varied (as shown in table 6 below) to exploit the error rate of *Taq* polymerase but keeping the total reaction mixture volume constant at 50 µl.

Table 6: Different conditions used for EP-PCR

Conditions	$MnCl_2$ (50 mM)		$MgCl_2$ (25 mM)		sdH_2O
	Concentration (mM)	Volume (µl)	Concentration (mM)	Volume (µl)	Volume added (µl)
1	0.5	0.5	1.0	2.0	31.25
2	0.5	0.5	1.5	3.0	30.25
3	0.5	0.5	2.0	4.0	29.25
4	0.5	0.5	7.0	14.0	19.25
5	1.0	1.0	1.5	3.0	29.75

Table 7: The temperature profiles used for EP-PCR

Cycle	Temperature (°C)	Duration (Sec)
1. *Taq* polymerase activation	95	180
2. Denaturation	95	30
3. Annealing	60	40
4. Extension	72	30
5. Final extension	72	600
6. Holding step	4	∞

3.10. **Minimum inhibitory concentrations (MIC)**

The minimum inhibitory concentration was determined by spot testing. Different concentrations of antibiotics (kasugamycin, spectinomycin, chlortetracycline, erythromycin and chloramphenicol) were prepared in a LA plate and dried for 48 hr at 37°C. A single colony from freshly grown clones/strains was placed in the wells of a spot test plate (replica plate) filled with 100 µl of sterile distilled water. A sterile 16 point inoculating pad was dipped into the plate and placed on the LA-antibiotic plate for 10 sec. This process was repeated for the other antibiotic

concentrations and all the LA-antibiotics plates were incubated at 37°C for up to 72 hr. The confluency of growth was assessed.

3.11. **Marker rescue technique**

Marker rescue technique was performed to screen a large number of clones for any increase in the minimum inhibitory concentration (MIC) of different antibiotics. Following transformation of *E. coli* MM294-4 with the PCR product of interest, the pool of colonies found on the selective media (LA supplemented with ampicillin) was washed off with 5-10 ml of LB into a sterile conical flask. The cells were grown at 37°C for 4-6. The plasmid DNA was extracted using the alkaline lysis method as stated in section 3.2. The isolated DNA was used to transform *E. coli* MM294-4 as stated in section 3.3. Aliquots of 100-500 µl of the cells were spread on increasing concentration of the antibiotic in question. A concentration of 100 µg/ml of ampicillin was added to all the LA plates to maintain the plasmid.

3.12. **DNA sequencing and analysis**

Sequencing was done by Inqaba Biotech and the sequencing results analyzed and edited by Finch TV (see section 4.5). To align regions with similarities between the clones and/or the parent strain, Basic Local Alignment Search Tool (BLAST) and Pairwise alignment tools (from sources listed in table 8 below) were used with the tools default parameters setup by the respective programmes. These programmes work solely by looking for sequences that match or closely resemble the query sequence.

The FASTA programme was used to search and compare the protein sequence specifically with those already on the database while the translation of the sequenced nucleotides to a protein sequence was done using ExPASy proteomics Server with the default parameters as well.

Table 8: Programmes used for DNA sequence analysis and alignments

Programme	Address
BLAST	http://www.ncbi.nlm.nih.gov/BLAST/
FASTA	http://www.ebi.ac.uk/fasta33/
ExPASy Proteomics Server (Translate tool)	http://www.expasy.ch/tools/dna.html
Pairwise alignment	http://www.ebi.ac.uk/Tools/emboss/align/index.html
Gene search	http://www.ncbi.nlm.nih.gov/

4. Results and Discussion

4.1. **Plasmid DNA isolation and purification**

This procedure was carried out as stated in section 3.2 above. The plasmid DNA isolated and used in for error prone PCR is pBSTN49 plasmid. It was derived from ligation of a *Mycobacterium smegmatis arr* gene into pGEM cloning vector (see appendix B). Upon completion of plasmid DNA isolation, as in section 3.2, the pBSTN49 plasmid was run on a 0.8% agarose gel electrophoresis and visualized under ultraviolet light using the Biorad Gel-Doc system. Upon visualization the present bands were compared to known standards from a Fermentas 1 kb gene ruler.

4.2. **Error prone PCR**

Error prone PCR was conducted as shown in table 5 and 6. The aim of this experiment was not to change the sequence such that it bears no similarity to the donor sequence but rather to simulate natural selection in introducing fewer random mutations. The correct error rate is crucial in a directed evolution project because protein functionality has to be maintained to some extent. In establishing the ideal/optimum EP-PCR condition in terms of mutations introduced, a 0.7% error rate was targeted for this study (Cline *et al.*, 1996). Given that the *arr* gene is about 432 bp, a 0.7% error rate would result in no more than 3 nucleotide changes per clone. This would result in a rather moderate probability of amino acid change. The total number of simultaneous base changes that results in amino acid change can be calculated by (Patrick *et al.*, 2003):

$$V_x = \frac{3^x N!}{x!(N - x)!}$$

were x is the number of nucleotides that will be changed simultaneously and N is the sequence length. It was found that the optimal condition in terms of inducing random mutagenesis was condition 2. However, condition 3 was found to be too drastic producing 13 to 16 nucleotide

changes. The results show that an increase in the concentration of Mn^{2+} ions compromises the fidelity of *Taq* polymerase. Concentrations above 0.5 mM decrease the amplification efficiency. A similar trend is observed with an increase of Mg^{2+} ions of which optimum concentration was observed to be around 1.5 mM. Concentration between 1.5 mM and 2.0 mM drastically increase the error rate to levels which the translated protein is too mutated and impairs its functionality.

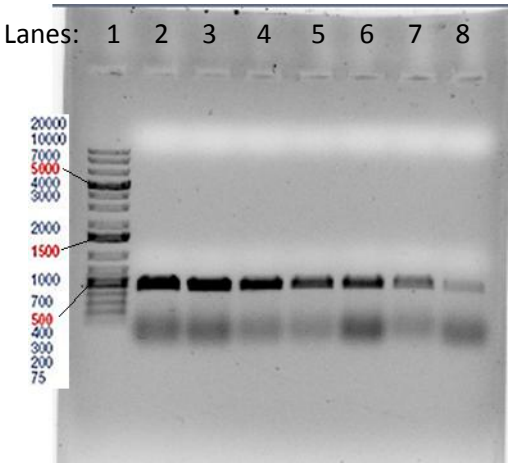

Figure 7: Effects of Mg^{2+} on error prone PCR. Lanes 1 contain molecular weight marker. Lane 2 contains 1.0mM Mg^{2+}. Lane 3 contains 1.5mM Mg^{2+}. Lane 4 contains 2.0mM. Lane 5 contains 2.5mM Mg^{2+}. Lane 6 contains 3.0mM Mg^{2+}. Lane 7 contains 4.0mM Mg^{2+} and lane 8 contains 7 mM Mg^{2+} respectively.

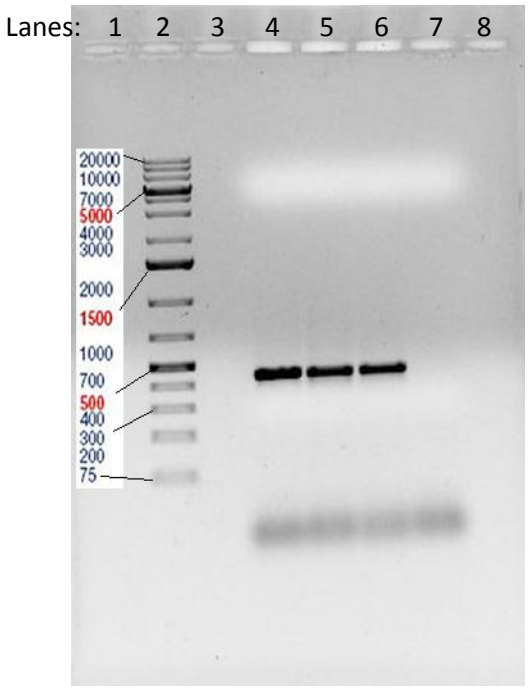

Figure 8: Effects of Mn^{2+} on error prone PCR. Lane 2 contains molecular weight marker. Lane 4 contains 0.3mM Mn^{2+}. Lane 5 contains 0.4mM Mn^{2+}. Lane 6 contains 0.5 mM Mn^{2+} and lane 7 contains 1.0 mM Mn^{2+} respectively.

The EP-PCR products were ligated into pGEM T-Easy vector (see appendix B) and transformed into *E. coli.* The clones produced were sent for sequencing to analyze the mutations that might have been introduced during EP-PCR. Figures 19-27 (see appendix B) indicate the results obtained from the sequencing of representative individual clones. The results are summarized in table 9.

4.3. **Marker rescue technique**

Marker rescue technique was used to screen for clones with a higher MIC. Approximately 4000 colonies were pooled and spread on plates with increased concentration of streptomycin, kasugamycin, erythromycin, chlortetracycline or chloramphenicol. However no colonies from the clones showed a significant increase in MIC on any of these antibiotics.

4.4. **Inactivation assays**

Clones generated were assayed for any change in rifampicin inactivating ability using *in vivo* and *in vitro* inactivation assays as described in section 3.7. From the clones generated (table 9), p8 and p20 showed no inactivation present. It is worth noting that bith this clones were generated using condition 3 from PCR which is rather drastic in terms of the number of mutations introduced. Clone p157 and p11 showed reduced resistance (about halved) when compared to the non-mutated variant. This could be due to the amino acid change which will affect the tertiary and folding kinetics of the protein and thus ultimately affect function. However the rest of the clones (p4, p7, p10, p12 and p15) retained the Rif^R activity and all showed evidence of inactivating rifampicin.

4.5. **Clones sequenced**

Clone generated through EP-PCR, were sent to Inqaba Biotech for sequencing. The raw data was viewed, analysed (or edited if necessary) and saved as a proper nucleotide sequence using FinchTV (downloaded from http://www.geospiza.com/Products/finchtvdlrequest.shtml). FinchTV is an analytic chromatogram trace viewer developed by Geospiza for genetic analysis. Using this software, the chromatogram generated during sequencing could be viewed as a linear nucleotide sequence.

Table 9: Clones sequenced

Clone	Mutation type	Nucleotide change	Amino acid change	Error rate (%)	Condition used in error prone PCR	Phenotype
p8	16 changes. Transversion and transition.			3.7	3	Loss of Rif^R
p11	Transition and transversion	A to G, T to A and A to G	D to G, Y to N and E to G	0.69	2	Reduced Rif^R
p12	Transition	C to T and C to T	H to Y and R to C	0.46	2	Rif^R
p15	Transition, Transversion	C to T, G to C(silent)	H to Y	0.46	2	Rif^R
p20	13 changes. Transversion and transition.			2.7	3	Loss of Rif^R
p157	Transversion	G to T	S to I	0.23	1	Reduced Rif^R
p4	None	None	None	0	4	Rif^R
P10	None	None	None	0	1	Rif^R
p7	None	None	None	0	5	Rif^R

4.6. **Minimum inhibitory concentration**

To establish the minimum concentration of various antibiotics required to inhibit growth, minimum inhibitory concentration tests were performed on the clones generated. To achieve this, LA plates supplemented with 100µg/ml of ampicillin were prepared with increasing concentrations of a specific antibiotic (erythromycin, chlortetracycline, kasugamycin, spectinomycin or chloramphenicol). An actively growing culture was streaked onto the LA-antibiotic plate then incubated further at 37°C for 72 hr. Erythromycin and kasugamycin concentrations were increased by 50µg/ml (starting from 50µg/ml). The maximum concentration for erythromycin was 300µg/ml while the maximum for kasugamycin was 350µg/ml. Chlortetracline and streptomycin concentrations were setup with increments of 5µg/ml (from 5µg/ml minimum) up to a maximum of 35µg/ml. Chloramphenicol concentrations however were only increased by 2.5µg/ml (starting from 5µg/ml) upto a maximum of 25µg/ml. The MIC value

was scored based on the confluence of growth on each antibiotic. Figures 9-11 represents the results obtained herein.

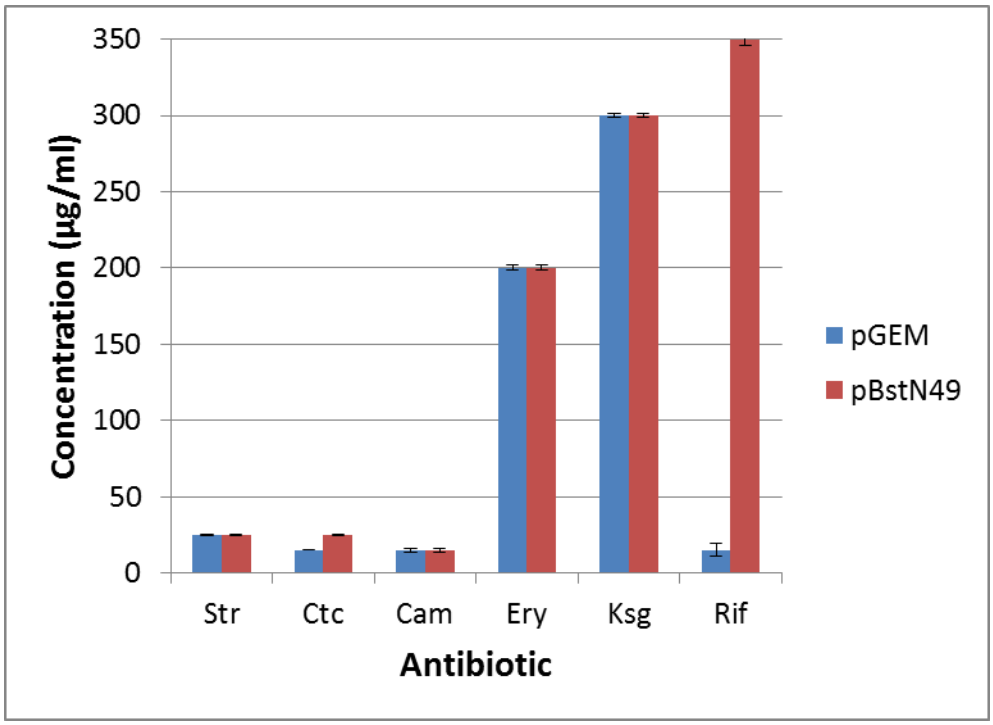

Figure 9: Representation of the MIC values obtain for vector only control (pGEM) and non-mutated clone (pBstN49).

From figure 9, it can be established that pBstN49 does not confer much increased in resistance to streptomycin, chloramphenicol, erythromycin and kasugamycin (the MIC values of the pGEM and pBstN49 clones are identical). However it confers about 25% increase in the resistance of chlortetracycline (the MIC value of pGEM was determined at 20µg/ml while the MIC value of pBstN49 was determined at 25µg/ml). The resistance of rifampicin conferred by pBstN49 is quite pronounced increasing to over 350µg/ml.

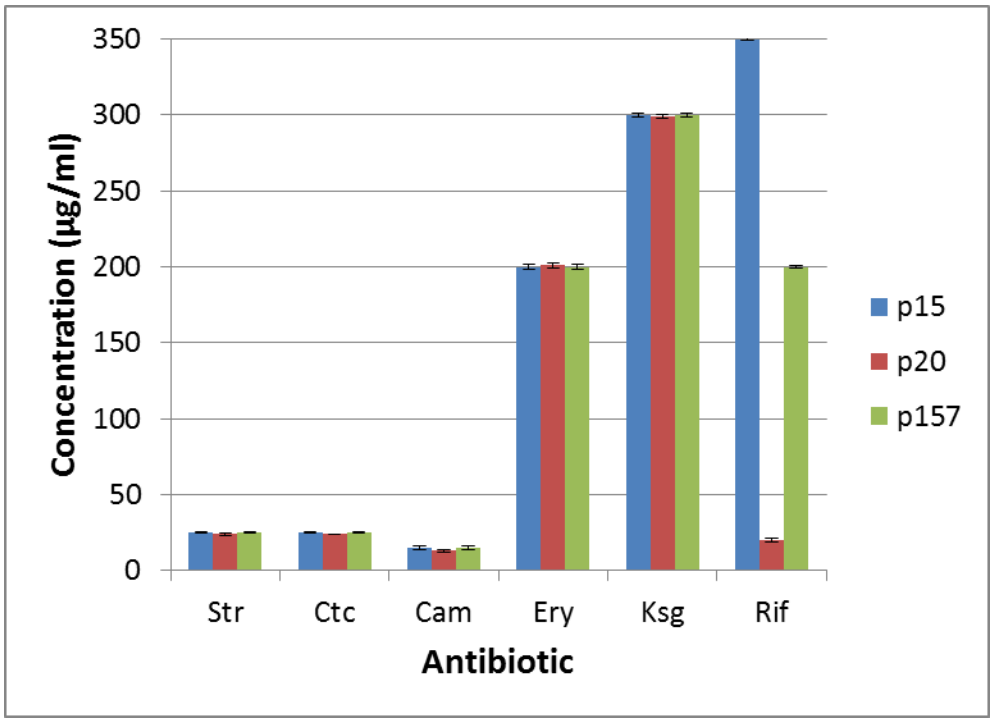

Figure 10: Representation of the MIC values obtained for clones p15, p20 and p157.

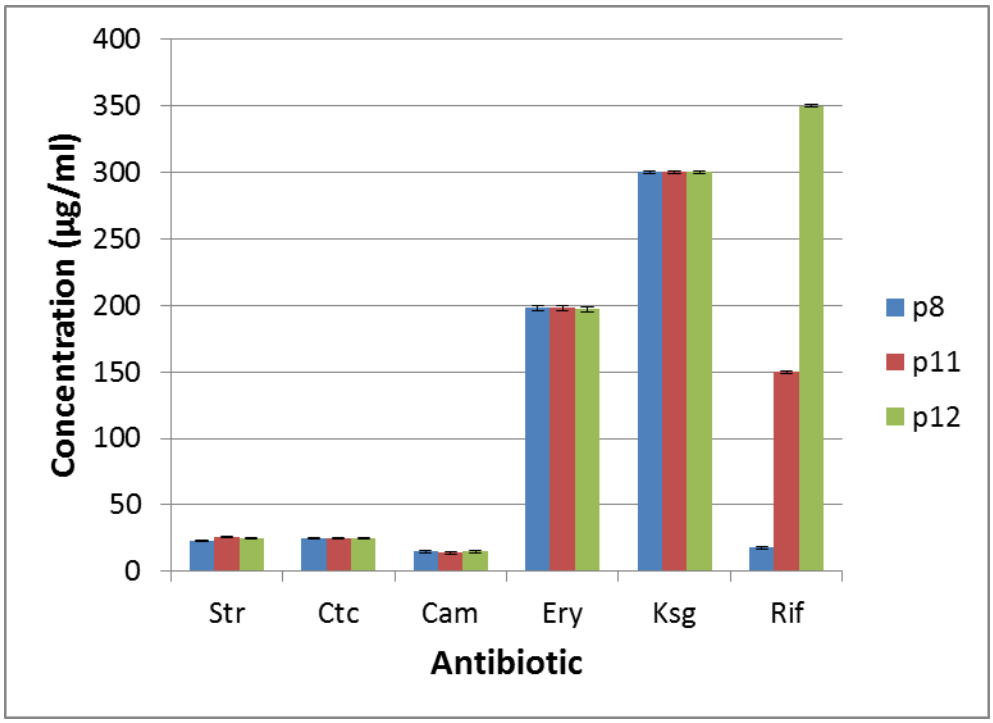

Figure 11: Representation of the MIC values obtained for clones p8, p11 and p12.

4.7. **Discussion**

4.7.1. Clones sequenced

ExPASy Proteomics Server (Translate tool) was used to predict the amino acid sequence of the sequenced clones and these clones were compared to the known sequence of the *arr* gene. Figure 12 to 18 below show the pairwise alignment files obtained.

```
arr              1 VVANPPKPFEVHESGAYLHGTKADLKVGDRLVPGRESNFEAGRIMKHVYI     50
                   ||||||||||:||||||||||||||||||||||||||||.|||||||
p12              1 VVANPPKPFEVYESGAYLHGTKADLKVGDRLVPGRESNFEAGCIMKHVYI     50

arr             51 TQTLDAAVWGAELAVGEGRGRIYIVEPEGEIEDDPNVTDKKLPGNPTRSY    100
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
p12             51 TQTLDAAVWGAELAVGEGRGRIYIVEPEGEIEDDPNVTDKKLPGNPTRSY    100

arr            101 RTREPVRIVGELTDWEGHSPEQIAAMREGLEDLRRKGLAVIYD    143
                   |||||||||||||||||||||||||||||||||||||||||||
p12            101 RTREPVRIVGELTDWEGHSPEQIAAMREGLEDLRRKGLAVIYD    143
```

Figure 12: Alignment of the p12 clone amino acid sequence with the Arr protein. The two nucleotide changes translated to two amino acid changes (H to Y and R to C).

Although the polar amino acids that were changed in clone p11 were replaced by polar amino acids the nature of the new amino acids is different in size and charge. The wild type has two extra charged units while the mutant protein has three smaller amino acids in their place. This change in amino acid sequence resulted in lower resistance to rifampicin

```
arr              1 VVANPPKPFEVHESGAYLHGTKADLKVGDRLVPGRESNFEAGRIMKHVYI     50
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
p11              1 VVANPPKPFEVHESGAYLHGTKADLKVGDRLVPGRESNFEAGRIMKHVYI     50

arr             51 TQTLDAAVWGAELAVGEGRGRIYIVEPEGEIEDDPNVTDKKLPGNPTRSY    100
                   ||||.||||||||||||||||||||||||||||||||||||||||||||||.
p11             51 TQTLGAAVWGAELAVGEGRGRIYIVEPEGEIEDDPNVTDKKLPGNPTRSN    100

arr            101 RTREPVRIVGELTDWEGHSPEQIAAMREGLEDLRRKGLAVIYD    143
                   ||||||||||.|||||||||||||||||||||||||||||||||
p11            101 RTREPVRIVGGLTDWEGHSPEQIAAMREGLEDLRRKGLAVIYD    143
```

Figure 13: Alignment of the p11 clone amino acid sequence with the Arr protein. A total of three amino acid changes were observed (D to G, E to G and Y to N).

The deletion of a thymidine residue at position 432 of the p8 clone sequence (figure 14) resulted in a frameshift mutation that changed the nature of the latter half of the protein. The first 11 nucleotide changes preceding the deletion resulted in a total of 8 amino acid changes.

```
arr              1 VVANPPKPFEVHESGAYLHGTKADLKVGDRLVPGRESNFEAGRIMKHVYI     50
                   ||||||||||||||||||.||||||||||||||||||.||||.|||
p8               1 -VANPPKPFEVHESGAYLHGAKADLKVGDRLVPGRESNFEAWRIMKLVYI     49

arr             51 TQTLDAAVWGAELAVGEGRGRIYIVEPEGEIEDDPNVTDKKLPGNPTRSY    100
                   ||||||||||||||.||||.|:|.||||||||||||||:|||||||||
p8              50 TQTLDAAVWGAELAVCEGRGWIHIDEPEGEIEDDPNVTDKRLPGNPTRS-     98

arr            101 RTREPVRIVGELTDWEGHSPEQIAAMREGLEDLRRKGLAVIYD-------    143
                     ..||.:.|.    .|:||.. ..:|.|......:| :.||.
p8              99 --TAPVSLCGS----SGNSPTG-GGIRRGRSLPCGRG-SRIYGTRGSRSS    140

arr            143 -------------------------    143

p8             141 MTRIEFPRPPWRPGACDVGXXXXXXX    166
```

Figure 14: Alignment of the p8 clone amino acid sequence with the Arr protein.

Clone p157 had a single notable change from S to I in the β4 subunit (refer to figure 6) of the protein. This mutation resulted in a reduced rifampicin resistance.

```
p157             1 LXANPPKPFEVHESGAYLHGTKADLKVGDRLVPGRESNFEAGRIMKHVYI     50
                   : ||||||||||||||||||||||||||||||||||||||||||||||||||
arr              1 VVANPPKPFEVHESGAYLHGTKADLKVGDRLVPGRESNFEAGRIMKHVYI     50

p157            51 TQTLDAAVWGAELAVGEGRGRIYIVEPEGESEDDPNVTDKKLPGNPTRSY    100
                   ||||||||||||||||||||||||||||||.||||||||||||||||||
arr             51 TQTLDAAVWGAELAVGEGRGRIYIVEPEGEIEDDPNVTDKKLPGNPTRSY    100

p157           101 RTREPVRIVGELTDWEGHSPEQIAAMREGLEDLRRKGLAVIYD    143
                   |||||||||||||||||||||||||||||||||||||||||||
arr            101 RTREPVRIVGELTDWEGHSPEQIAAMREGLEDLRRKGLAVIYD    143
```

Figure 15: Alignment of the p157 clone amino acid sequence with the Arr protein. The nucleotide change resulted in a change from S to I.

The 13 nucleotide changes that occurred in clone p20 resulted in a loss of resistance. This can be contributed to the radical change that happened to the amino acid sequence which ultimately changes the folding thermodynamics of the tertiary structure and possibly even the physicochemical properties of the protein.

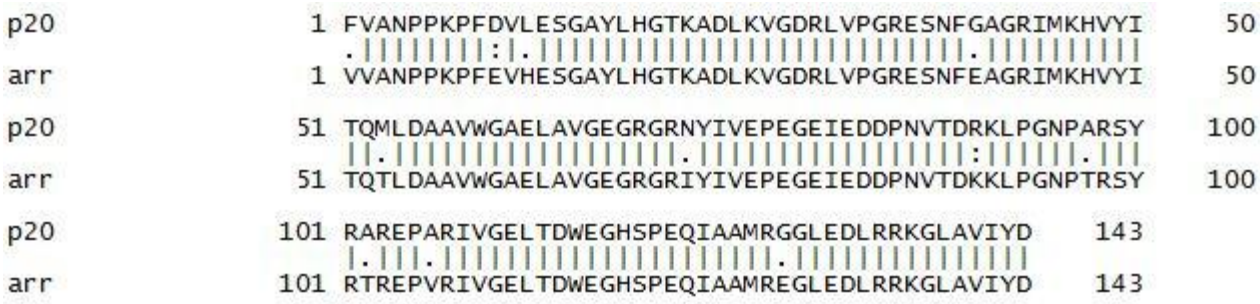

Figure 16: Alignment of the p20 clone amino acid sequence with the Arr protein. The 13 changes in the nucleotide sequence produced a total of 11 amino acid changes.

The change from tyrosine (Y) to histidine (H) in clone p15 had little effect on the activity of the Arr protein. This can be due to the polar nature of the two amino acids hence substituting Y with H does not drastically change the conformational structure of the protein and it can retain its original function.

Figure 17: Alignment of the p15 clone amino acid sequence with the Arr protein. The change from G to C on the nucleotide sequence produced a silent mutation while the second change (C to T) led to a change in the protein sequence from H to Y.

Clones p4, p7 and p10 had no changes to the nucleotide sequence from EP-PCR so it was expected that there will be no change in the protein sequence. Figure 18 below represent the results from the alignment of each of these sequences with the Arr protein sequence.

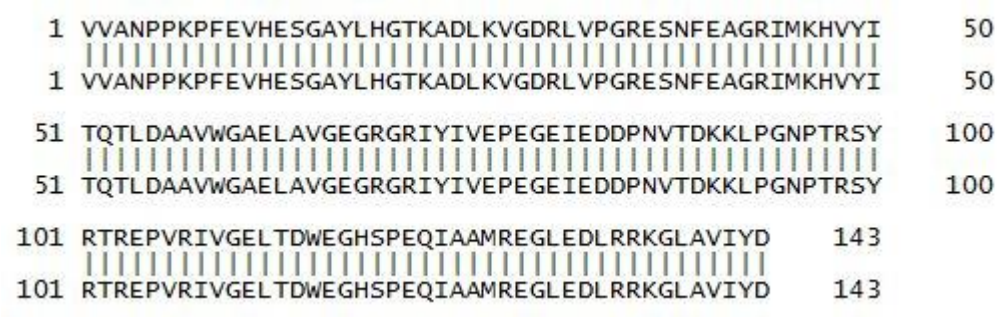

Figure 18: Alignment of the p10, p4 and p7 clone amino acid sequence with the Arr protein. As expected there were no changes to protein sequence since there were no changes in the nucleotide sequences.

4.7.2. Conclusion

By producing a sequence with a mutation that conferred resistance to a novel antibiotic, a new clone could be available to study the effects and/or mechanisms of antibiotic resistance and perhaps develop novel, semi synthetic antibiotics to treat infection before they become a worldwide pandemic. The introduction of new drugs to the market would be of great benefit to treat infection, for instance tuberculosis has been treated with the same drug for about 40 years now (Andries *et al.*, 2005).

Although error prone PCR was highly effective in generating mutations in the *arr* gene, based on this work evolving novel antibiotic resistance is a difficult task to accomplish. The process of evolution of antibiotic resistance is driven by the selective pressure placed on microbes by the presence of antimicrobial agents and this constant selective pressure is difficult to replicate in the lab. More effective techniques as well as the production and sequencing of more clones should be implemented in future to accomplish this task.

5. References

Allard, J.D. and Bertrand, K.P. 1992. Membrane topology of the pBR322 tetracycline resistance protein: *tetA-phoA* gene fussions and implication for the mechanism of the TetA membrane insertion. *The American Society for Biochemistry and Molecular Biology* **267**: 17809-17819.

Alexander D.C., Jones J.R.W., and Lui J. 2003. A Rifampin- hypersensitive mutant reveals differences between strains of *Mycobacterium smegmatis* and presence of a novel transposon, IS*1623. Antimicrobial Agents and Chemotherapy* **47**: 3208-3213.

Andersen, S.J., Quan, S., Gowan, B. and Dabbs, E.R. 1996. Monooxygenase-like sequence of a *Rhodococcus equi* gene conferring increased resistance to rifampin by inactivating this antibiotic. *Antimicrobial Agents and Chemotherapy* **41**: 218-221.

Andries, K., Verhasselt, P., Guillemont, J., Gohlmann, H.W.H., Neefs, J., Winkler, H., van Gestel J., Timmerman, P., Zhu, M., Lee, E., Williams, P., de Chaffoy, D., Huitric, E., Hoffner, S., Cambau, E., Truffot-Pernot, C., Lounis, N. and Jarlier, V. 2005. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis. Science Magazine* **307**: 223-227.

August, P.R., Tang, L., Yoon, Y.J., Ning, S., Muller, R., Yu, T.W., Taylor, M., Hoffmann, D., Kim, C.G., Zhang, X., Hutchinson, C.R. and Floss H.G. 1998. Biosynthesis of the ansamycin rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amycolatopsis mediterranei* S699. *Chemistry and Biology* **5**: 69-79.

Baysarowich, J., Koteva, K., Hughes, D.W., Ejim, L., Griffiths, E., Zhang, K., Junop, M., and Wright, G.D. 2008. Rifamycin antibiotic resistance by ADP-ribosylation: Structure and diversity of Arr. *Proceedings of the National Academy of Science* **105**: 4886-4891.

Brogden, R.N. and Fitton, A. 1994. Rifabutin. A review of its antimicrobial activity, pharmacokinetic properties and therapeutic efficacy. *Anti-cancer Drugs* **47**: 983-1009.

Cadwell, R.C. and Joyce, G.F. 1994. Randomization of genes by PCR mutagenesis. *PCR Methods and Applications* **2**: 28-33.

Campbell, E.A., Korzheva, N., Mustaev, A., Murakami, K., Nair, S., Goldfrab, A. and Darst, S.A. 2001. Structural mechanism for inhibition of bacterial RNA polymerase. *Cell Press* **104**: 901-912.

Chi, Z., Lui, R., Yang, H., Shen, H. and Wang, J. 2011. Binding o tetracycline and chlortetracycline to the enzyme trypsin: spectroscopic and molecular modeling investigations. *Public Library of Science* **16**: 1-9.

Cline, J., Braman, J.C. and Hogrefe. HH. 1996. PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Research* **24**: 3546-3551

Dabbs E.R., Yazawa K., Mikami Y., Miyaji M., Morisaki N., Iwasaki S., and Furihata K. 1995. Ribosylation by mycobacterial strains as a new mechanism of rifampin inactivation. *Antimicrobial Agents and Chemotherapy* **39**: 1007-1009

Daley, C.L. and Caminero, J.A. 2013. Management of multidrug resistant tuberculosis. *Seminars in Respiratory and Critical Care Medicine* **34**: 044-059.

Davies, J. and Davies, D. 2010. Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews* **74**: 417-433.

Honjo, T., Nishizuka, Y., Hayaishi, O. and Kato, I. 1968. Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyl transfer II and inhibition of protein synthesis. *The Journal of Biological Chemistry* **243**: 3553-3555.

Honore, N. and Cole, S.T. 1993. Molecular basis of rifampicin resistance in *Mycobacterium leprae*. *Antimicrobial Agents and Chemotherapy* **37**: 414-418.

Hoshino, Y., Fujii, S., Shinonaga, H., Arai, K., Saito, F., Fukai, T., Satoh, H., Miyazaki, Y. and Ishikawa, J. 2009. Monooxygenation of rifampicin catalyzed by the *rox* gene product of *Nocardia farcinica*: structure elucidation, gene identification and role in drug resistance. *The Journal of Antibiotics* **63**: 23-28.

Hottiger, M.O., Hassa, P.O., Luscher, B., Schuler, H. and Koch-Nolte, F. 2010. Toward a unified nomenclature for mammalian ADP-ribosyltransferases. *Trends in Biochemical Sciences* **35**: 208-219.

Imai, T., Watanabe, K., Mikami, Y., Yazawa, K., Ando, K., Nagata, Y., Morisaki, N., Hashimoto, Y., and Dabbs, E.R. 1999. Identification and characterization of a new intermediate in the ribosylative inactivation of rifampicin by *Mycobacterium smegmatis*. *Microbial Drug Resistance* **5**: 259-264.

Jin, D.J. and Gross, C.A. 1988. Mapping and sequencing of mutations in *Escherichia coli rpo*B gene that leads to rifampicin resistance. *Journal of Molecular Biology* **202**: 45-58.

Kehrenberg, C. and Schwarz, S. 2007. Mutations in 16S rRNA and ribosomal protein S5 associated with high-level spectinomycin resistance in *Pasteurella multocida. Antimicrobial Agents and Chemotherapy* **51**: 2244-2246.

Lakadong, O.R., Kataki, A.C. and Sharan, R.N. 2010. ADP-ribose polymer- a novel and general biomarker of human cancers of head & neck, breast and cervix. *Molecular Cancer* **9**: 286.

Laport, M.S., Santos, O.C.S. and Muricy, G. 2009. Marine sponges: Potential sources of new antimicrobial drugs. *Current Pharmaceutical Biotechnology* **10**: 86-105.

Ludden, PW. 1994. Reversible ADP-ribosylation as a mechanism of enzyme regulation in prokaryotes. *Molecular and Cellular Biochemistry* **138**: 123-129.

Mathews, C.K., Van Holde, K.E. and Ahern, K.G. 2000. Biochemistry. Addison-Wesley publishing company. USA. Page 588 and 887.

Mayers, D.L. 2009. Antimicrobial drug resistance. Volume 1: Mechanisms of drug resistance. Springer. London. Page 92.

Morisaki N., Hashimoto Y., Furihata K., Imai T., Watanabe K., Mikami Y., Yazawa K., Ando A., Nagata Y., Dabbs ER. 2000. Structure of ADP-ribosylated rifampicin and its metabolite: Intermediates of rifampicin to ribosylated rifampicin by *Mycobacterium smegmatis* DSM 43756. *Journal of Antibiotics* **53**: 269-275.

Moss, J. and Vaughan, M. 1997. Mechanism of action of choleragen: evidence for ADP-ribosyltransferase activity with arginine as an acceptor. *The Journal of Biological Chemistry* **22**: 2455-2457.

Olson, M.W., Ruzin, A., Feyfant, E., Rush, T.S., O'Connell, J. and Bradford, P.A. 2006. Functional, biophysical, and structural bases for antibacterial activity of tigecycline. *American Society for Microbiology* **50**: 2156-2166.

Patrick, W.M., Firth, A.E. and Blackburn J.M. 2003. User-friendly algorithms for estimating completeness and diversity in randomized protein-encoding libraries. *Protein Engineering* **16**: 451-457.

Pearson, J.P., Van Delden, C. and Iglewski, B.H. 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *Journal of Bacteriology* **181**: 1203-1210.

Quan, S., Imai, T., Mikami, Y., Yazawa, K., Dabbs, E. R., Morisaki, N., Iwasaki, S., Hashimoto, Y. and Furihata, K. 1999. ADP-ribosylation as an intermediate step in inactivation of rifampicin by a mycobacterial gene. *Antimicrobial Agents and Chemotherapy* **43**: 181-184.

Quan, S., Venter, H., and Dabbs, E.R. 1997. Ribosylative inactivation of rifampin by *Mycobacterium smegmatis* is a principal contributor to its low susceptibility to this antibiotic. *Antimicrobial Agents and Chemotherapy* **41**: 2456-2460.

Sato, K. and Nakae, T. (1991). Outer membrane permeability of Acinetobacter calcoaceticus and its implication in antibiotic resistance. *The British Society for Antimicrobial Chemotherapy* **28**: 35-45.

Senda, K., Arakawa, Y., Ichiyama, S., Nakashima, K., Ito, H., Ohsuka, S., Shimokata, K., Kato, N. and Ohta, M. 1996. PCR detection of metallo-beta-lactamase gene (blaIMP) in gram-negative rods resistant to broad-spectrum beta-lactams. *Journal of Clinical Microbiology* **34**: 2909-2913.

Tait-Kamradt, A., Davies, T., Cronan, M., Jacobs, M.R., Appelbaum, P.C. and Sutcliffe, J. 2000. Mutation in 23S rRNA and ribosomal protein L4 account for resistance in Pneumococcal strains selected *In vitro* by macrolide passage. *Antimicrobial Agents and Chemotherapy* **44**: 2118-2125.

Tanaka Y., Yazawa K., Dabbs E.R., Nishikawa K., Komaki H., Mikami Y., Miyaji M., Morisaki N., and Iwasaki S. 1996. Different rifampicin inactivation mechanisms in *Nocardia* and related taxa. *Microbiology and Immunology* **40**: 1-4.

Telenti, A., Imboden, P., Marchesi, F., Lowrie, D., Cole, S.T., Colston, L., Matter, K., Schoolfer, K. and Bodmer, T. 1993a. Detection of rifampicin resistant mutations in *Mycobacterium tuberculosis*. *Lancet* **341**: 647-650.

Vernon, A., Burman, W., Benator D., Khan A. and Bozeman, L. 1999. Acquired rifamycin monoresistance in patients with HIV-related tuberculosis treated with once-weekly rifapentine and isoniazid. *Lancet* **353**: 1843-1847.

Wallace Jr, R.J., Brown, B.A., Griffith, D.E., Girard, W. and Tanaka, K. 1995. Reduced serum levels of clarithromycin in patients treated with multidrug regimens including rifampicin or

rifabutin for *Mycobacterium avium- M. intracellulare* infection. *Oxford University Press* **171**: 747-750.

Xu, J., Mahmud, T. and Floss, H.G. 2003. Isolation and characterization of 27-O-demethylrifamycin SV methyltransferase provides new insights into the post-PKS modification steps during the biosynthesis of the antitubercular drug rifamycin B by *Amycolatopsis medditteranei* S699. *Archives on Biochemistry and Biophysics* **411**: 277-288.

Yawaza, K., Mikami, Y., Sakamoto, T., Ueno, Y., Morisaki, N., Iwasaki S, and Furihata K. 1994. Inactivation of the macrolide antibiotics erythromycin, midecamycin and rokitamycin by pathogenic *Norcadia* species. *Antimicrobial Agents and Chemotherapy* **38**: 2197.

Zhang, T., Li, S., Williams, K.N., Andries, K. and Nuermberger, E.L. 2011. Short-course chemotherapy with TMC-207 and rifapentine in a murine model of latent tuberculosis infection. Department of medicine, center for tuberculosis research. 1-33.

6. Appendix A

Media

LA ½ Agar

1 g tryptone
0.5 g NaCl
0.5 g yeast extract
0.75 g agar
100 ml distilled water

LA

1 g tryptone
0.5 g NaCl
0.5 g yeast extract
1.5 g agar
100 ml distilled water

LB

1 g tryptone
0.5 g NaCl
0.5 g yeast extract
100 ml distilled water

Plasmid isolation

Solution I

0.9 g glucose
2.5 ml 1 M Tris-HCl pH 8.0

2 ml 0.5 M EDTA

95.5 ml distilled water

Solution II

0.8 g NaOH

1 g SDS

100 ml distilled water

Solution III

60 ml 5 M potassium acetate

11.5 ml glacial acetic acid

28.5 ml distilled water

0.5M EDTA pH 8.0

18.61 g EDTA

pH adjusted to 8.0

Filled up with distilled water to 100 ml

TE

1 ml 1 M Tris-HCl pH 8.0

2 ml 0.5 M EDTA pH 8.0

100 ml distilled water

5M potassium acetate pH 6.0

4.9 g potassium acetate

pH adjusted to 6.0 with acetic acid

Distilled water added to 10 ml

1% RNAse

0.01 g RNAse

1 ml distilled water

Boiled for 10 min

Electrophoresis

5x TBE

54 g Tris base

27.5 g boric acid

20 ml 0.5 M EDTA pH 8.0

Distilled water to 1000 ml

0.5x TBE

20 ml 5x TBE

180 ml distilled water

0.4% Agarose

0.4 g agarose

20 ml 5x TBE

180 ml distilled water

0.8% Agarose

0.8 g agarose

20 ml 5x TBE

180 ml distilled water

0.8% Low gelling agarose

0.8 g low gelling agarose

20 ml 5x TBE

180 ml distilled water

1.0% Agarose

1.0 g agarose

20 ml 5x TBE

180 ml distilled water

Running buffer

20 ml 5x TBE

180 ml distilled water

20 µl 1% ethidium bromide

1% Ethidium bromide

0.1 g ethidium bromide

10 ml distilled water

Tracking dye

10 mg bromophenol blue

10 mg xylene cyanol

10 ml 30% glycerol in TE

Transformation

Calcium chloride transformation buffer

4.38 g calcium chloride hexahydrate

2 ml 1 M Tris-HCl pH 8.0

198 ml distilled water

20% Glucose

4 g glucose

20 ml distilled water

Table 10: Solvents used to make the antibiotic stock solutions

Antibiotic	Stock solution (mg/ml)	Solvent (% per 100 ml)

Amp	100	70:30 (Ethanol:dH_2O)
Rif	10	Methanol
Ctc	10	dH_2O
Kan	10	70:30 (Ethanol:dH_2O)
Str	10	dH_2O
Spc	10	dH_2O
Nal	10	70:30 (Ethanol:dH_2O)
Ksg	100	dH_2O
Ery	100	70:30 (Ethanol:dH_2O)
Cam	10	Ethanol

In vivo/ In vitro inactivation assay

Sonication buffer

2.0 ml 1 M Tris-HCl pH 8

100 µl 0.5 M EDTA

100 µl 1 M DTT

97.8 ml distilled water

Co-factor NAD

51 mg NAD

10 ml distilled water

Co-enzyme A

15 mg coenzyme A trilithium salt

10 ml distilled water

Glutathione

0.102 g glutathione

10 ml distilled water

LDR

1 µg lysozyme

1 µg DNAse

1 µg RNAse

1 ml distilled water

UDPG

12 mg UDPG disodium salt

10 ml distilled water

7. Appendix B

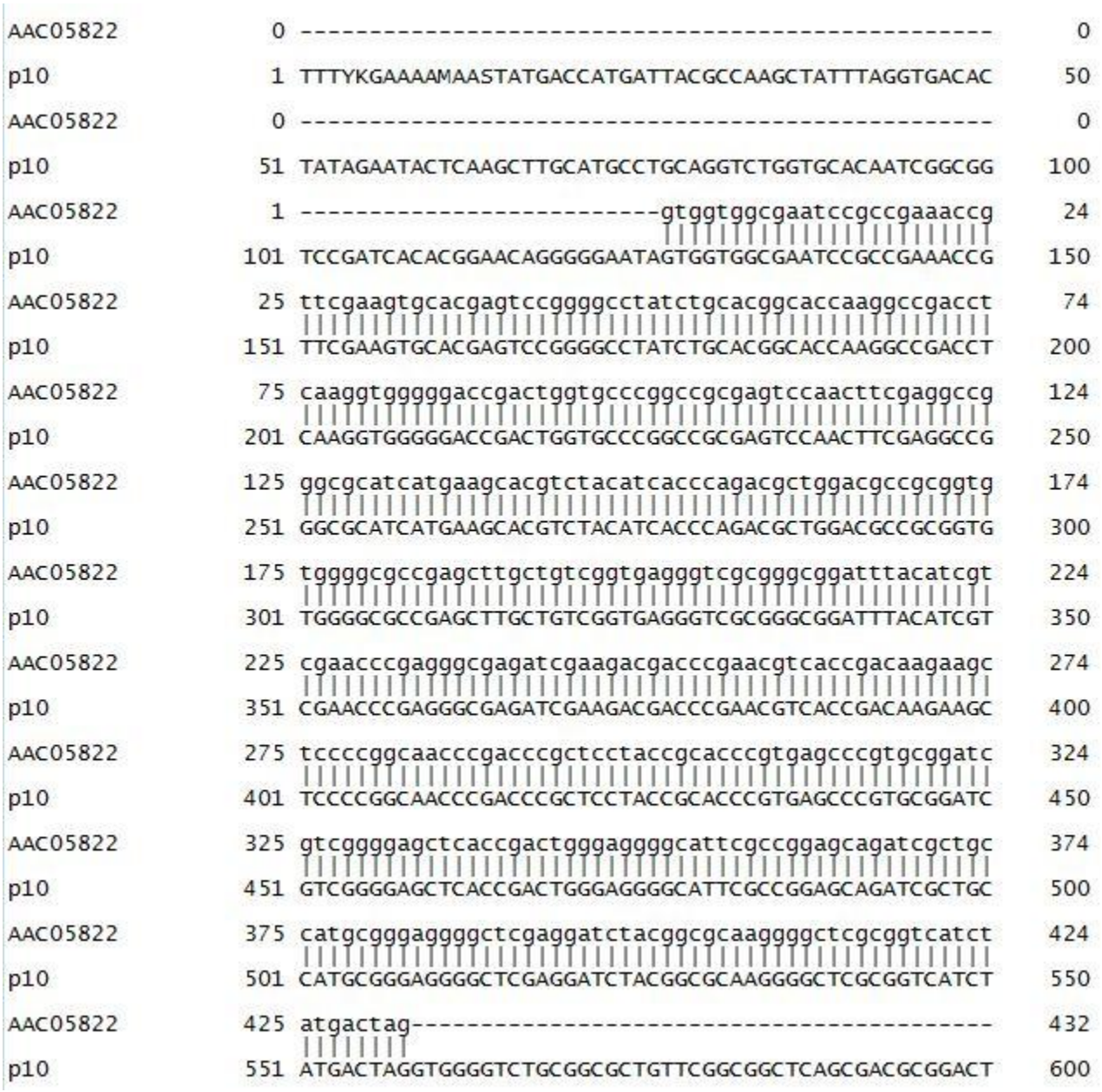

Figure 19: Alignment of clone p10 with *arr* gene open reading frame. No nucleotide changes were detected.

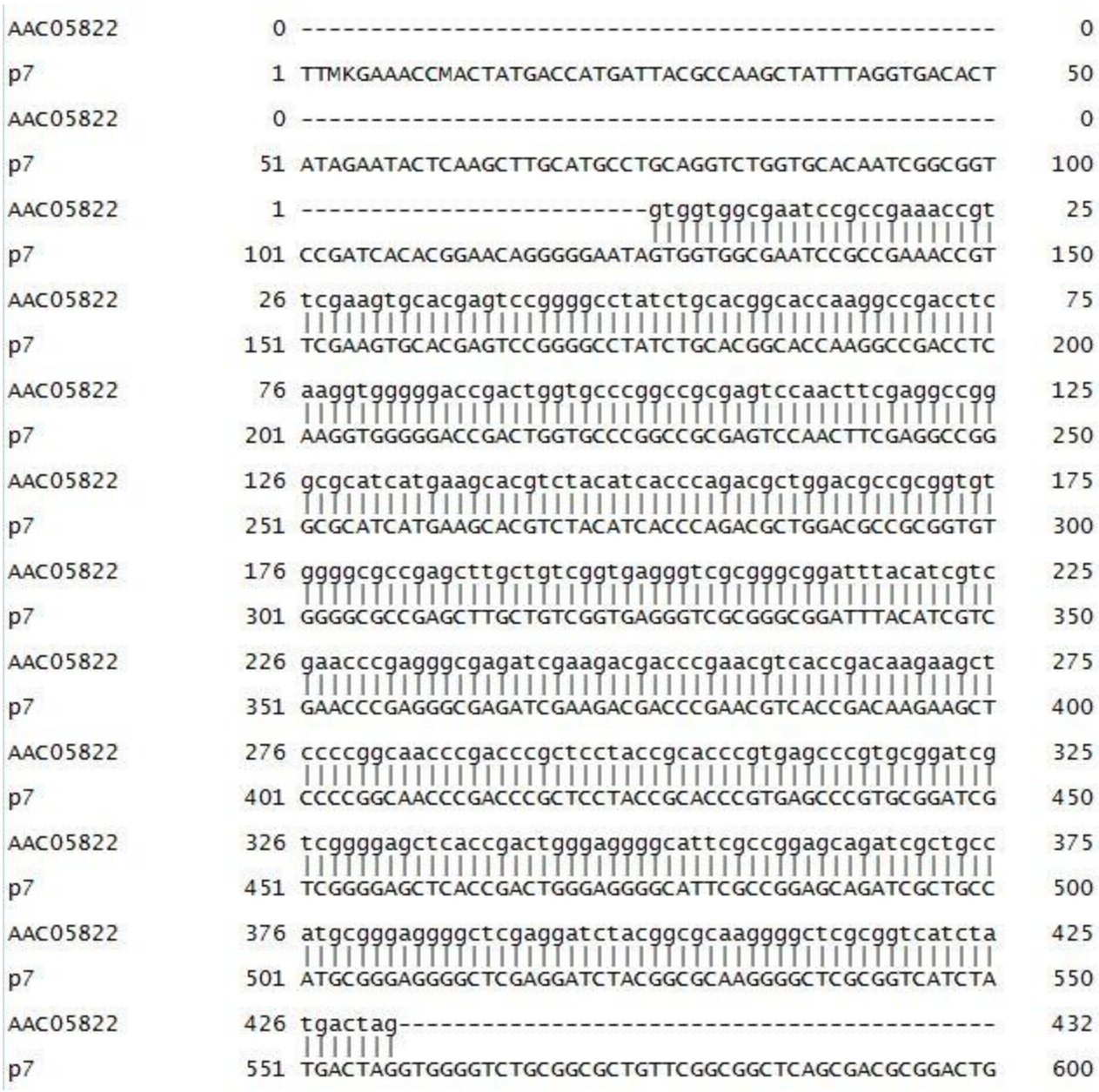

Figure 20: Alignment of clone p7 with *arr* gene open reading frame. No nucleotide changes were detected.

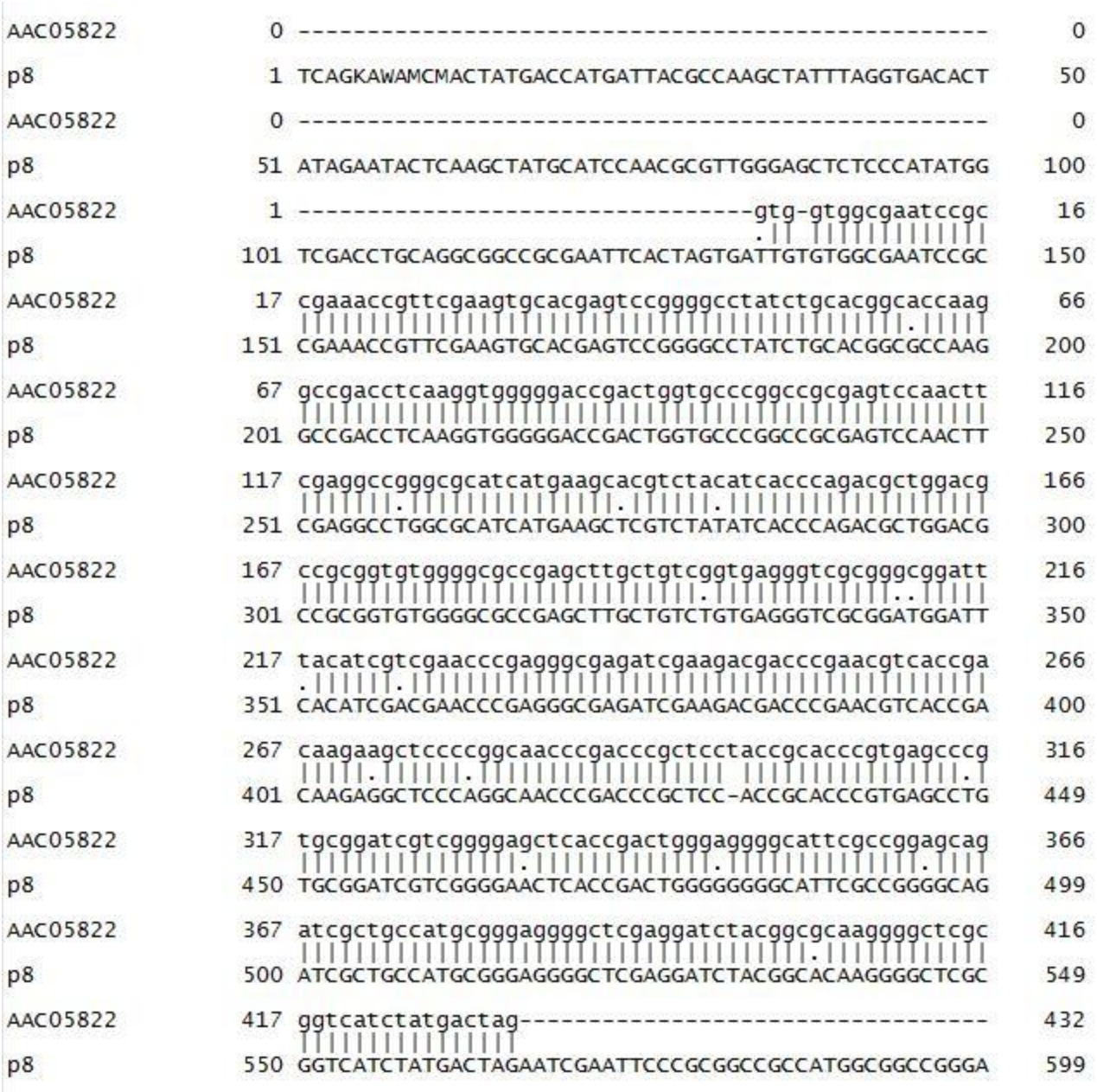

Figure 21: Alignment of clone p8 with *arr* gene open reading frame. A total of 16 nucleotide changes and a single base deletion were obtained.

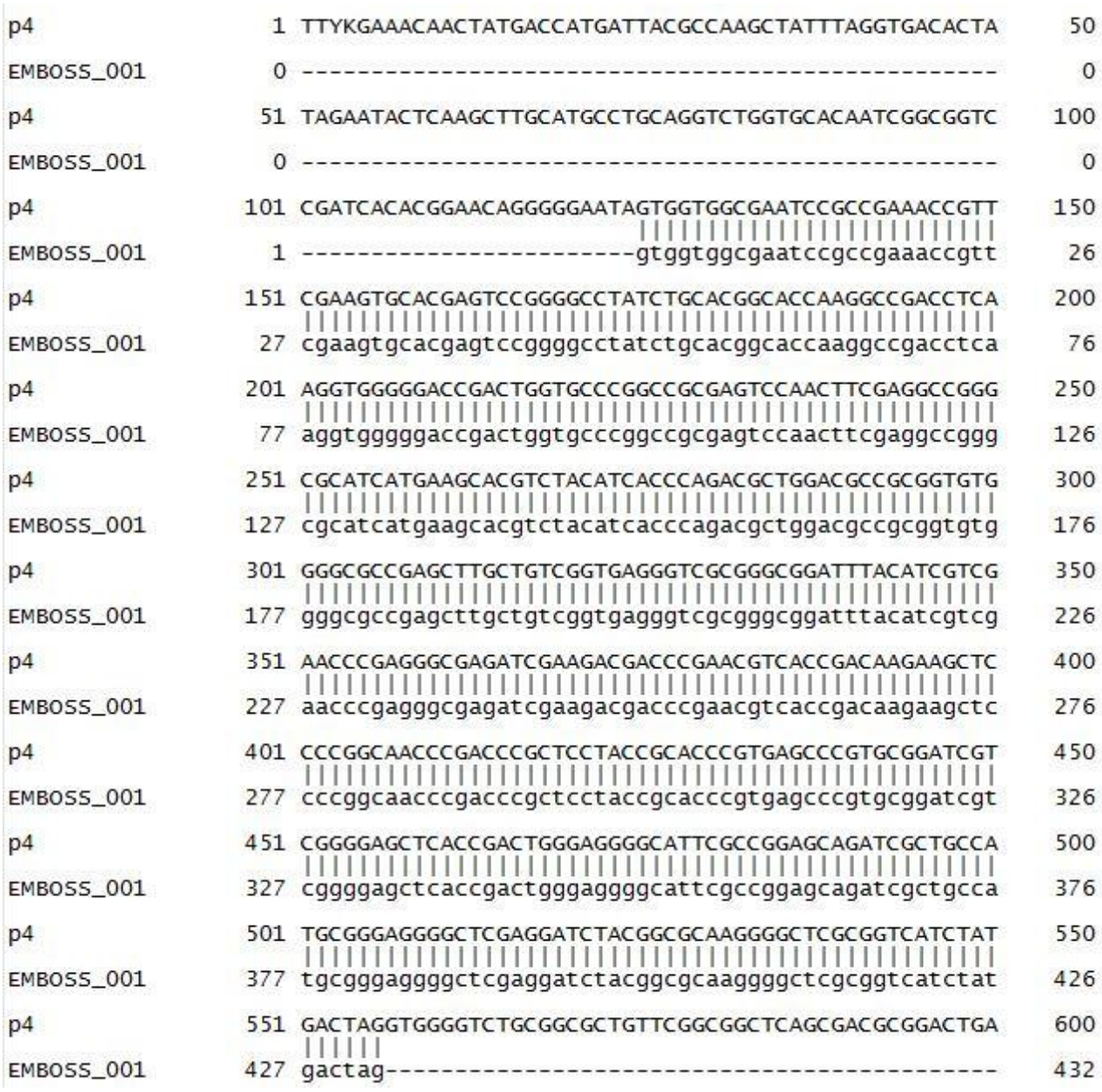

Figure 22: Alignment of clone p4 with *arr* gene open reading frame. No nucleotide changes were detected.

Figure 23: Alignment of clone p157 with *arr* gene open reading frame. A single nucleotide change was detected. This change from T to G resulted in an amino acid change from S to I.

Figure 24: Alignment of clone p20 with *arr* gene open reading frame. A total of 13 nucleotide changes were detected.

Figure 25: Alignment of clone p15 with *arr* gene open reading frame. Two nucleotide changes were detected. The change from C to T resulted in an amino acid change from H to Y while the second mutation (G to C) was silent.

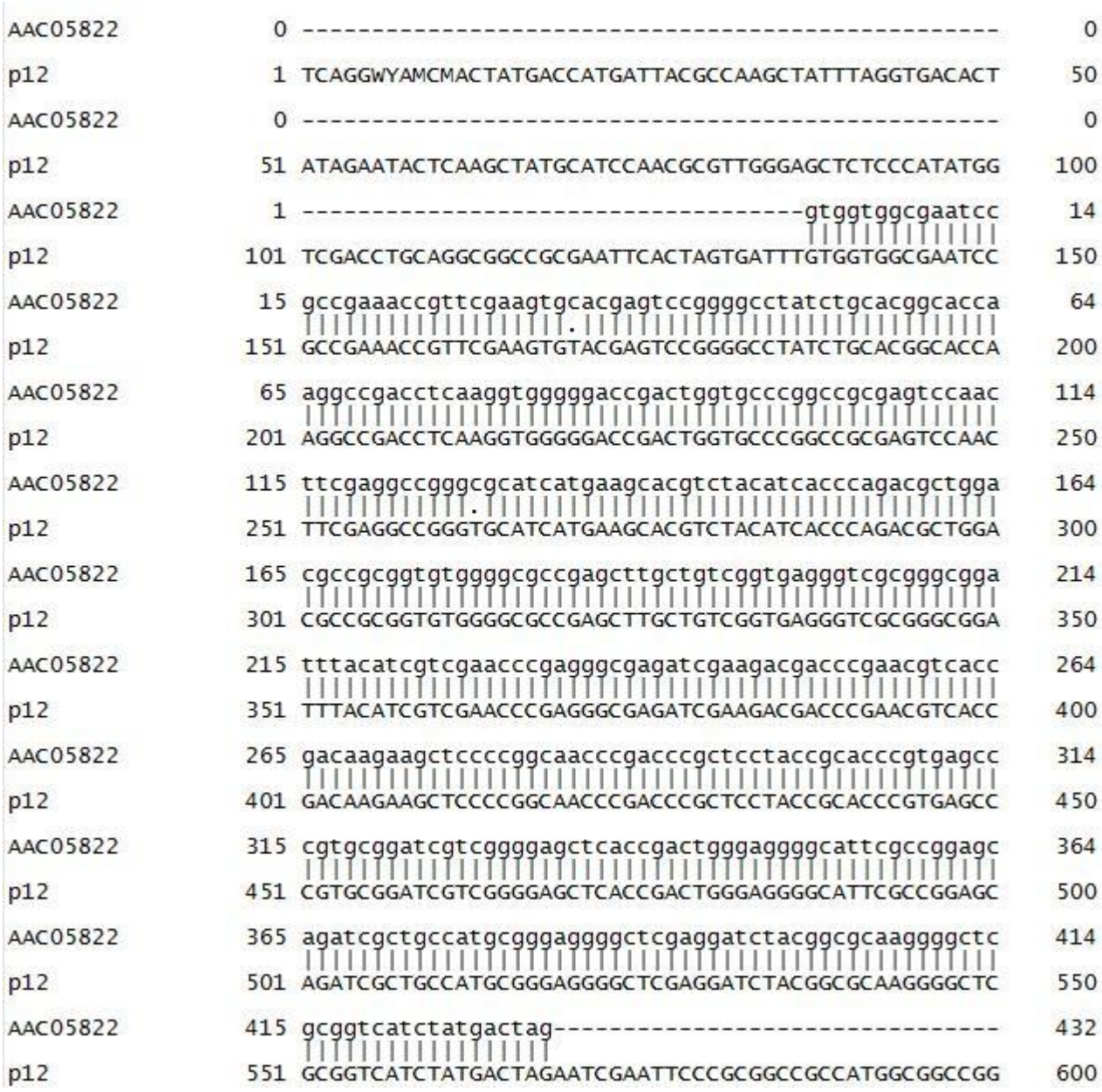

Figure 26: Alignment of clone p12 with *arr* gene open reading frame. Two nucleotide changes were detected and two amino acid changes were introduced (H to R and R to C).

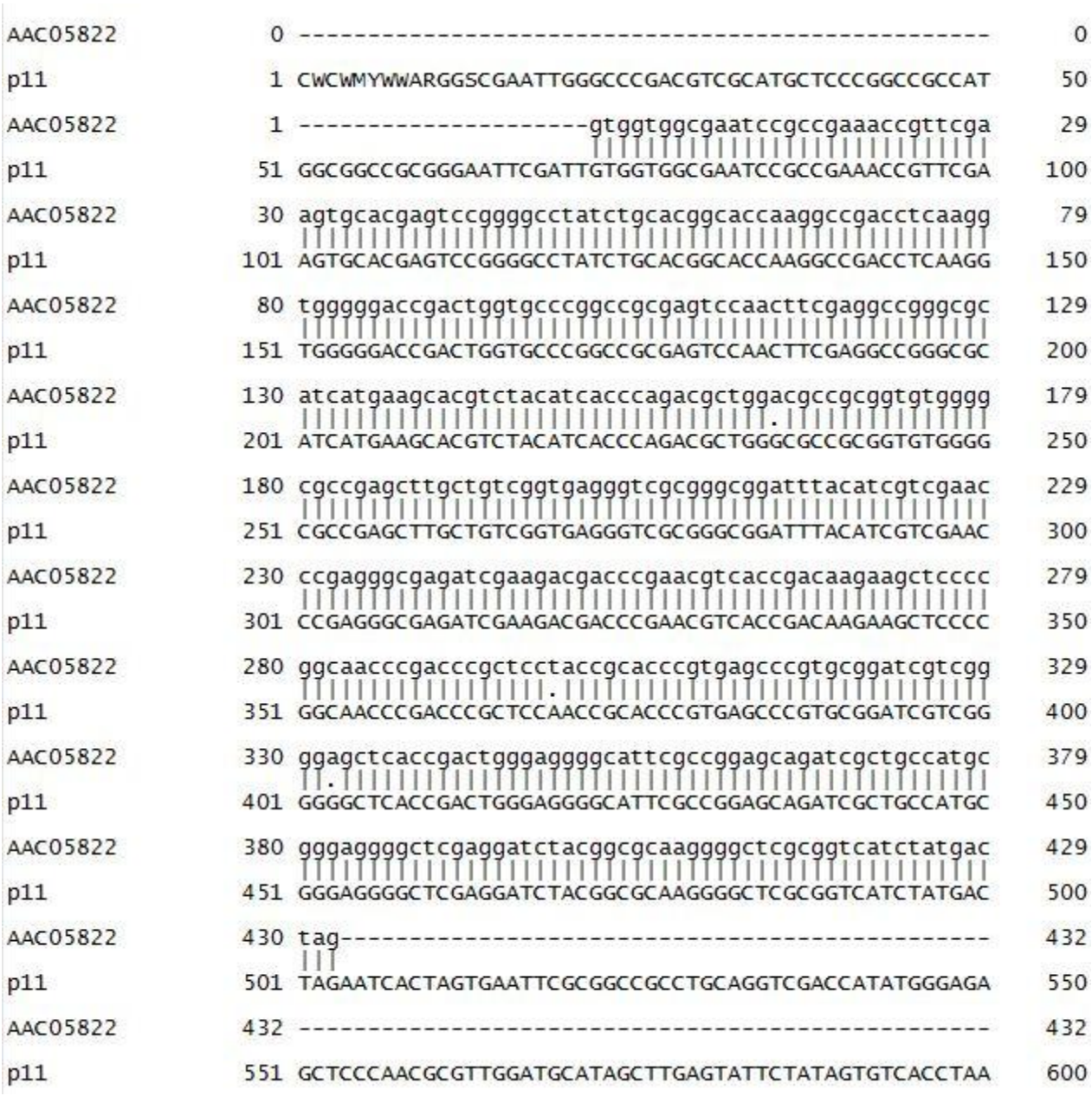

Figure 27: Alignment of clone p11 with *arr* gene open reading frame. Three nucleotide changes were detected and three amino acid changes were made (D to G, Y to N and E to G).

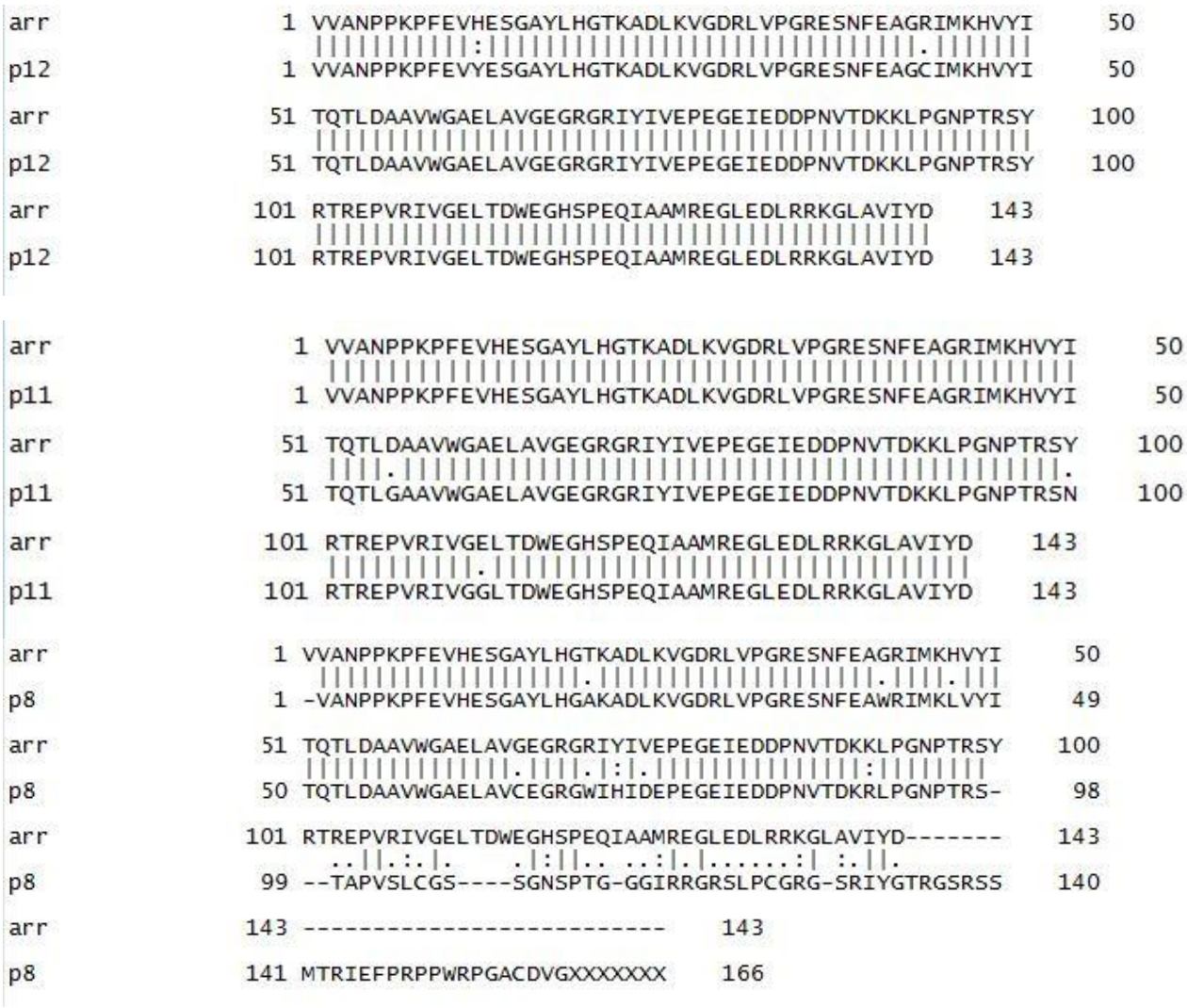

Figure 28: Alignment of the p11, p12 and p8 clones amino acid sequences with the Arr protein.

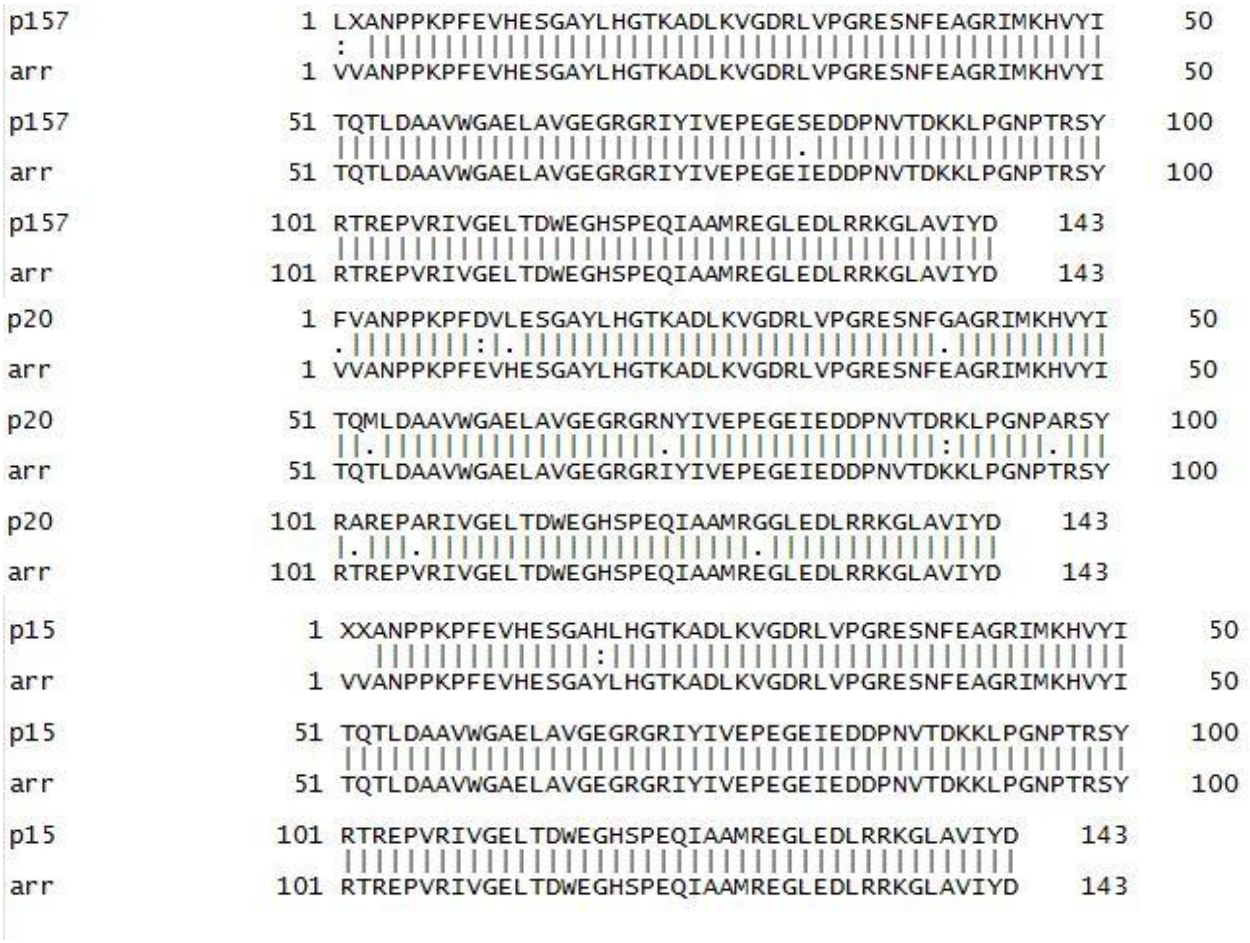

Figure 29: Alignment of the p157, p20 and p15 clones amino acid sequences with the Arr protein.

Figure 30: Alignment of the p4, p7 and p10 clones amino acid sequences with the Arr protein.

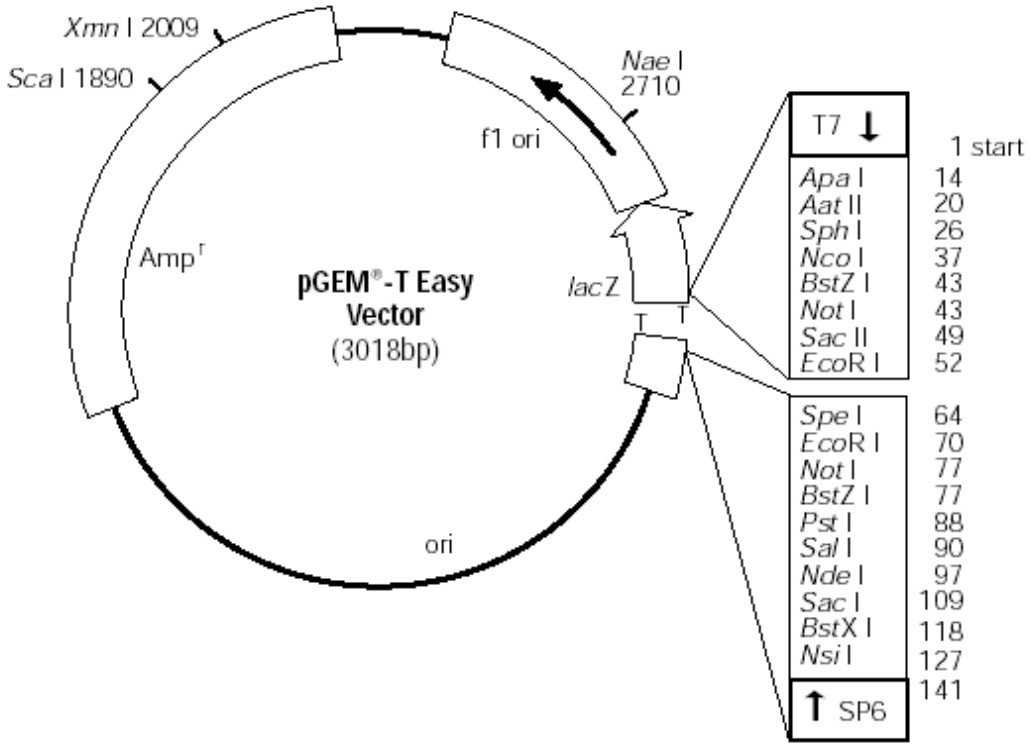

Figure 31: pGEM T-Easy cloning vector adapted from www.promega.com, technical manual No.042