UNDERSTANDING HIGH SPECIFICITY OF ACQUIRED 16S rRNA METHYLTRANSFERASES OF AMR PATHOGENS

DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS

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CERTIFICATE

This is certified that the thesis entitled '**Understanding High Specificity of acquired 16S rRNA Methyltransferases of AMR pathogens**' submitted by **Sahibleen Kaur** (Roll No. 302101020), a postgraduate student of the Department of Biotechnology in partial fulfilment for the award of the degree of Masters of Science at Thapar Institute of Engineering and Technology, Patiala, Punjab 147004, India, is a record of student's own work carried out under my supervision and guidance. The student has declared that this report has not been submitted to any other university or institute for the award of any degree in India or abroad.

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DECLARATION

I hereby declare that the work presented in the dissertation entitled "**Understanding High Specificity of acquired 16S rRNA Methyltransferases of AMR pathogens**" in partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology (TIET) Deemed to be University, Patiala, is a genuine record of my own work during the period from January 2023 to June 2023, under the supervision of Dr. Bhupesh Taneja, Senior Principal Scientist, Genomics and Integrative Biology (CSIR–IGIB). The matter incorporated in this thesis has not been submitted to any other university or institute for the award of any degree in India or abroad.

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ABSTRACT

Antimicrobial Resistance is one of the major causes of concern around the world and is projected to be even more prevalent in years to come. The study of this ability of bacteria to tolerate the presence of the existent antimicrobials by different mechanism, is important for understanding the cause of this problem. This study is focused on one such resistance mechanism, i.e. the alteration of target site of antimicrobials, specifically aminoglycosides that use 30S subunit of rRNA as a substrate for their antimicrobial action. The 16S RNA Methyl Transferases alter the A site of translation by methylation and provide resistance to the bacteria. These RMTases consist of an N-terminal domain and a C-terminal domain each. In this study, three different RMTases namely, NpmA, ArmA and RmtA are studied as two different chimeras, namely, $NpmA^{N}$_$ArmA^{C}$ and $NpmA^{N}$_$RmtA^{C}$ in the hope of understanding the specificity of the domains towards ribosome binding (and methylation). The chimeras were constructed by joining the N-terminal domain of NpmA to the C-terminal domains of ArmA or RmtA. The effect of change in binding site of the RMTase was seen by quantifying the change in the tolerance of wild type strain to the new transformed strain. The protein expression of the newly constructed RMTases were also checked by different techniques.

Keywords: *Antibiotic resistance, ESKAPE pathogens, Aminoglycoside, Aminoglycoside resistance, 16S rRNA Methyltransferases, Extrachromosomal DNA, Protein chimera, Cloning and Transformation, Aminoglycoside susceptibility assay, Bioscreen*.

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LIST OF ABBREVIATIONS

ABBREVIATION	FULL FORM
%	Percent
µL	Microliter
mL	Milliliter
mM	Millimolar
µg	Microgram
AdoHcy	S-adenosyl homocysteine
AdoMet	S-adenosyl Methionine
AMR	Antimicrobial Resistance
APS	Ammonium persulfate
AWaRe	Access, Watch, or Reserve
BSA	Bovine serum albumin
CA	Community-acquired
cAMP	Cyclic Adenosine Monophosphate
CD	Circular Dichroism
CDC	Centre for Disease Control
CLSI	Clinical and Laboratory Standards Institute
CRP	cAMP-catabolite repressor protein
CTD	C-terminal domain
DOS	Deoxystreptamine
EDTA	Ethylenediaminetetraacetic acid
FPLC	Fast protein liquid chromatography
FT	Flow Through
HA	Hospital Acquired
His	Polyhistidine
IMAC	Immobilized Metal Affinity Chromatography
IPTG	Isopropyl β- d-1-thiogalactopyranoside
kDa	Kilo Dalton
LA	Luria Bertani Agar
LB	Luria Bertani Broth
log	Logarithmic
LPS	Lipo-Polysaccharide

MCT	Micro-Centrifuge Tube
MDR	Multi-Drug Resistance
MGEs	Mobile Genetic Elements
MIC	Minimum Inhibitory Concentration
NTD	N-terminal domain
NFW	Nuclease Free Water
NTA	Nitrilotriacetic acid
O.D	Optical Density
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl Fluoride
PMQR	Plasmid-mediated Quinolone Resistance
PPL	Priority Pathogen List
PVDF	Polyvinylidene difluoride
RMTases	RNA Methyl Transferases
RT	Room Temperature
rSAP	Shrimp Alkaline Phosphatase
SAM	S-adenosyl Methionine
SDS	Sodium Dodecyl Sulphate
TBST	Tris Buffered Saline with Tween 20
TEMED	N, N, N′, N′-Tetramethylethylenediamine
UTIs	Urinary Tract Infections
WB	Wash Buffer
WHO	World Health Organisation
XDR	eXtensively Drug Resistant

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CHAPTER I

INTRODUCTION

The ability of microbes to survive or grow in the presence of antibiotics designed to stop them or kill them is known as AMR (antimicrobial resistance). AMR emerges when microorganisms adapt over time and stop responding to medications, making it harder to treat illnesses, raising the death rate, and spreading disease. It is common for microorganisms to develop antibiotic resistance. However, the development and dissemination of the resistance may be accelerated by our activities. Use of antibiotics hastens this natural progression. While some bacteria die when humans take antibiotics, resistant bacteria can live and even proliferate. Antibiotic usage increases the prevalence of microorganisms with resistance. Bacteria are more likely to develop antibiotic resistance the more frequently we use antibiotics. This means that in the future, when we require antibiotics to treat infections, they will not be effective. The next generation of antibiotics may be more successful in killing germs if we reduce the use of antibiotics (Murray et al., 2022).

As antibiotics lose their effectiveness, a rising number of infections, including gonorrhoea, blood poisoning, pneumonia, and tuberculosis, are become difficult to cure and occasionally becoming incurable.

Six extremely virulent and antibiotic-resistant bacterial pathogens are referred to as "**ESKAPE**" pathogens by the World Health Organization. These pathogens include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp. Escherichia coli* is occasionally added to the abbreviation, making it ESKAPEE. Due to their rising multi-drug resistance, these group of Gram-positive and Gram-negative bacteria can avoid or "escape" from routinely used antibiotics. Because of this, they are the main contributor to fatal hospital-acquired infections around the globe, especially in severely ill and immunocompromised patients who are most vulnerable (Prestinaci et al., 2015).

Four of the pathogens from the ESKAPE group are on the critical priority pathogen list, while the other two pathogens that make up the group are on the high priority pathogen list, according to the WHO's global priority pathogen list (PPL), which divides pathogens into three categories: **critical**, **high**, and **medium**.

Methyl-Transferases are enzymes that transfer a methyl group onto their substrate by using S-Adenosine Methionine (SAM) as a cofactor. They are divided into 3 classes as –

- Class I – The methyl transferases that contain the Rosmann fold
- Class II – Contain a SET domain
- Class III – Membrane associated methyl transferases (Zhi-Gang et al., 2009)

One such type Class I Methyl Transferase include 16S rRNA Methyl-Transferases (RMTases) which bind to the 30S subunit of rRNA. These methyl transferases, methylate the A site of translation and act as a defense mechanism of bacteria against antimicrobials.

The mechanism of resistance caused by the presence of the genes encoding for 16S RMTases are important to study as these are coded extra chromosomally and are co-located with other resistant genes giving rise to Multi Drug Resistant (MDR) strains or eXtensively Drug Resistant (XDR) strains in the bacteria. The other resistance genes are co-located as well but the presence of these genes has been shown to enhance the resistance by 5X to 10X folds. This is why the study of this mechanism in detail is crucial.

The class I methyl transferases consist of two terminals – The N-terminal domain, which binds to the substrate at the target site and the C-terminal domain which is the catalytic domain and has the classic Rosmann fold. The resistance imparted by the different RMTases is seen by the difference in the site of action/methylation. Depending upon the binding of the RMTase to either the G1405 or the A1408 site in the A site, the bacteria shows resistance to different aminoglycosides.

By introducing a MTase in the bacteria and its overexperssion, the transformed bacteria should show a higher tolerance for the target group of antibiotics at higher concentrations than the wild type bacteria of the same strain. In this proposal, the role of N- or C-terminal domains of the MTases towards imparting specificity is studied by monitoring the MICs upon transformation with designed constructs.

CHAPTER II

REVIEW OF LITERATURE

2.1 Antimicrobial Resistance

Antimicrobial Resistance (AMR) refers to the capability of microorganisms such as bacteria, viruses, fungi, and parasites to adapt and thrive under antimicrobial agents that are intended to eradicate them (Dadgostar P, 2019)

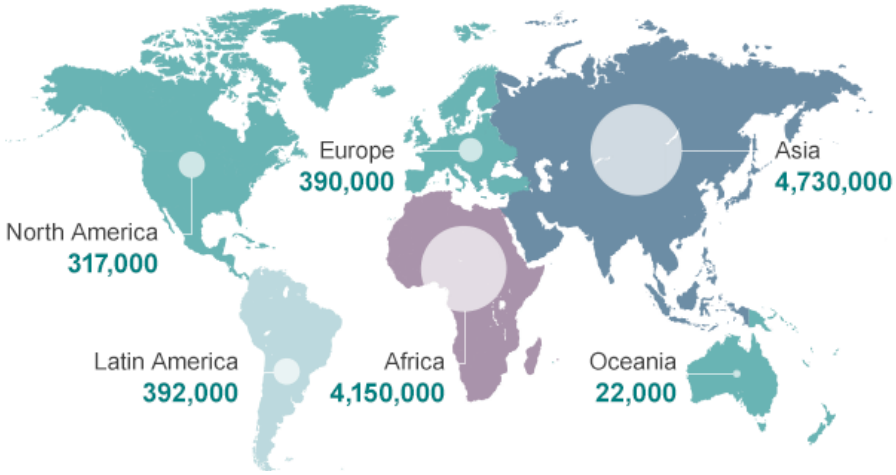


Figure 1- Deaths attributed to AMR every year by 2050 (Review on Antimicrobial Resistance 2014)

This phenomenon poses a significant threat to global healthcare, as it renders existing antibiotics ineffective against life-threatening infectious diseases, thereby creating an uncertain future for healthcare. The co-occurrence of AMR and existing infections results in increased hospital admissions, prolonged hospital stays, higher costs for second-line drugs, and a high likelihood of treatment failures. In Europe alone, AMR has been estimated to contribute to an annual expenditure of over nine billion euros.

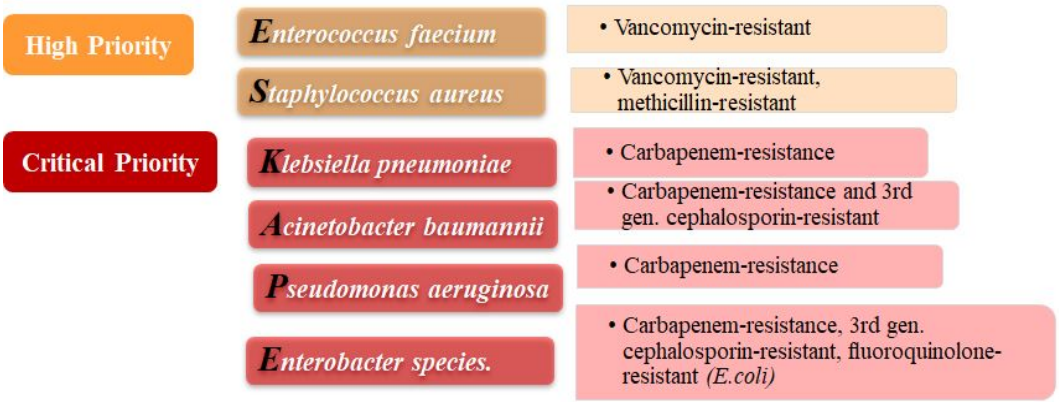


Figure 2 - WHO Priority pathogens list for new antibiotics (WHO 2017)

Table 1: Mortality Rates by 2050 by Condition

Cancer	8.2 Million
Cholera	100,000–120,000
Diabetes	1.5 Million
Diarrheal Disease	1.4 Million
Measles	130,000
Road Traffic Accidents	1.2 Million
Tetanus	60,000
Antimicrobial Resistance	10 Million

(Adapted from Antimicrobial resistance: tackling a crisis for the health and wealth of nations. 2014)

A decrease in the production of novel antibiotics has corresponded with the increase of multidrug-resistant (MDR) bacteria, which are resistant to more than three different classes of antibiotics. Antibiotic-resistant (AMR) microorganisms are a serious threat to human health, according to the World Health Organization (WHO) and the U.S. Centers for Disease Control and Prevention (CDC) (De Oliveira et al., 2020). Despite the lack of an organised international surveillance system for AMR, information now available indicates that over 2 million AMR infections occur in the United States each year, causing 29,000 deaths and costing the country's healthcare system more than $4.7 billion. AMR infections acquired in hospitals (HA) and the community (CA) are responsible for about 33,000 fatalities, 874,000 years of life with a handicap adjusted for age, and $1.5 billion in direct and indirect expenditures in Europe. Emerging and reemerging infectious illnesses have considerable impact on developing countries, where communicable diseases are the primary cause of death. Lack of quick diagnostic tools for identifying bacterial infections and AMR genes in clinical settings frequently contributes to the overuse of broad-spectrum antibiotics (Dadgostar P, 2019).

Antibiotics that are the last line of defense, such carbapenems, glycopeptides, and polymyxins, have been rendered ineffective by ESKAPE pathogens due to the development of resistance mechanisms. Lipoglycopeptide resistance is uncommon, probably because lipoglycopeptides suppress peptidoglycan formation while also disrupting bacterial cell membranes. It is extremely concerning that more bacterial species with these processes are seen in infections contracted in hospitals. (Gür et al., 2020)

AMR In both community and healthcare settings, *E. coli* is a significant contributor to worldwide bloodstream and urinary tract infections (UTIs). A typical symptom of *E. coli* UTI is sepsis (Galimand et al., 2003). Over the past ten years, MDR uropathogenic *E. coli* clones like ST131 and ST95 have spread across the globe. Through horizontal gene transfer, *E. coli* often obtains resistance genes from other Enterobacterales members. In Europe, there are numerous antibiotics with high rates of resistance (Al-Guranie et al., 2020).

The clinical burden of AMR E. coli on human and animal health is substantial. To prevent further escalating these issues, organisations involved in AMR policy, research and development, and surveillance must view this pathogen as a crucial public health risk.

2.2 Watch Group of Antibiotics

The development of antibiotic stewardship at the local, national, and international levels has been aided by the implementation of a classification system, according to the World Health Organization (WHO). 180 common antibacterials are divided into three groups: access, watch, or reserve (AWaRe) in a categorization database that was updated and created in October 2019. The 49 antibiotics in the access-group have a wider spectrum of efficacy against frequently encountered susceptible infections and a lower risk of developing resistance than those in the other groups. 110 antibiotics in the watch-group have a higher risk of developing resistance. Finally, 22 reserve-group antibiotics should only be used as a very last resort for very specific patients and situations after all other options have been exhausted or are not suitable. (Pauwels et al., 2021). The database also lists antibiotics that are not advised by the WHO, such as fixed-dose combinations of several broad-spectrum antibiotics without recommendations in high-quality international guidelines or evidence-based indications for usage.

- **Access** antibiotics are drugs with a limited spectrum of activity, with fewer side effects, a reduced risk of selecting for antimicrobial resistance, and a lower price. They ought to be readily accessible and are advised for the empiric treatment of the majority of common infections.
- **Watch** antibiotics are typically used in sicker patients in the hospital setting and have a larger potential for the selection of antimicrobial resistance. To prevent abuse, their use should be closely monitored.
- **Reserve** antibiotics are antibiotics of last resort that should only be used to treat severe infections brought on by microorganisms that are drug-resistant to several drugs. (Zanichelli et al., 2023)

2.3 Aminoglycosides

The ESKAPE pathogens, which possess multidrug-resistant (MDR) genes, are responsible for causing infections that are challenging to treat and are one of the main causes of morbidity and

mortality in the world. These infections occur due to the ability of pathogens to evade the effects of antibiotics by utilizing various resistance mechanisms.

Aminoglycosides are a commonly used broad-spectrum antimicrobial class that is highly effective against infections. They work synergistically with other antibiotics and are considered ideal for treating nosocomial infections due to their predictable pharmacokinetics and bactericidal properties (Mingeot-Leclercq et al., 1999). Aminoglycosides are classified into three groups based on their chemical structures: 4,6-disubstituted 2-deoxystreptamine (DOS), 4,5-disubstituted DOS, and 4-monosubstituted DOS. The 4,6-disubstituted DOS agents, including gentamicin, tobramycin, and amikacin, are commonly used intravenously or nebulized to treat infections caused by gram-negative bacteria, gram-positive bacteria, and atypical mycobacteria. Due to their toxicity, the 4,5-disubstituted DOS agents, like neomycin, have limited applications and are only given topically or orally. The mono-substituted DOS agent, apramycin, is used in veterinary medicine (Krause et al., 2016).

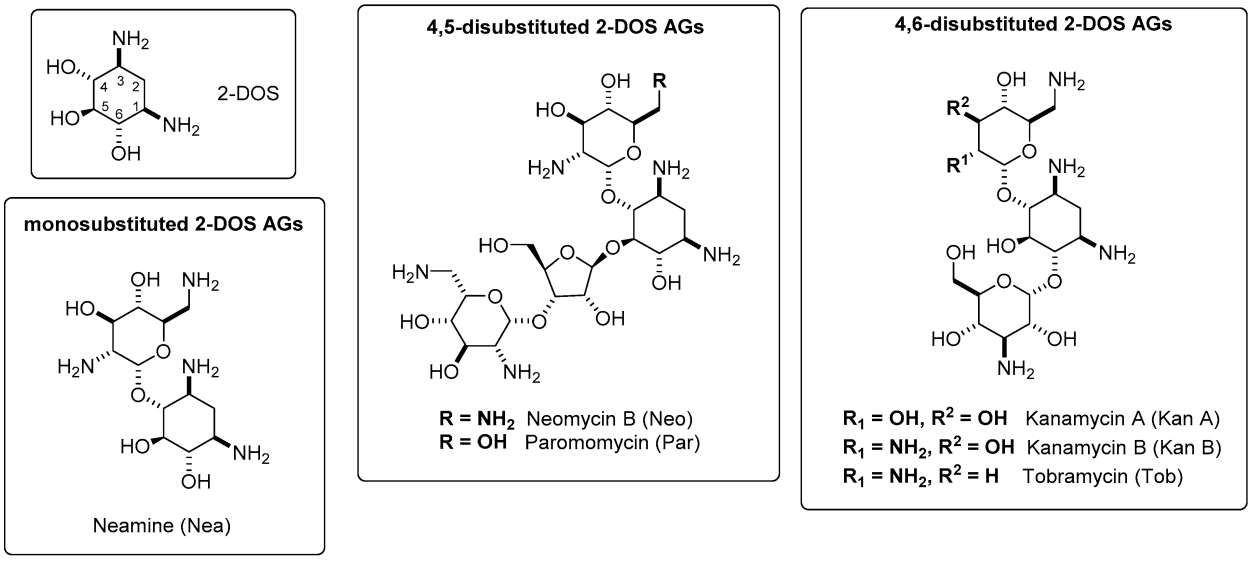


Figure 3- Structures of some aminoglycosides (Bellucci et al., 2020)

2.4 Antimicrobial Resistance Mechanisms

General mechanisms of resistance in bacteria include four main categories:

2.4.1 Decreased Uptake:

Inherent differences exist among bacteria in their ability to restrict the uptake of antimicrobial agents. Gram-negative bacteria possess a barrier to certain types of molecules due to the LPS layer's structure and functionality, which provide intrinsic resistance to specific classes of powerful antibacterial drugs like Rifampicin.

In bacteria with large outer membranes, substances typically enter the cell through porin channels that allow access to hydrophilic molecules. Porin changes can limit drug uptake in two primary ways: the absence of more porins and mutations that affect the selectivity of the porin channel. These mutations have been observed in *E. aerogenes*, resulting in resistance to imipenem and certain cephalosporins, and in Neisseria gonorrhoeae, leading to resistance to β-lactams and tetracycline (Reygaert W, 2018).

2.4.2 Drug Inactivation:

Inactivation of drugs by bacteria can occur through two primary mechanisms: degradation of the antibiotics or incorporation of a chemical group to the antimicrobials. The β-lactamases, a group of enzymes, are responsible for hydrolyzing a wide range of drugs. Additionally, the tetX gene can lead to the hydrolyzation of tetracycline. Transfer of chemical groups, such as acetyl, phosphoryl, and adenyl groups, is another common method of drug inactivation. Numerous transferases have been identified, with acetylation being the most widely used mechanism against drugs such as aminoglycosides, chloramphenicol, streptogramins, and fluoroquinolones. To combat aminoglycosides, phosphorylation and adenylation are primarily used. To combat aminoglycosides, phosphorylation and adenylation are primarily used (Munita et al., 2016).

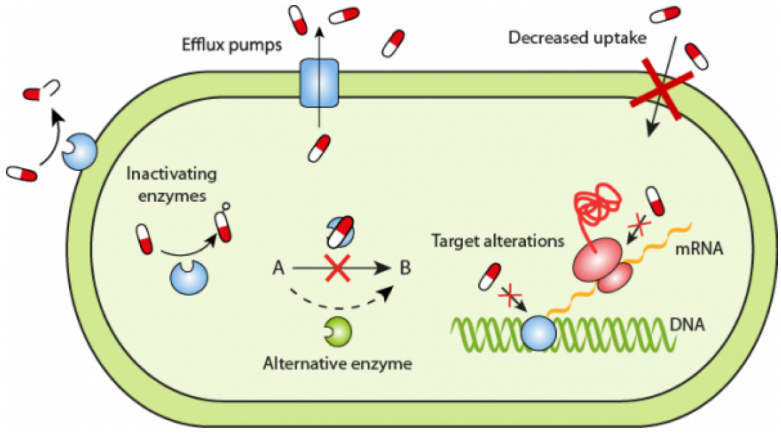


Figure 4 - Antibiotic Resistance Strategies in Bacteria (Wistrand-Yuen et al., 2018)

2.4.3 Efflux pumps:

Bacteria possess genes for efflux pumps that are encoded in their chromosomes. These pumps can be expressed constitutively or induced/excessively expressed in response to specific environmental cues or the presence of an appropriate substrate.

These efflux pumps' fundamental purpose is to remove harmful substances from the bacterial cell, and some of them can transport a variety of substances, making them multi-drug efflux pumps. It has been observed that the resistance capacity of these pumps is influenced by the availability of carbon

sources (Peterson et al., 2018). Additionally, high-level resistance can be achieved through mutations that modify the transport channel.

2.4.4 Target alteration:

The bacteria that exhibit resistance to antimicrobial agents are characterized by possessing either a modified *rrs* gene that encodes for 16S rRNA or undergoing post-transcriptional modifications in 16S rRNA, which hinder the binding of the drug to the ribosomal substrate. The primary area of interest in this research project is the category of 'Target Alterations,' with a specific focus on post-transcriptional modifications that prevent the binding of antimicrobial agents to the substrate.

Linezolid and aminoglycoside resistance in ESKAPE organisms is mediated at the ribosome level. Both *S. aureus* and *Enterococcus spp*. are susceptible to linezolid resistance due to mutations in the genes encoding the 23S rRNA and/or 50S ribosomal subunit complexes or *Cfr*-mediated methylation of the 23S rRNA at residue A2503. Staphylococcal strains with additional linezolid resistance mechanisms have been shown to carry the *cfr* gene, which is transferable within mobile genetic elements (MGEs) and frequently connected to other antimicrobial resistance (AMR) determinants like erm. (De Oliveira et al., 2020).

Additionally, a key acquired AMR mechanism in Gram-negative ESKAPE infections has recently been identified as the enzymatic methylation of 16S rRNA, which imparts high-level aminoglycoside resistance to all aminoglycosides, including plazomicin. 11 distinct types of 16S rRNA methyltransferases, including ArnA, RmfA to RmtI, and NmpA, have been identified globally. The number of viable treatments is further diminished by the fact that these enzymes are frequently found on plasmids that contain genes for additional multidrug resistance (MDR) determinants, such as blaOXA-23 and blaNDM (S. Lioy et al., 2014).

2.5 Aminoglycoside Resistance Mechanism – 16S rRNA Methylation

The interaction mechanism between aminoglycosides and 16s rRNA, and its subsequent impact on translation, has been extensively researched. The findings suggest that these molecules exhibit a strong affinity towards RNA substrates, particularly specific sites within the rRNA. The binding sites vary depending on the aminoglycoside class and the structural compatibility between the two. For instance, neomycin and kanamycin bind to the A-site on the 16S rRNA in E. coli, and chemical footprinting experiments have demonstrated their ability to protect bases A1408 and G1494 (F. Liou et al., 2006). Aminoglycoside binding to the A-site in the decoding region, where codon and anticodon recognition takes place, is thought to have an impact on how accurately homologous tRNA

is recognised by rRNA during translation. Additionally, it is believed that these interactions prevent tRNA from moving from the A-site to the peptidyl-tRNA site (P-site) (Doi et al., 2016).

Table 2: Resistance mechanisms of different types of aminoglycosides

AMINOGLYCOSIDES	N7-G1405 16S RMTases	N1-A1408 16S RMTases
4,6-Disubstituted DOS (gentamicin, tobramycin, amikacin)	Resistant	Resistant
4,5-Disubstituted DOS (neomycin)	Susceptible	Resistant
Monosubstituted DOS (apramycin)	Susceptible	Resistant
No DOS ring (streptomycin)	Susceptible	Susceptible

Posttranscriptional modification of the 16S rRNA occurs often in *Actinobacteria* that make aminoglycosides, including *Streptomyces* species and *Micromonospora* species. In this alteration, different 16S rRNA methyltransferases methylate either the N-7 site of nucleotide G1405 or the N-1 site of nucleotide A1408 on the 16S rRNA (16S-RMTases). While intrinsic N1-A1408 16S-RMTases have only been discovered in *Streptomyces spp*., intrinsic N7-G1405 16S-RMTases are found in both *Streptomyces spp*. and *Micromonospora spp*. The former does not transfer resistance to 4,5-disubstituted and mono-substituted DOS agents, but does confer resistance to 4,6-disubstituted DOS agents such gentamicin, tobramycin, and amikacin. On the other hand, the latter has the ability to grant all of these groups resistance (Kotra et al., 2000).

2.6 RNA Methyl-Transferases

RMTases are a class of enzymes that utilize S-Adenosine Methionine (SAM) as a co-factor to transfer a methyl group to RNA substrates. The study of 16S RMTases is crucial due to their extra-chromosomal coding and co-localization with other resistance genes, causing the emergence of eXtensively drug-resistant (XDR) and multi-drug-resistant (MDR) strains of bacteria. Acquired 16S RMTases are categorized into two families based on the site of modification. ArmA and RmtA-I methylate N7 of Guanine at position 1405, while NpmA is the sole known RMTase responsible for methylation of N1 of Adenine at position 1408 (Nosrati et al., 2019).

The ArmA (aminoglycoside resistance methyltransferase) genes were commonly found in the clinical isolates of *Enterobacteriaceae spp* as well as *Klebsiella pneumoniae* strains. These acquired methyl transferases were seen to be responsible for production of MDR strains in conjugation with plasmid-mediated quinolone resistance (PMQR) genes, thus decreasing the susceptibility of the strains towards multiple drugs (Yang et al., 2011). The first methyltransferase discovered in a clinical isolate that can methylate 16S rRNA at m1A1408 is NpmA from *Escherichia coli* clinical strain ARS3. In

addition, the NpmA gene was discovered on a plasmid, indicating its capacity for quick horizontal transfer between bacterial pathogens, posing a risk to the effectiveness of aminoglycoside antibiotics in the treatment of infectious disorders (Shibayama et al., 2007).

Methylation of these sites results in a conformational change, rendering the antimicrobial unable to bind and induce therapeutic effects. The A-site, where the amino-acyl tRNA binds for protein translation, cannot recognize the altered conformation, resulting in the inhibition of subsequent protein synthesis.

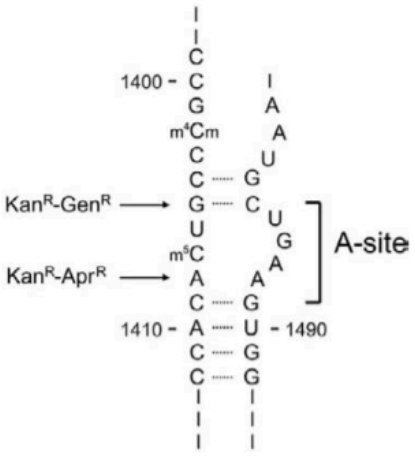


Figure 5 - Positions of modificationas in A site in decoding region in 16S rRNA (Doi et al., 2007)

Molecular replacement techniques were used to identify the structure of RmtB complexed with S-adenosyl homocysteine (AdoHcy) in two distinct crystalline forms. The structure of RmtB is well defined and is divided into two parts:

- C Terminal domain, which has a classical fold of Class I Methyl transferases consisting of seven stranded β sheets with helices on each side.
- N Terminal domain, further consisting of two groups N1 and N2, each having three α helices each (Schmitt et al., 2009).

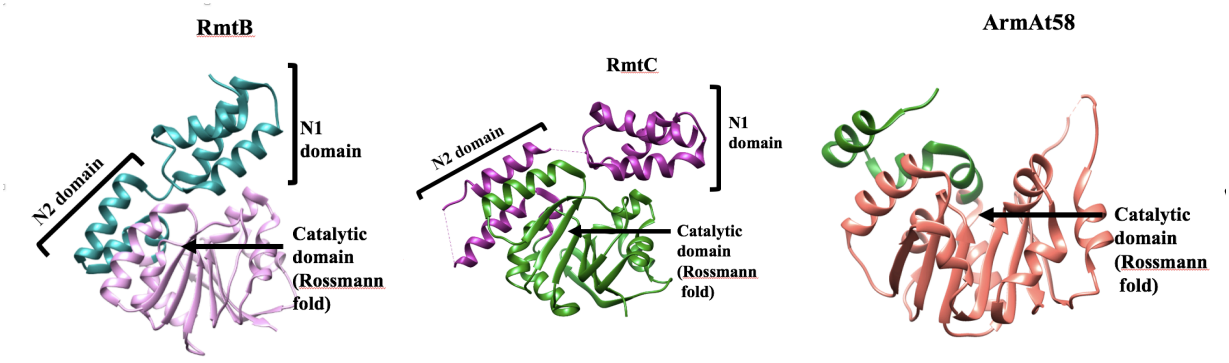


Figure 6 – Structures of RmtB, ArmA58, RmtC (Bogaerts et al., 2007)

This was crucial in defining the catalytic and the binding domains of the enzyme.

ArmA, as isolated in the experiments, was seen as a truncated structure with truncation starting at 58th residue. Thus, this variant was termed as ArmAt58. According to the 3D structures, this family of methyltransferases' unique N-terminal domain is necessary for enzymatic activity. The N7 group of G1405, which is located on helix 44 of 16 S RNA, is methylated by the actions of RmtB and ArmA. It was established that the 30 S ribosomal subunit is the only one for which the ArmA methylation process occurs.

The N-terminal domain surface of RmtC, which is made up of basic residues from the N1 and N2 subdomains, directly contributed to the protein's ability to bind 30S. The C-terminal domain's additional residues were crucial for the 16S rRNA alteration, but they had no direct impact on the binding affinity (Nosrati et al., 2019).

The outcomes of these experiments outlined the essential elements of m7G1405 methyltransferase substrate recognition and identified at least two distinctive, crucial contributions of the enzyme residues under test: Stabilizing a binding-induced 16S rRNA conformation required for G1405 modification and increasing 30S-binding affinities (Schmitt et al., 2009).

2.6.1 NpmA(Novel Plasmid - Mediated aminoglycoside–resistance 16S rRNA Methyltransferase A)

Clinical isolates of E. coli had the methyltransferase NpmA, which imparts resistance to aminoglycoside antibiotics. NpmA adopts a seven-stranded -sheet structure for its Class I methyltransferase fold. In contrast to other enzymes of this fold, NpmA's N-terminal helix (corresponding to motif X) folds onto the opposite portion of the central β-sheet (Obranic et al., 2010).

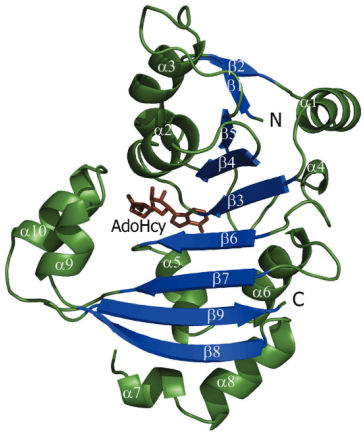


Figure 7 - Structure of NpmA bound to AdoHcy (Hussain et al., 2010)

The 30S ribosomal subunit's 16S rRNA contains the nucleotide residue A1408, which is a critical "hot spot" for aminoglycoside resistance. The generally conserved A1408 is crucial for aminoglycoside antibiotic binding to the 16S rRNA's A region. The hydroxyl group on sugar ring I of the 4,6- and 4,5-disubstituted 2-deoxystreptamines establishes hydrogen bonds with the N1 and N6 atoms of A1408 according to structural investigations. According to footprinting investigations, NpmA's N1 methylation of A1408 impairs the 16S rRNA's interaction with the medication, resulting in resistance (Hussain et al., 2010).

2.6.2 ArmA (Aminoglycoside Resistance Methylase A)

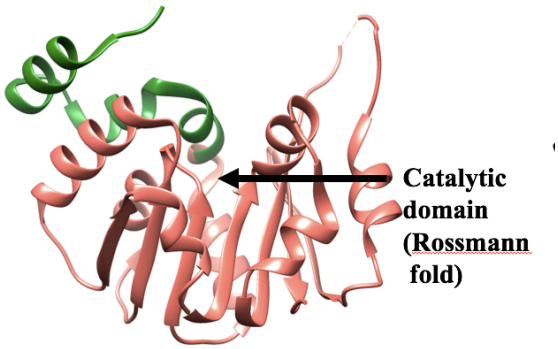


Figure 8 - Truncated structure of ArmA at position 58 (Shmitt et al., 2009)

ArmA has been shown to methylate the N7 position of G1405 16 S rRNA. The basic structure of ArmA consists of an N-terminal domain for binding and a C-terminal domain which is catalytic in nature and consists of the classical Rossmann Fold. A truncated version of the ArmA protein (ArmAt58), starting at position 58, was crystallised in the presence of AdoMet in order to explore the crystalline structure of ArmA. ArmA is responsible for catalysing the methylation of the N7 group of G1405 on helix 44 of 16S RNA. It was discovered that the 30S ribosomal subunit is the only one that can undergo the ArmA methylation reaction; neither the 16S rRNA nor the 70 S ribosomes can (Shmitt et al., 2009).

2.7 Transformation of Competent Cells

A small extrachromosomal DNA molecule called a plasmid is physically distinct from chromosomal DNA and has the ability to replicate on its own in a cell. Although plasmids are most frequently discovered in bacteria as tiny, circular, double-stranded DNA molecules, they can also be found in archaea and eukaryotic cells.

Plasmids that are utilized experimentally for research purposes are referred to as vectors. By incorporating DNA snippets or genes into a plasmid vector, scientists can produce a recombinant plasmid. Through the process of transformation, a method of horizontal gene transfer whereby some bacteria take up foreign genetic material from the environment, this plasmid can then be inserted into a bacterium (Barretto et al., 2014).

Bacterial transformation is facilitated by the use of competent cells, which have been altered to make their membranes more porous. Chemical competence is commonly achieved by incubating bacteria on ice in a solution containing calcium chloride, which neutralizes the negative charges on the bacterial lipid bilayer and DNA. The bacteria are then subjected to a heat shock, which further increases their porosity to external DNA and enhances the chances of successful uptake of the plasmid. This process of forcing foreign DNA into a host is known as transformation (Li et al., 2010).

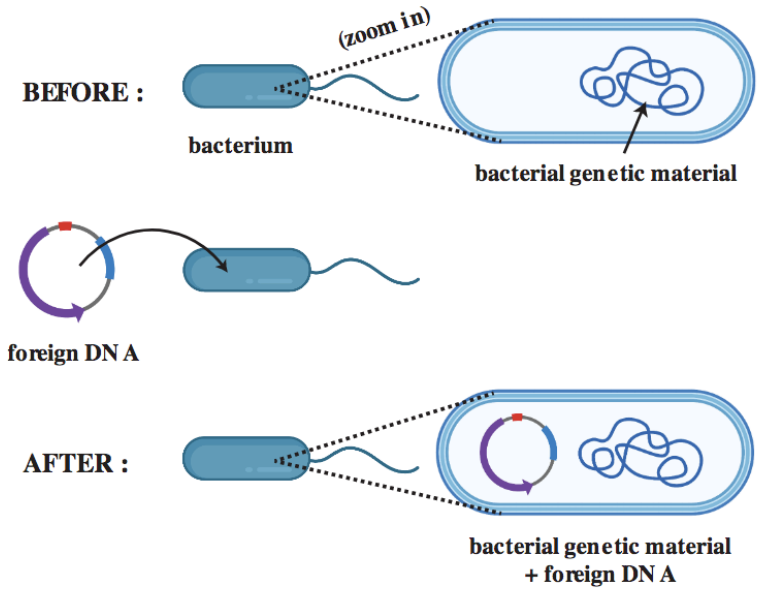


Figure 9 - General process of transformation in bacteria (Zhang et al., 2022)

In the process of heat shock transformation, the application of a heat pulse results in a reduction of the membrane potential of competent cells, thereby facilitating the entry of DNA, which is negatively charged, into the cytoplasm by lowering the potential barrier. The preparation of chemically competent cells typically involves the treatment of cell pellets with salts such as $CaCl_2$ or $MgCl_2$, which serve to neutralize the negative charges associated with both the phospholipid bilayer and DNA, thereby promoting the proximity of DNA to the cell (Panja et al., 2006).

2.7.1 Controlled Expression

An expression vector, also referred to as an expression construct, is a genetic tool commonly used for gene expression in cells. Usually, a virus or plasmid is used to introduce a particular gene into a target

cell. The vector can then use the cell's system for synthesising proteins to make the protein that the gene encodes. The expression vector comprises sequences upstream of the cloned gene that regulate transcription and translation of the cloned gene. A gene's transcription into mRNA and subsequent translation into protein are referred to as the process of gene expression. The primary control of gene expression occurs at the transcription level, mainly due to the binding of proteins to specific sites on DNA (Old et al., 1994).

An "**operon**," a group of linked genes on the chromosome that includes an operator, a promoter, a regulator gene, and structural genes, controls the synthesis of the enzyme

- The *structural genes* encompass the genetic information necessary for the synthesis of protein products. The control of protein production is primarily accomplished through the regulation of RNA polymerase's ability to access the structural gene undergoing transcription.
- The *promoter gene* is a non-coding DNA sequence that serves as the primary binding site for RNA polymerase, without encoding any functional protein or molecule.
- The *operator gene*, which serves as the binding site for the repressor, is a non-coding DNA sequence.
- The *regulator gene* responsible for regulation encodes the production of a repressor molecule that binds to the operator, thereby impeding the transcription of the structural genes by RNA polymerase (Picknett et al., 2001).

The promoter pBAD, which is activated by arabinose, causes cells to either be fully induced or uninduced, even at intermediate concentrations of arabinose. To create a system where control is more consistent in a single population of cells, the gene *araE* from *E. coli* was inserted into a plasmid derived from RSF1010, with regulation controlled by the inducible promoters P_{tac} and P_{taclac}. This gene codes for a protein that transports arabinose with low affinity but high capacity, and is naturally regulated by an arabinose-inducible promoter (Guzman et al., 1995).

2.7.1.1 pBAD Vector

The L-arabinose and glucose-free conditions in which the PBAD promoter is most strongly activated in *E. coli*. When L-arabinose is absent, *E. coli AraC* binds to sequences upstream of pBAD, inhibiting araBAD production and activating it when it is present (Schleif, 1996; Gallegos et al., 1997). The cAMP-catabolite repressor protein (CRP) is also needed for the araBAD genes to be fully induced (Hahn et al., 1984). A 1000-fold range of L-arabinose concentrations is controlled by the araC-PBAD regulatory system, which also offers tight suppression in the absence of L-arabinose and high induction in its presence.

The *AraC* protein has the ability to regulate both positively and negatively. When arabinose is present, transcription from the pBAD promoter is activated, but when it is absent, transcription occurs at very low levels. Glucose can further diminish transcription levels by inhibiting the catabolite-repressed pBAD promoter's ability to express itself by lowering levels of cyclic AMP. *AraC*'s role in the expression and regulation of pBAD has been thoroughly investigated, and their molecular interactions have been examined. Previous studies using plasmids with transcriptional or translational fusions to araB have shown that the expression from this system is tightly regulated (Guzman et al., 1995).

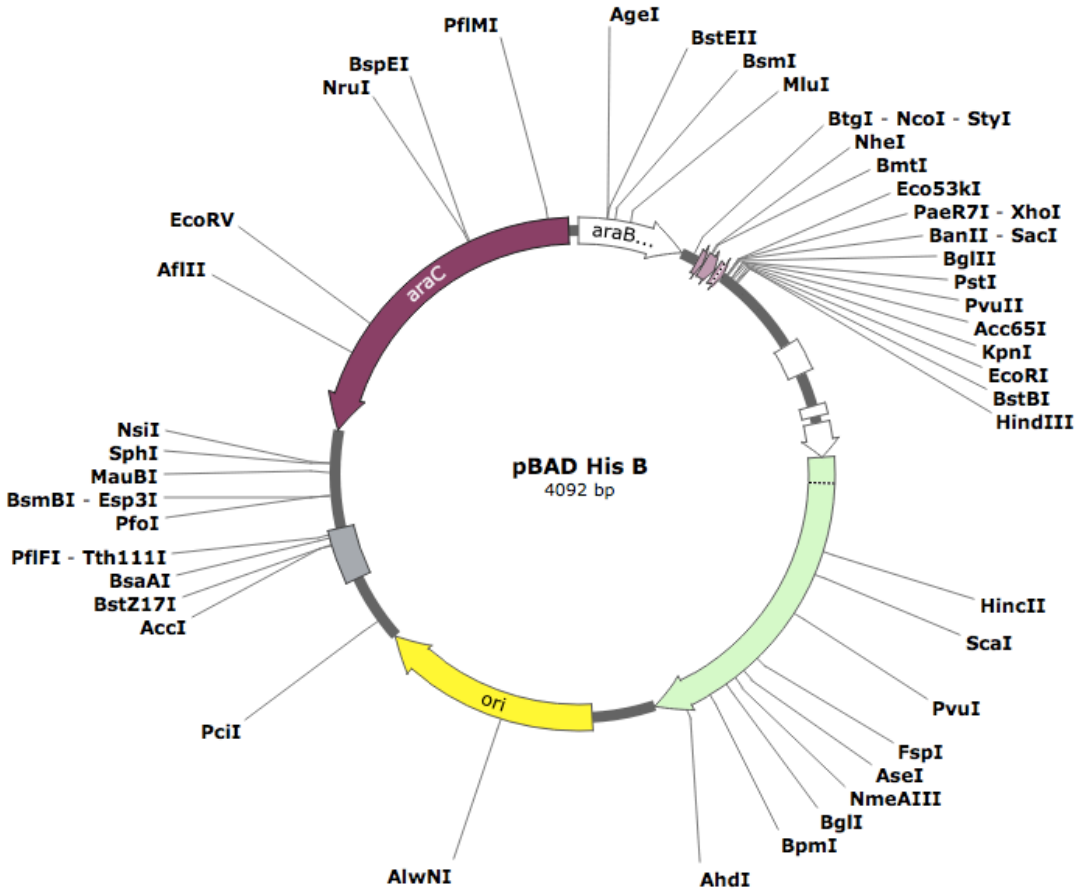


Figure 10 - Vector Map for pBAD HisB expression vector

2.7.1.2 6x His-Tag

A polyhistidine-tag is a sequence of amino acids found in proteins that usually contains six or more histidine (His) residues, usually located at either end of the protein. This tag, also known as 6xHis-tag, is a popular and uncomplicated purification tag that can easily bind to transition metal ions like Ni^{2+} or Co^{2+} that are attached to beads or a resin for purification purposes (Bornhorst et al., 2000).

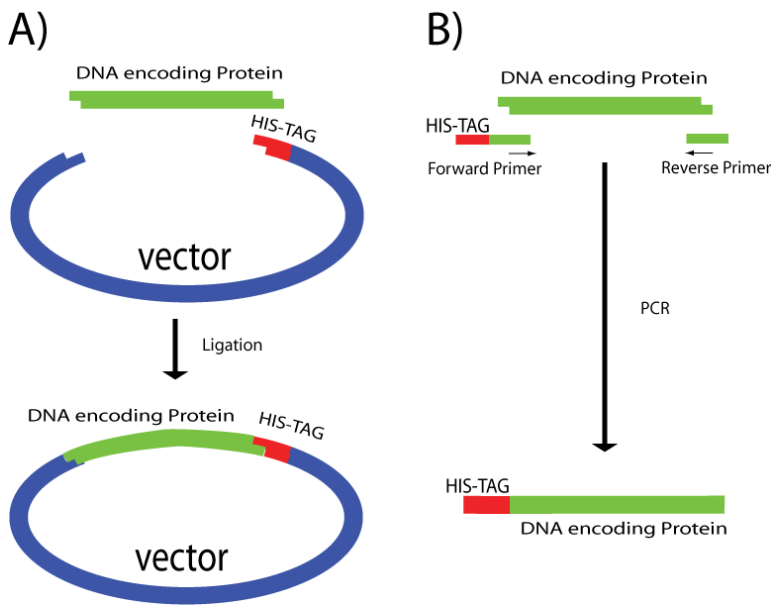


Figure 11 - Poly-Histidine Tagged protein expression in vector (Mierendorf, R C et al., 1998)

2.7.2 TOP10 Cells

One of the most widely utilised competent cells in the lab is the TOP10 strain, which was developed from the MC1061 strain. TOP10 grows quicker (slower than mach1-t1 but comparatively faster than DH5α). In 10 hours, the clone will be apparent. The stability of inserted DNA and the extraction of high purity plasmid DNA are both benefited by recA1 and endA1 alterations. The TOP10 Chemically Competent Cell exhibits streptomycin resistance and can be utilised for cloning and blue and white spot screening.

Competent *E. coli* TOP10 cells are prepared for heat shock transformation with vector DNA and its subsequent proliferation for cloning and transfection purposes (Khodabakhsh et al., 2013).

2.8 Growth curve of bacteria

To study the growth of bacterial populations, it is necessary to introduce viable cells into a sterile broth medium and incubate them under optimal conditions of temperature, pH, and gas. This allows the cells to reproduce quickly, and their growth can be tracked using a population growth curve. This curve shows the increase in cell numbers over time and can be used to identify different stages of the growth cycle (Paulton et al., 1991). Additionally, it allows for the assessment of an organism's generation time, which is the amount of time needed for the population to double, as well as cell

counts and the rate of growth of that organism under standardised settings. The growth curve typically consists of several stages:

- **Lag phase**: In this phase, the cells are adapting to their fresh surroundings. The pace of cellular metabolism is hastened, leading to swift production of cellular macromolecules, particularly enzymes, to get ready for the following stage of the cycle. Despite the cells growing in size, there is no cell replication, and as a result, there is no rise in the number of cells.

- **Logarithmic (log) phase**: When provided with ideal nutrition and physical environment, strong cells reproduce quickly and uniformly through binary fission, leading to a rapid and exponential growth in population until it reaches its maximum capacity. The time taken for the population to double is known as the generation time, and the duration of the log phase varies based on the organism and the medium's composition. On average, the log phase lasts between 6 to 12 hours.

- **Stationary phase**: In this stage, the number of cells dividing is balanced with the number of cells dying, resulting in no net increase in cell count. This maintains the population at its highest level for a certain duration. The main reasons for this phase are the exhaustion of crucial nutrients and the buildup of harmful acidic or alkaline substances in the environment.

- **Decline or death phase**: The decline in the number of individuals caused by mortality is similar to the rise observed during the logarithmic phase. In theory, the entire population should perish within the same time frame as the logarithmic phase. Nonetheless, this is not the case because a few extremely resilient organisms endure for an unknown period (Zwietering et al., 1990).

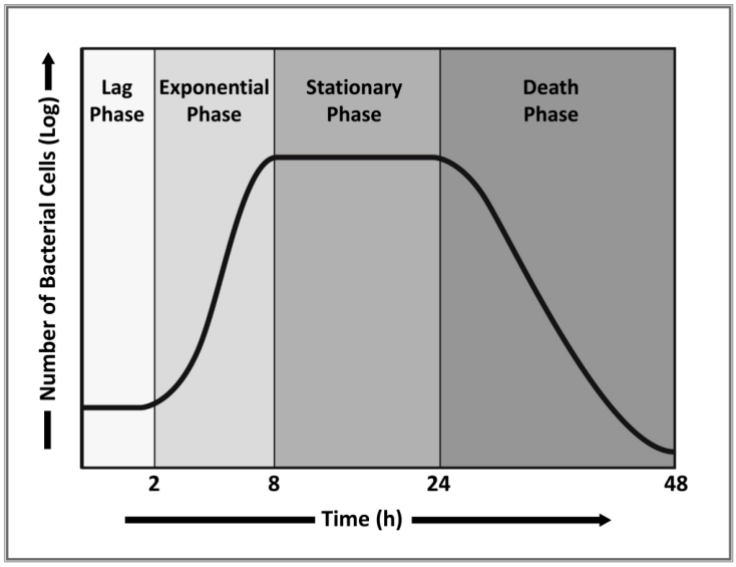


Figure 12- Growth curve of bacteria (Garrison et al., 2016)

2.8.1 Broth Microdilution Assay (Bioscreen)

The minimum inhibitory concentration (MIC) is a measure of the susceptibility or resistance of particular bacterial strains to antibiotics applied in vitro. Accurate determination of MIC is crucial in selecting an effective treatment strategy, which can greatly impact the success of infection therapy (Kowalska-Krochmal et al., 2021).

A technique used to determine an organism's sensitivity to antibiotics is called broth microdilution. According to the requirements of the target bacterium, several microtiter plates are filled with a specific broth during testing. The test bacteria and various antibiotic concentrations are then introduced to the plate. The plate is then put into a non-CO_2 incubator and incubated for 16 to 20 hours at 37° C. The plate is taken out and examined for bacterial growth after the stipulated amount of time has passed. Bacterial growth was evident if the broth become hazy or if a layer of cells grew at the bottom. Minimum Inhibitory Concentration (MIC), or the lowest concentration of antibiotics that inhibited bacterial proliferation, is used to describe the outcomes of the broth microdilution procedure (Lorian V, 2005).

2.9 Protein Expression

Polyhistidine-tags are commonly used to purify recombinant proteins that are expressed in bacteria like E. coli. The cells are collected and then broken open using physical methods or detergents and enzymes. The resulting lysate contains the recombinant protein as well as other proteins from the host cells. The lysate is then exposed to a resin that is bound to a carrier matrix and contains a divalent cation. This resin is either added directly to the lysate or passed over in a column format (Bornhorst et al., 2000). The protein of interest is then eluted using a solution with a high imidazole concentration or a lower pH after the resin has been washed to eliminate proteins that do not particularly interact with the cation. The purity and quantity of the protein can be determined using techniques like SDS-PAGE and Western blotting (Mahmood et al., 2012).

A relatively pure protein is typically produced by affinity purification using a polyhistidine-tag. By incorporating a small amount of imidazole (20–40 mM) into the binding and wash solutions, protein purity can be increased. However, additional purification procedures like ion exchange or size exclusion chromatography can be required depending on the downstream application. IMAC resins frequently retain some indigenous proteins as impurities, but these can be removed by taking further steps for purification or by producing the recombinant protein in cells with a deficiency. An alternative is to employ cobalt-charged IMAC resins, which have a lower affinity for endogenous proteins (Block et al., 2009).

2.9.1 Western Blotting

Protein separation and identification in research often include the Western blot method. It involves employing gel electrophoresis to separate a mixture of proteins according to their molecular weight. After being separated, the proteins are put onto a membrane, where each protein forms a band. The membrane is then in contact with certain antibodies that bind to the target protein. Only the antibodies bound to the protein of interest are left after the unbound antibodies have been removed. Then a film is developed to find these attached antibodies. Exclusively one band should be noticeable because the antibodies only bind to the target protein. A standard can be used to calculate the protein quantity because the thickness of the band shows the amount of protein present (Mahmood et al., 2012).

Sample preparation - The most commonly used type of sample for western blot are cell lysates. Protein extraction aims to gather all the proteins present in the cytosol of the cell. To avoid damaging the proteins, this process should be carried out at a low temperature and with the addition of protease inhibitors. Tissue samples, which have a more organized structure, require mechanical methods like homogenization or sonication to extract the proteins (Posch et al., 2014).

Once the appropriate sample volume is determined, it is mixed with a loading buffer that includes glycerol to facilitate the sinking of the samples into the gel wells. The loading buffer also contains a tracking dye called bromophenol blue, which helps the researcher monitor the progress of separation. To denature the higher order structure while preserving sulfide bridges, the diluted sample is heated after being mixed with the loading buffer. This denaturation procedure guarantees that the amino acids' negative charges are preserved, enabling the protein to move in an electric field used during electro transfer (Taylor et al., 2014).

Gel electrophoresis - Stacking gel and separating gel are two different kinds of agarose gel used in the Western blot method. The separating gel is placed beneath the stacking gel, which has a pH of 6.8 that is somewhat acidic and a lower acrylamide content. This composition results in a porous gel that exhibits poor protein separation but facilitates the formation of thin, well-defined bands (Scofield et al., 2006). Conversely, the separating gel, located below the stacking gel, possesses a basic pH of 8.8 and a higher content of polyacrylamide, leading to narrower pores. Consequently, protein separation in this gel primarily occurs based on size, with smaller proteins traversing the gel more easily and rapidly than larger proteins. Prior to loading onto the gel, the proteins are denatured through heating, resulting in a negative charge. Upon the application of a voltage, the proteins migrate towards the positive electrode. The gel is typically made by pouring the gel solution between two glass/plastic plates. The selected wells are loaded with the samples and a marker, and sample buffer is added to

the empty wells. The gel is then linked to a power source and left to run after that. It is crucial to carefully regulate the voltage, as excessive voltage can lead to overheating and distortion of the protein bands (Johansson et al., 1972).

Blotting Following the separation of the protein mixture, it is subsequently transferred onto a membrane. This transfer process involves the application of an electric field that is positioned perpendicular to the surface of the gel. Consequently, the proteins migrate out of the gel and onto the membrane. A sandwich arrangement is used to assist this exchange, with the membrane positioned between the gel surface and the positive electrode (Kurien et al., 2006).

Washing, blocking and antibody incubation Blocking is a crucial stage in the western blotting process, as it serves to prevent non-specific binding of antibodies to the membrane. Typically, blocking is achieved by using a solution containing 5% bovine serum albumin (BSA), which is diluted by using Tris-buffered saline with Tween 20 (TBST). This blocking step is employed to minimize background noise and enhance the specificity of antibody binding (Yang et al., 2012).

Quantification It is important to recognise that the information obtained from a western blot examination is typically thought of as semi-quantitative. This is because, rather than providing an absolute measurement of quantity, it might provide a relative estimate of protein levels (Kurien et al., 2006).

2.9.2 Ni-NTA Affinity Chromatography

Affinity chromatography is a versatile technique that can provide valuable insights into the specific binding partner of a protein of interest and the relative strength of their interaction. One such method is immobilized metal ion affinity chromatography (IMAC), which is a straightforward approach for studying protein-protein interactions and determining their binding affinities. A specialised FPLC equipment enclosed in an anaerobic chamber can be utilised to perform a particular type of IMAC known as Ni-NTA affinity chromatography in anaerobic circumstances (Block et al., 2009).

A protein of interest with a polyhistidine tag, sometimes known as the "bait protein," is immobilised onto the Ni-NTA resin to study protein-protein interactions. The "prey proteins," or prospective interacting proteins without a His motif and unable to interact directly with the Ni-NTA resin, are next put into the column and incubated. Prey proteins may interact with the bait protein throughout this incubation time, and as a result, the prey proteins may be retained on the Ni-NTA column. A wash step is used after the incubation to get rid of any prey proteins that didn't interact with the bait

protein. Finally, when all proteins have been eluted from the column, downstream analysis using SDS-PAGE is performed on the load, wash, and elution fractions (Gaberc-Porekar et al., 2001).

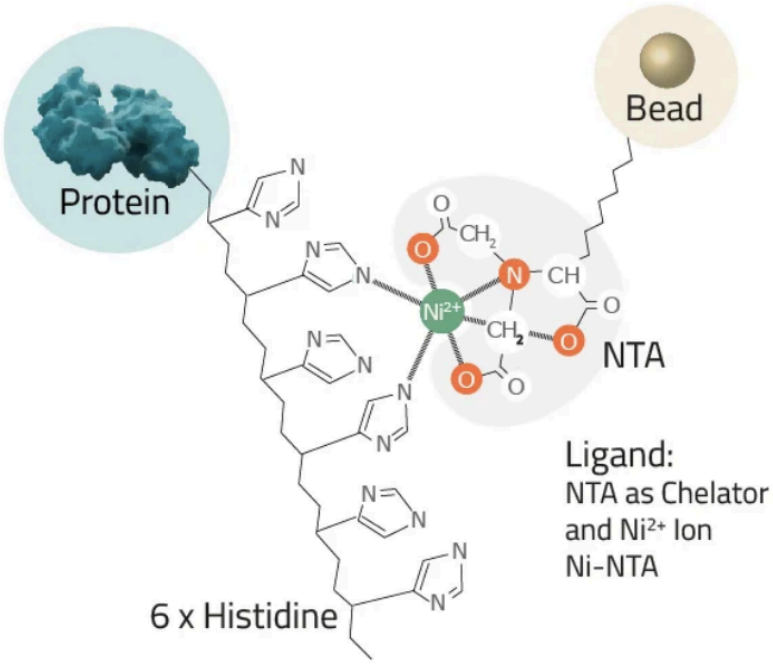


Figure 13- Schematic depiction of a chelator complex consisting of NTA and Ni^{2+}. This nickel ion binds two imidazole rings of a His-tag that has been added to a protein (Hochuli, E. et al., 2004)

CHAPTER III

HYPOTHESIS

N-Terminal Domain (NTD) of NpmA ligated to the catalytic domains(CTD) of ArmA and RmtA should validate the importance of NTD in recognition and binding of substrate (30S subunit).

The acquired RMTases which were hypothesized, should, in principle, be able to impart resistance against both 4,5- and 4,6- DOS groups of aminoglycosides. If there is resistance in the transformed bacteria, it shows that the N-terminal Domain (NTD) is important for recognition and binding of 30S subunit.

OBJECTIVES

- Construct Chimera of $NpmA^{N}$_$ArmA^{C}$ and $NpmA^{N}$_$RmtA^{C}$.
- Evaluate the *in vivo* mechanism assay of the chimera transformed strains.
- Enzyme isolation and purification of Protein for Structural Analysis.

CHAPTER IV

MATERIALS AND METHODS

1. Primer Construction

The chimera sequences were analyzed and the forward and reverse primers to be used were designed accordingly for further use.

- **$NpmA^{N}$-$ArmA^{C}$**

Nucleotide sequence (534 bp)
ATGTTGTTAATACTCAAAGGAACAAAGACGGTTGATTTATCAAAAGATGAATTG
ACAGAAATAATAGGTCAGTTTGATCGTTCATCTATTTTAGATTTTGGTTGTGGCT
TCAATCCATTAGCTTTATACCAATGGAATGAAAATGAAAAAATAATATATCATG
CATACGATATTGATAGAGCTGAGATAGCTTTTTTGAGTAGCATTATTGGGAAGT
TAAAGACGACGATAAAGTATAGGTTTTTGAATAAAGAGAGTGATGTCTACAAA
GGTACTTATGATGTAGTATTCCTTTTAAAGATGCTTCCTGTGCTAAAACAGCAAG
ATGTAAATATCTTGGATTTCCTACAGCTTTTTCATACTCAAAACTTTGTAATATC
TTTTCCAATAAAGTCTTTATCTGGAAAGGAGAAGGGAATGGAAGAGAATTACCA
GCTATGGTTTGAATCTTTTACAAAAGGTTGGATAAAAATCCTTGATTCGAAGGT
TATAGGGAATGAGTTAGTATATATTACTAGTGGATTTCAGAAATAA

Protein sequence (177 amino acids)
MLLILKGTKTVDLSKDELTEIIGQFDRSSILDFGCGFNPLALYQWNENEKIIYHAYDI
DRAEIAFLSSIIGKLKTTIKYRFLNKESDVYKGTYDVVFLLKMLPVLKQQDVNILDFL
QLFHTQNFVISFPIKSLSGKEKGMEENYQLWFESFTKGWIKILDSKVIGNELVYITSG
FQK

- **$NpmA^{N}$-$RmtA^{C}$**

Nucleotide sequence (525 bp)
ATGTTGTTAATACTCAAAGGAACAAAGACGGTTGATTTATCAAAAGATGAATTG
ACAGAAATAATAGGTCAGTTTGATCGTCATCGTGTGTTGGATATCGCTTGCGGC
CTAAACCCGCTGGCCCTCTTTATACGTGACATAACATCTGTATGGGCGTGCGAC
ATCCATCAGGGGTTGGGCGATGTGATCACCCCCTTTGCCCATCATCAGGGATTG
GACTTCACGTTCGCCCTGCAGGATGTGATGTGTACGCCGCCCACTGAGACGGGG
GATTTGGCACTGGTATTTAAATTACTGCCTTTGCTGGAGCGAGAGCAAGCTGGC
GCCGCCATGGCGCTACTGCAGGCACTAGCTACCCCTCGGATTGCCGTCAGCTTC
CCCACCCGCAGTTTAGGCGGGCGCGGCAAGGGCATGGAAGCAAACTATTCCGC
ATGGTTCGAGGGGGCACTGCCTGATGAATTTGAAATTGAGGATACCAAGACCAT
TGGAATAGAGCTTGTGTACATGATAAAAAGGAATAAGTGA

Protein sequence (175 amino acids)
MLLILKGTKTVDLSKDELTEIIGQFDPRHRVLDIACGLNPLALFIRDITSVWACDIHQ
GLGDVITPFAHHQGLDFTFALQDVMCTPPTETGDLALVFKLLPLLEREQAGAAMAL
LQALATPRIAVSFPTRSLGGRGKGMEANYSAWFEGALPDEFEIEDTKTIGIELVYMI
KRNK

2. Gene Amplification using PCR

PCR was carried out to amplify NpmA, ArmA and RmtA in given templates by using primers with an amplicon size of 74 bp, 453 bp and 444 bp respectively.

Sequence of primers used:

NA_NTD_FP	GCGCAGATCTATGTTGTTAATACTCAAAGGAACAA
NA_NTD_RP	TATACCGCGGATCAAACTGACCTATTATTTCTGTC
AA_CTD_FP	TATACCGCGGTCATCTATTTTAGATTTTGGTTGTG
AA_CTD_RP	GCGCGAATTCTTATTTCTGAAATCCACTAGTAATATATAC
RA_CTD_FP	TATACCGCGGCATCGTGTGTTGGATATCGC
RA_CTD_RP	GCGCGAATTCTCACTTATTCCTTTTTATCATGTACA

PCR mixture:

Composition	Concentration
Pfu Buffer (Invitrogen)	10 X
dNTPs	10mM
Forward primer	10 µM
Reverse primer	10 µM
Template DNA	100 ng
Pfu Polymerase	2.5 u/µL
Nuclease free water (NFW)	-

Steps (NA_NTD)	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	45
Annealing	56°C	30 sec	
Extension	72°C	30 sec	
Final Extensions	72°C	7 min	1

Steps (RA_CTD/AA_CTD)	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	35
Annealing	58°C	30 sec	
Extension	72°C	30 sec	
Final Extensions	72°C	7 min	1

3. Gel Extraction of Amplified PCR products

- In a conical flask with 1X TAE buffer, 1.5% agarose gel was made and microwaved to dissolve. Ethidium Bromide was added.

- A gel casting tray with the required number and sizes of wells was filled with dissolved agarose and left to set at room temperature.
- The comb was removed from the gelcomb and put in an electrophoresis tank with 1X TAE buffer once it had solidified.
- Using a micropipette, the PCR product and loading dye (6X) were combined and added to the wells.
- To determine the size of the PCR products, 2 µl of DNA ladder [1 kb bp plus] was also placed in one of the wells.
- The gel was examined and photographed using the GelDoc (Bio-Rad) system after the electrophoresis procedure.
- Under the UV transilluminator, the required amplicons (PCR products) were seen as a brilliant band of the appropriate size and were then removed from the gel for additional processing.

❖ **Wizard r SV Gel and PCR Clean-up System**

- Excision of the band was done by visualization on transilluminator.
- Membrane binding buffer was added to the Eppendorf according to the weight of the band as 10µl of buffer/10mg of gel.
- The mixture was vortexed and incubated at 50-65°C till it melted completely.
- Transferred the mixture into the provided spin column and incubated it at room temperature for 5 minutes.
- This column was centrifuged for 1 minute at 13000 rpm; the flow through was discarded.
- Added 700µL of wash solution and centrifuged at 13000 rpm for 1 minute. Repeated this step.
- Transferred the column onto a fresh 1.5mL MCT and left the membrane for drying.
- Added 30µL of NFW and incubated for 5 minutes. Centrifuged for 1 minute at 13000rpm and stored at -20°C for further use.

4. Restriction Digestion of $NpmA^N$, $ArmA^C$, $RmtA^C$ and pBAD vector

- Required quantity of DNA inserts were digested using enzymes suitable to the restriction sites present on the whole DNA segment.

The reaction mixtures were prepared as follows:

Composition ($NpmA^N$)	**Concentration**
Cutsmart Buffer	10 X
Template	1 µg
SacII HF	10 units/µg
NFW	-

Composition ($ArmA^C$/$RmtA^C$)	Concentration
Cutsmart Buffer	10 X
Template	1 μg
EcoR1 HF	10 units/μg
SacII HF	10 units/μg
NFW	-

Composition (pBAD)	Concentration
Cutsmart Buffer	10 X
Template	1 μg
EcoR1 HF	10 units/μg
NFW	-

- These reaction mixtures underwent a two-hour incubation period at 37°C.
- After 2 hours, second set of enzymes were added to pBAD and $NpmA^N$ reaction mixtures.
- NaCl was added first in small quantity and then BglII was added for sequential digestion of the inserts.

5. **Clean-up of salt from sequentially digested product (**using the Wizard r SV Gel and PCR Clean-up System)
   - Membrane binding buffer was added to the Eppendorf according to the quantity of the sample as 1:1.
   - The mixture was vortexed and transferred into the provided spin column and incubated it at room temperature for 5 minutes.
   - This column was centrifuged for 1 minute at 13000 rpm; the flow through was discarded.
   - Added 700μL of wash solution and centrifuged at 13000 rpm for 1 minute. Repeated this step.
   - Transferred the column onto a fresh 1.5mL MCT and left the membrane for drying.
   - Added 30μL of NFW and incubated for 5 minutes. Centrifuged for 1 minute at 13000 rpm and stored at -20°C for further use.

6. **Estimate of DNA content by Nanodrop Spectrophotometer**
   - Calibrated the spectrophotometer by using 1 μL of NFW as blank.
   - Set parameters for DNA-50 protocol and loaded 1 μL of sample onto the spectrophotometer.
   - Noted the estimated DNA content as well values of 260/280 and 260/230 to check for purity of the sample.

7. rSAP treatment of vector

- The gel extracted and digested vector pBAD was subjected to rSAP treatment. After addition of enzyme, the mixture was incubated at 37°C for 40 minutes.
- After incubation, the mixture was kept at 65°C for 10 minutes to inactivate rSAP and its dephosphorylating action.

Composition	Concentration
rSAP	10 units/µg
pBAD	1 µg
NFW	-

8. Ligation (of both terminals)

- Using NEBioCalculator and using the estimate of DNA by nanodrop, the ligation of insert ($Npma^{N}$) was done to vector ($ArmA^{C}$/$RmtA^{C}$) as follows.

Composition	Concentration
T4 DNA ligase Buffer	10 X
Insert: Vector	3:1
T4 DNA Ligase	10 units/µg
NFW	-

- For one hour, this mixture was incubated at 37°C.

9. Ligation (of insert into vector)

- Using NEBioCalculator and using the estimate of DNA by nanodrop, the ligation of insert ($NpmA^{N}$-$ArmA^{C}$/ $NpmA^{N}$-$RmtA^{C}$) was done to vector (pBAD) as follows:

Composition	Concentration
T4 DNA ligase Buffer	10 X
Insert: Vector	3:1
T4 DNA Ligase	10 units/µg
NFW	-

- For one hour, this mixture was incubated at 37°C.

10. Competent Cell Preparation

- Incubated *E.coli* TOP10 in LB broth. Kept at 37°C in an orbital shaker for overnight incubation at 180 rpm.
- Incubated secondary culture from overnight primary culture in fresh LB media in ratio 1:100. Grown till absorbance at 600nm reached an O.D of 0.4.

- Aliquoted into 50mL falcons and centrifuged at 5000rpm for 10 minutes at 4°C. Decanted the media and dried the pellet.
- Re-suspended the pellet in 100mM $CaCl_2$ (15 mL per 100mL cell pellet). Mixed properly and incubated on ice for 15 minutes.
- Pelleted the cells down at 3000rpm for 5 mins at 4°C and decanted the supernatant. Re-suspended the pellet 100mM $CaCl_2$. Incubated in ice bath for 30 minutes.
- Pelleted the cells down at 3000 rpm for 10 mins at 4°C and decanted the supernatant. Added 600µL of 100mM $CaCl_2$ and 50% glycerol for each 100mL of pellet. Aliquoted 100µL onto each microfuge tube.
- Stored at -80°C for further usage.

11. Transformation of *E.coli* TOP10 cells

- Added 5-10 ng of DNA to 100µL of competent cells and incubated for 30 minutes on ice. Heat shock was given for 90 seconds at 42°C and then back on ice for 5 minutes.
- Added 900µL of LB broth and incubated at 37°C at 180rpm for 1 hour.
- LB agar plates were prepared using required concentration of antibiotic (Ampicillin 100mg/µL)
- Centrifuged the culture at 3000rpm for 10 minutes after the incubation period ended.
- Re-suspended the pellet with 100µL of fresh LB broth and plated onto LB agar plates.
- Incubated this plate at 37°C for 8-12 hours.

12. Plasmid isolation of transformed cells (Alkaline Lysis Method)

- **Solutions for plasmid extraction**

Alkaline lysis solution 1

Components	Final concentration
Glucose	50 mM
Tris-Cl (pH-8.0)	25 mM
EDTA (pH- 8.0)	10 mM

Alkaline lysis solution 2 (Prepared freshly)

Components	Final concentration
NaOH (0.2 N stock)	0.2 N
SDS	1.0 %

Alkaline lysis solution III

Components	Final Concentration
5M potassium acetate	5 M
Glacial acetic acid	11.5%

- Overnight grown bacterial culture was Pelleted down. The pellet was re-suspended in 250µL of ice cold solution 1.
- 250µL of solution 2 was added and mixed by inverting. This mixture was incubated on ice for 5 minutes.
- 350µL of ice-cold solution 3 was added, and it was left to incubate for five minutes on ice.
- Centrifuged at 13000 rpm for 15 minutes. Transferred supernatant into a fresh micro centrifuge tube.
- Precipitated plasmid from above obtained supernantent by adding equal volume of chilled isopropanol. Incubated at -20°C for 60 minutes.
- Centrifuged at 13000 rpm for 5 minutes at 4°C and removed supernatant.
- Added 1mL of chilled ethanol and mixed by inversion. Centrifuged at 13000 rpm for 2 minutes at 4°C.
- Air dried the pellet till ethanol smell goes off. Added 50µL of nuclease free water. Stored at -20°C for further use.

13. PCR confirmation of Clones

- PCR was carried out to confirm presence of $NpmA^N$-$ArmA^C$/ $NpmA^N$-$RmtA^C$ in the transformed cells by using forward primer of $NpmA^N$ and reverse primer of $ArmA^C$/ $RmtA^C$ as follows:

Sequence of primers used:

NA_NTD_FP	GCGCAGATCTATGTTGTTAATACTCAAAGGAACAA
AA_CTD_RP	GCGCGAATTCTTATTTCTGAAATCCACTAGTAATATATAC
RA_CTD_RP	GCGCGAATTCTCACTTATTCCTTTTTATCATGTACA

Composition	Concentration
5x Master Mix	1X
Template	100 ng
Forward Primer	0.4 µM
Reverse Primer	0.4 µM
NFW	-

- This PCR product was run on agarose gel (1.5%) and analyzed using ladder (1kb).

14. Broth Microdilution Assay (using CLSI guidelines)

- **Preparation of antibiotic dilutions**
    - Initial concentrations of Kanamycin and Neomycin were 100mg/mL and 25.5mg/mL respectively.
    - Initially, 1.28 μL of kanamycin and 5 μL of neomycin were taken in 1mL of NFW to make 128 μg/mL dilution for each.
    - Subsequently, 500 μL of these initial dilutions were taken in 500 μL of NFW up until the concentration was diluted to 0.25 μg/mL.
    - These serial dilutions were taken as –
        - 0.25 μg/mL, 0.5 μg/mL, 1.0 μg/mL, 2.0 μg/mL, 4.0 μg/mL, 8.0 μg/mL, 16.0 μg/mL, 32.0 μg/mL, 64.0 μg/mL and 128 μg/mL.
- **Assay in MIC plate**
    - Each well was loaded as shown in the table for both the test and control culture against both antibiotics.
    - This plate was read under the bioscreen apparatus for 24 hours with optical density of the growth of bacteria being checked every hour.
    - The results were further plotted as a growth curve at different intensities of antibiotic for comparison.

Table 3: Layout for MIC assay

	10	9	8	7	6	5	4	3	2	1
1	50µL Kanamycin (1.0)	50µL Kanamycin (0.5)	50µL Kanamycin (0.5)	50µL Kanamycin (0.5)	50µL Kanamycin (0.25)	50µL Kanamycin (0.25)	50µL Kanamycin (0.25)	50µL Media	50µL Media	50µL Media
2	50µL Kanamycin (8.0)	50µL Kanamycin (8.0)	50µL Kanamycin (4.0)	50µL Kanamycin (4.0)	50µL Kanamycin (4.0)	50µL Kanamycin (2.0)	50µL Kanamycin (2.0)	50µL Kanamycin (2.0)	50µL Kanamycin (1.0)	50µL Kanamycin (1.0)
3	50µL Kanamycin (64.0)	50µL Kanamycin (64.0)	50µL Kanamycin (64.0)	50µL Kanamycin (32.0)	50µL Kanamycin (32.0)	50µL Kanamycin (32.0)	50µL Kanamycin (16.0)	50µL Kanamycin (16.0)	50µL Kanamycin (16.0)	50µL Kanamycin (8.0)
4					100µL of media only	100µL of media only	100µL of media only	50µL Kanamycin (128.0)	50µL Kanamycin (128.0)	50µL Kanamycin (128.0)
1	50µL Neomycin (1.0)	50µL Neomycin (0.5)	50µL Neomycin (0.5)	50µL Neomycin (0.5)	50µL Neomycin (0.25)	50µL Neomycin (0.25)	50µL Neomycin (0.25)	50µL Media	50µL Media	50µL Media
2	50µL Neomycin (8.0)	50µL Neomycin (8.0)	50µL Neomycin (4.0)	50µL Neomycin (4.0)	50µL Neomycin (4.0)	50µL Neomycin (2.0)	50µL Neomycin (2.0)	50µL Neomycin (2.0)	50µL Neomycin (1.0)	50µL Neomycin (1.0)
3	50µL Neomycin (64.0)	50µL Neomycin (64.0)	50µL Neomycin (64.0)	50µL Neomycin (32.0)	50µL Neomycin (32.0)	50µL Neomycin (32.0)	50µL Neomycin (16.0)	50µL Neomycin (16.0)	50µL Neomycin (16.0)	50µL Neomycin (8.0)
4					100µL of media only	100µL of media only	100µL of media only	50µL Neomycin (128.0)	50µL Neomycin (128.0)	50µL Neomycin (128.0)

15. Western Blotting for Protein Expression

SDS PAGE

Prepared the Resolving gel (12%) in the following order:

H_2O	3.2 mL
Acrylamide/bis (30%)	4.0 mL
Tris–HCl (1.5 M, pH 8.8)	2.6 mL
SDS 10%	100 µL
N, *N*, *N*′, *N*′-tetramethylethylene-diamine (TEMED)	14 µL
Ammonium persulfate (APS), 10%	100 µL

- Poured gel, leaving about two centimeters below the comb's base for the stacking gel. bubbles were carefully removed.
- Isopropanol was layered over the top of the gel. This will eliminate the gel's top bubbles and prevent the polymerized gel from drying out. The gel was entirely polymerized in about 30 minutes.
- Eliminated the isopropanol and flushed any lingering residue with distilled water.
- Prepared the stacking gel (12%) in the following order:

H_2O	1.5 mL
Acrylamide/bis (30%)	0.5 mL
Tris–HCl (0.5 M, pH 6.8)	0.75 mL
SDS 10%	30 µL
TEMED	6 µL
Ammonium persulfate (APS), 10%	30 µL

- After adding combs to create wells, stacking gel was added on top of the separation gel. The stacking gel fully polymerized in about 30 minutes.
- Gel was crammed into the device, and gel running buffer was added to both buffer chambers.
- Placed samples and protein markers with a high molecular mass into wells for electrophoresis separation.

Blot Transfer from SDS gel

- **Materials**
  - Blotting paper
  - Transfer membrane (PVDF Membrane)
  - Transfer buffer (for each 1L):

	Quantity (Per liter)
Tris HCl	3 g
Glycine	14.4 g
Methanol	100 mL
Autoclaved water	To make up volume to 1L

  - Methanol
  - Tank transfer system

- **Setup**
  - Prepared enough transfer buffer to fill the transfer tank, plus an extra 200 mL to moisten the filter paper and equilibrate the gel and membrane.
  - Took the gel out of its cassette and removed any wells and stacking gel.
  - Submerged the gel for 10 to 30 minutes in the transfer buffer.
  - Transfer buffer soaked filter sheets for at least 30 seconds.
  - The membrane was prepared by 15 seconds of methanol soaking. Membrane ought to uniformly transition from being opaque to being translucent.
  - Carefully insert the membrane into the transfer buffer, and then wait at least five minutes for the membrane to equilibrate.
- **Transfer Stack Assembly**
  - Placed a foam (fibre) pad on one side of the cassette after opening the cassette holder.
  - Placed the gel on top of one sheet of filter paper, which was then placed on top of the pad.
  - Stacking a second piece of filter paper on top of the membrane and gel was done.
  - After setting a second foam pad on top of the filter paper, the cassette holder was closed.
- **Method for Protein Transfer (Tank Transfer)**
  - Set up the cassette holder in the transfer tank with the membrane side facing the anode (+) and the gel side facing the cathode (-).
  - Enough buffer was added to the tank to completely enclose the cassette holder.
  - On the transfer unit, insert the red anode lead (+) into the anode jack and the black cathode lead (-) into the cathode jack.
  - Joined the cathode and anode leads to the appropriate power outputs.
  - Started the system at 90V for one to one and a half hours.
  - Take off the cassette holder from the tank once the transfer was finished.
  - Carefully disassembled the transfer stack with forceps.

- **Antibody Incubations**
    - Incubated the blot with agitation for an hour after placing it in the blocking solution.
    - The blot was submerged in the primary antibody solution (antibody to the His tag) and incubated at 4°C with stirring overnight. The membrane's surface should not restrict the solution's ability to move.
    - I washed the blot in PBS for ten minutes. twice more, this time using a new buffer.
    - Submerged the blot in the secondary antibody solution, which was then incubated for an hour at either RT or 37 °C with agitation.
    - I washed the blot in PBS for ten minutes. twice more, this time using a new buffer.
    - Chemiluminescent detection was carried out for visualization and quantification.
- **Chemiluminescent detection**
    - Added substrate to completely cover the membrane on the blot before placing it in a container and incubating it for one minute.
    - Checked the blot by exposure for 5 minutes in chemilumniscence detector.

16. Protein Purification Ni-NTA chromatography

- **Solutions to be prepared (freshly)**

Resuspension Buffer

Components	**Final concentration**
NaCl	300 mM
Tris-Cl (pH-8.5)	20 mM
Glycerol	5%
PMSF	5 mM

Equilibration Buffer

Components	**Final concentration**
NaCl	300 mM
Tris-Cl (pH-8.5)	20 mM
Glycerol	5%
Imidazole	5 mM

Wash Buffer

Components	**Final concentration**
NaCl	300 mM
Tris-Cl (pH-8.5)	20 mM

Glycerol	5%
Imidazole	20 mM

Elution Buffer

Components	**Final concentration**
NaCl	300 mM
Tris-Cl (pH-8.5)	20 mM
Glycerol	5%
Imidazole	300 mM

- Use LB medium to set up the desired culture volume for protein expression. Use the starter culture produced in the previous step at a 1:100 dilution to inoculate a bigger culture. Make sure the culture is in a suitable container that allows for efficient aeration.
- Induced protein expression by activating the arabinose promoter with 0.02% of 20% L-arabinose.
- Cells were centrifuged at 4°C for 10 minutes at 1500 rcf to pellet them. To check for protein expression, remove 1 mL.
- Supernatant was removed, and cells were then rinsed in re-suspension buffer. As before, pelleted, and supernatant discarded.
- Moved the cell suspension for sonication of the probe to a clean microfuge tube.
- Samples that were sonicated using an ultrasonicator (15 kHz, 30% amp). kept samples chilled both during and in between sonication cycles.
- Spun for 30 minutes at 4°C at 60,000g. supernatant recovered and in a clean tube. The pellet should be kept at -20°C to preserve the insoluble protein portion.
- A suitable quantity of Ni-NTA resin slurry (50 percent slurry in 20 percent ethanol) was added to the gravity-flow column. 500 mL of resin slurry are added to 15 mL of bacterial cultures that have been re-suspended in 2 mL of lysis buffer.
- Maintained column upright and let resin settle. once everything was in place, opened the cap and recorded the flow-through (FT) twice as FT1 and FT2.
- Used 500 mL of Wash buffer to wash the sample, collecting the first and last wash elutes as W1 and LWB.
- Elute ten times with one mL of elute buffer, and collect each time individually (i.e., E1, E2, E3, E4, et cetera).
- To evaluate the quality of purification, run SDS-PAGE on pre-FT (10 μL each), FT 1 and FT 2 (10 μL), W1–W3 (20 μL), and E1–E10 (30 μL).

CHAPTER V

RESULTS AND DISCUSSION

RMTases are a group of enzymes that uses 30S ribosomal subunit as a substrate. They methylate different positions of the A site of translation, thus inhibiting attachment of aminoacyl tRNA by using SAM as the cofactor. The presence of these RMTase genes is an important area of study in AMR.

Hypothesis: The acquired RMTases which were hypothesized, should, in principle, be able to impart resistance against both 4,5- and 4,6- DOS groups of aminoglycosides. If there is resistance in the transformed bacteria, it shows that the N-terminal Domain (NTD) is important for recognition and binding of 30S subunit.

Both the RMTases were processed using the same pipeline of workflow and have responded as follows:

1. $NpmA^{N}\_ArmA^{C}$

This RMTase chimera follows the following construct formation:

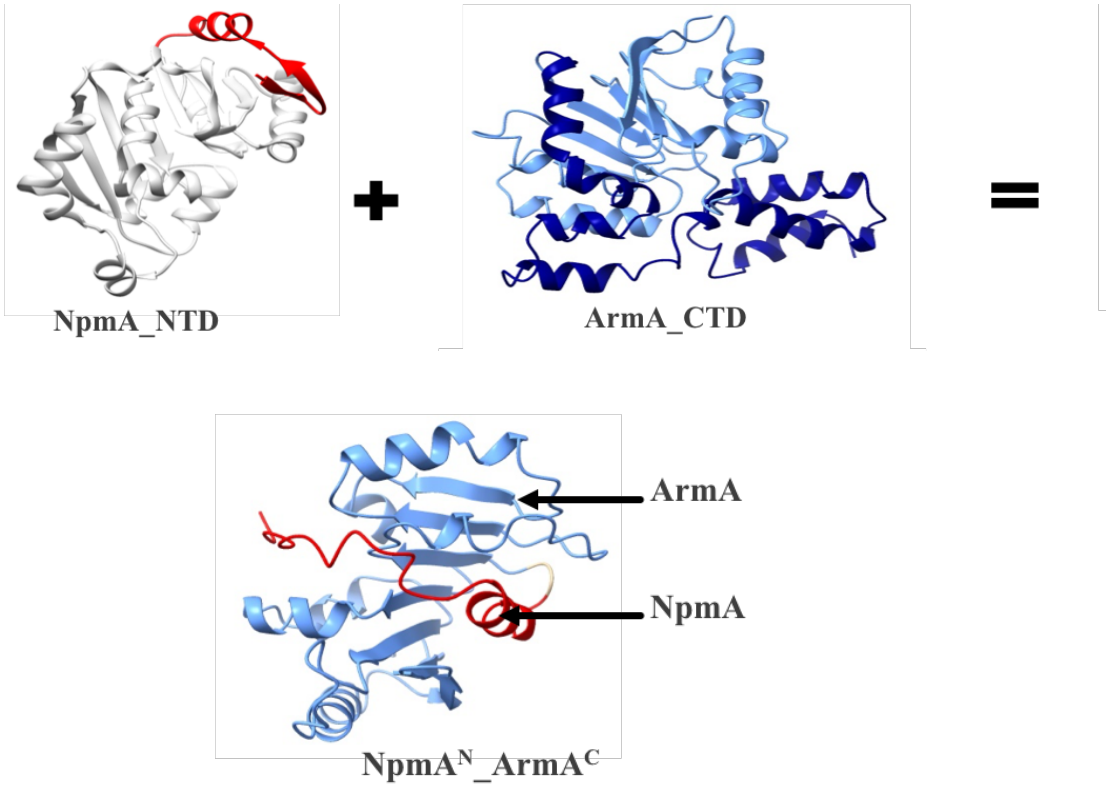


Figure 14 - Alphafold predicted structure of $NpmA^{N}\_ArmA^{C}$

a. Gene Amplification of Templates

PCR amplification of $NpmA^N$ and $ArmA^C$ was done successfully by using the required forward and reverse primers.

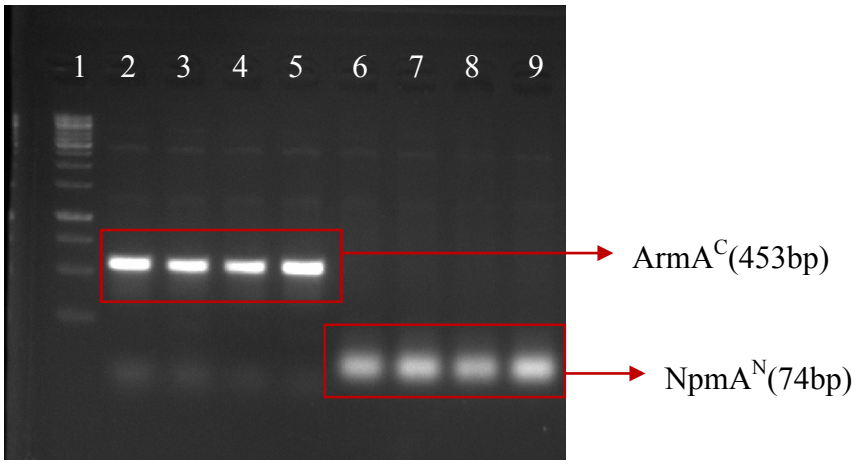


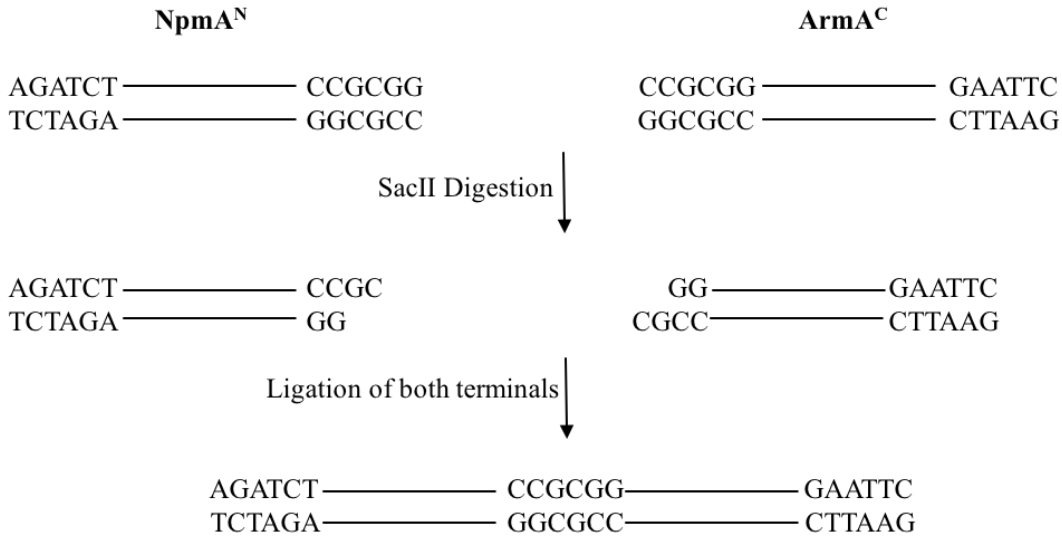
Figure 15 - PCR amplification of N terminal domain of NpmA and C terminal domain of ArmA

- Lane 1 : 1 Kb ladder
- Lanes 2-5 : $ArmA^C$
- Lanes 6-9 : $NpmA^N$

b. Restriction Digestion by SacII and Ligation of Terminals

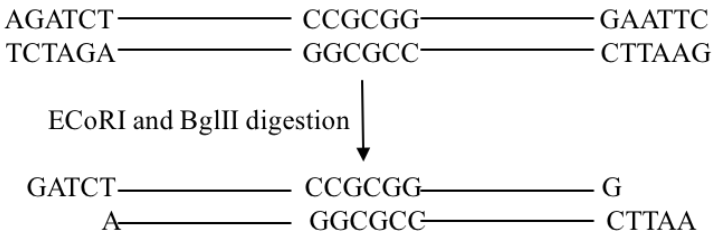
Upon quantification, it was seen that there was not a significant difference in the resistance of the TOP10 cells with $NpmA^N$_$ArmA^C$ as compared to the TOP10 cells with only pBAD His B vector.

This showed that loss of NTD in N^7G1405 16S RMTases indicates loss of mechanism for binding to 30S subunit, as protein expression of the chimera was already confirmed. Alternately, some C-terminal residues may also be required for specificity.



c. Restriction Digestion by ECoRI and BglII

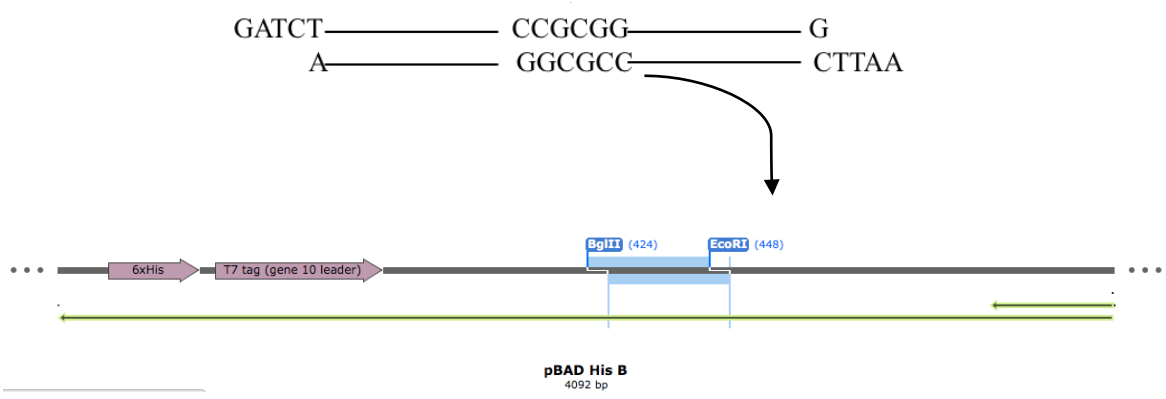
The so formed chimera was digested using ECoRI HF for 2 hours and then subsequently with BglII after adding NaCl to the mixture as BglII works in the presence of salt and cutsmart buffer is completely salt free. This led to formation of sticky ends for further ligation in vector. 37°C Both steps were performed for 2 hours each at 37°C.



d. Ligation in pBAD vector

pBAD vector which has a controlled expression is used in the experiment. The sticky ends formed by restriction digestion of pBAD vector by ECoRI and BglII are used to insert the chimera into the vector.

The vector is treated with rSAP (Shrimp Alkaline Phosphatase) before this ligation. This is done to remove the phosphorylated 5' and 3' ends. This prevents the religation of the linearized pBAD.



e. Transformation in *E. coli* TOP10 and plasmid isolation

Competent cells of TOP10 strain of *E. coli* were used for transformation. The pBAD vector, upon transformation, imparted ampicillin resistance to the TOP10 cells which would otherwise not be seen on an LA Amp plate. The presence of colonies on this plate show successful transformation.

These colonies were then screened through PCR amplification to further confirm the presence of chimera in the extrachromosomal plasmid of the transformed cells.

Figure 16 - Transformed colonies of *E. coli* TOP10 cells with $NpmA^{N}$_$ArmA^{C}$

f. PCR confirmation of clones

Confirmation of the clones was done using PCR amplification with forward primer of NA_NTD
(5'GCGCAGATCTATGTTGTTAATACTCAAAGGAACAA3')
and reverse primer of RA_CTD
(5'GCGCGAATTCTCACTTATTCCTTTTTATCATGTACA3').
The presence of band when compared to 500bp on 1 kb ladder shows a positive clone which contains $NpmA^{N}$_$ArmA^{C}$, thus showing successful transformation.

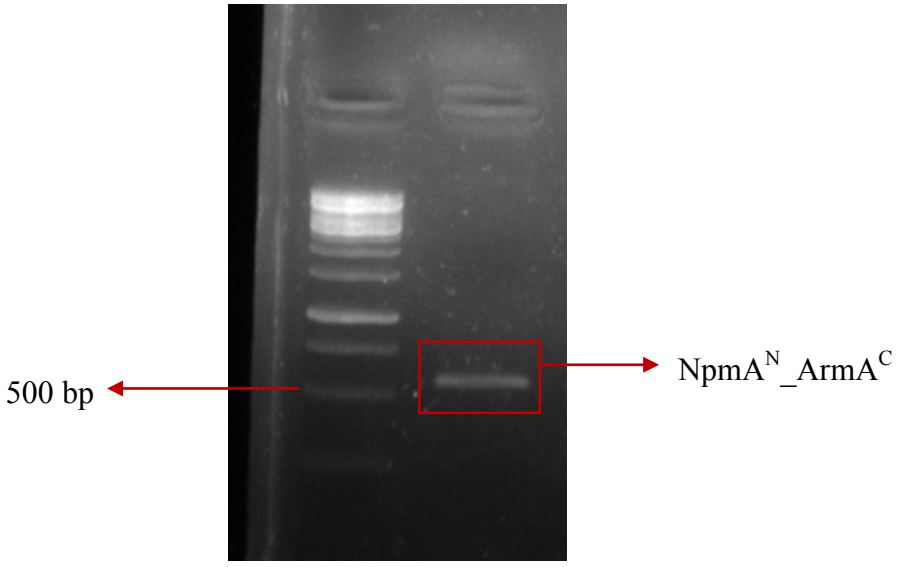


Figure 17 - Confirmation of clones of $NpmA^{N}$_$ArmA^{C}$ using PCR amplification

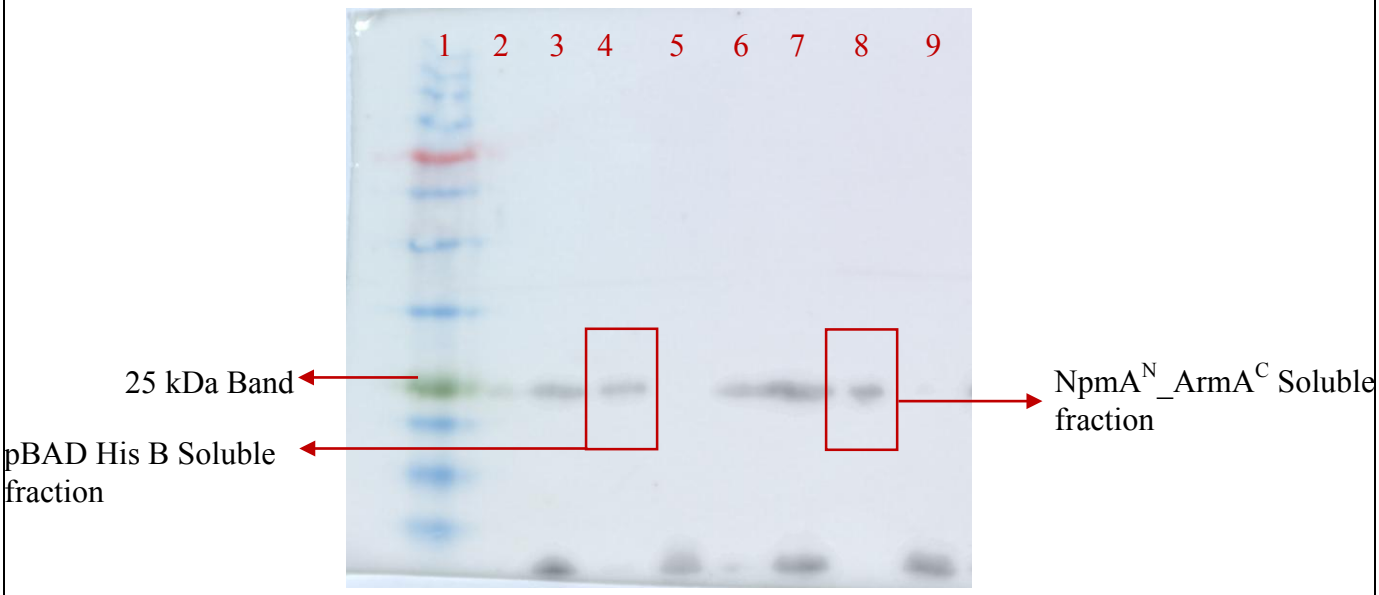
g. Western Blotting for protein Expression

Further testing of the protein formation was done for the TOP10 cells. Western blotting was done to check specific protein expression.

The antibody used for primary binding was Anti-His antibody which meant that only specific proteins with 6xHis tag would be seen on the blot. The same was done for a control that consisted of TOP10 cells with empty pBAD His B vector. This was done because there is some expression of His tagged proteins in empty vector as well and that has to be eliminated to check protein expression of the transformed cells.

The estimated size of expressed protein was 20.63 kDa and the His-tag is 3 kDa. This meant that the band size for expressed protein was 23.63 kDa.

The un-induced fraction of both the samples were also run alongside. This was done for comparison to the induced fraction in which arabinose was used as an inducer to induce the operon in the vector and subsequent protein expression.



Lane 1 – Protein Ladder
Lane 2 – pBAD His B uninduced fraction
Lane 3 - pBAD His B induced fraction
Lane 4 - pBAD His B Soluble fraction
Lane 5 - pBAD His B pellet fraction
Lane 6 – $NpmA^N\_ArmA^C$ uninduced fraction
Lane 7 - $NpmA^N\_ArmA^C$ induced fraction
Lane 8 - $NpmA^N\_ArmA^C$ Soluble fraction
Lane 9 - $NpmA^N\_ArmA^C$ pellet fraction

Figure 18 - Western Blot showing protein expression of $NpmA^N\_ArmA^C$

The blot obtained was analyzed for protein expression and compared against the empty vector's protein expression. The results were plotted as follows:

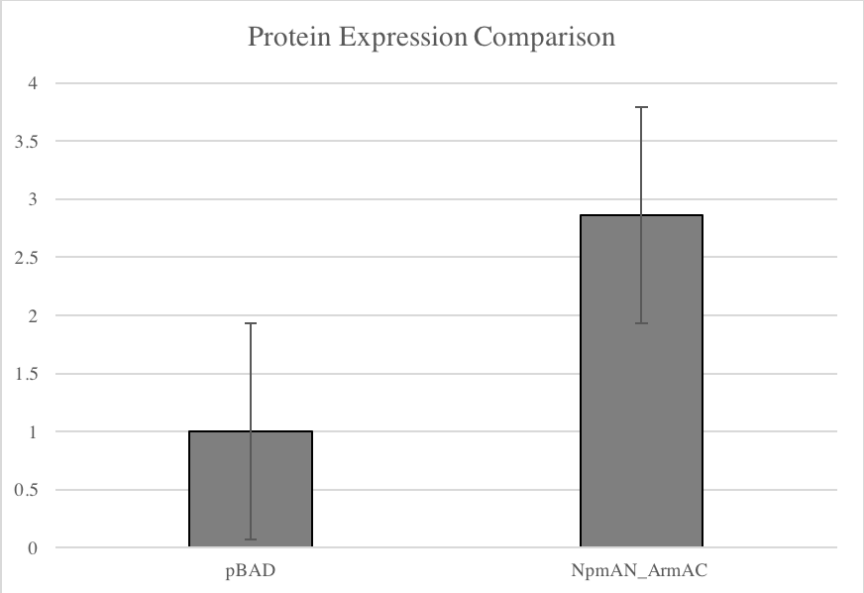


Figure 19 - Quantification of protein expression of $NpmA^{N}$_$ArmA^{C}$ with empty vector pBAD HisB

h. MIC assay

The confirmed positive clone was then checked for resistance against antibiotics Kanamycin and Neomycin at varying concentrations. These concentrations were ranging from 0 µg/mL to 128 µg/mL for both the antibiotics.

The same experiment was also done for pBAD His B vector as a control so that the inherent resistance of pBAD can be compared to the resistance imparted by presence of the chimera.

The experiment was conducted through bioscreen equipment which takes spectrophotometer readings of the culture in MIC plate every hour for 24 hours.

The O.D was then plotted against time to quantify the results.

Upon quantification, it was seen that there was no significant difference in the resistance of the TOP10 cells with $NpmA^{N}$_$ArmA^{C}$ as compared to the TOP10 cells with only pBAD His B vector.

This showed that loss of NTD in N^{7}G1405 16S RMTases indicates loss of mechanism for binding to 30S subunit, as protein expression of the chimera was already confirmed. Alternately, some C-terminal residues may also be required for specificity.

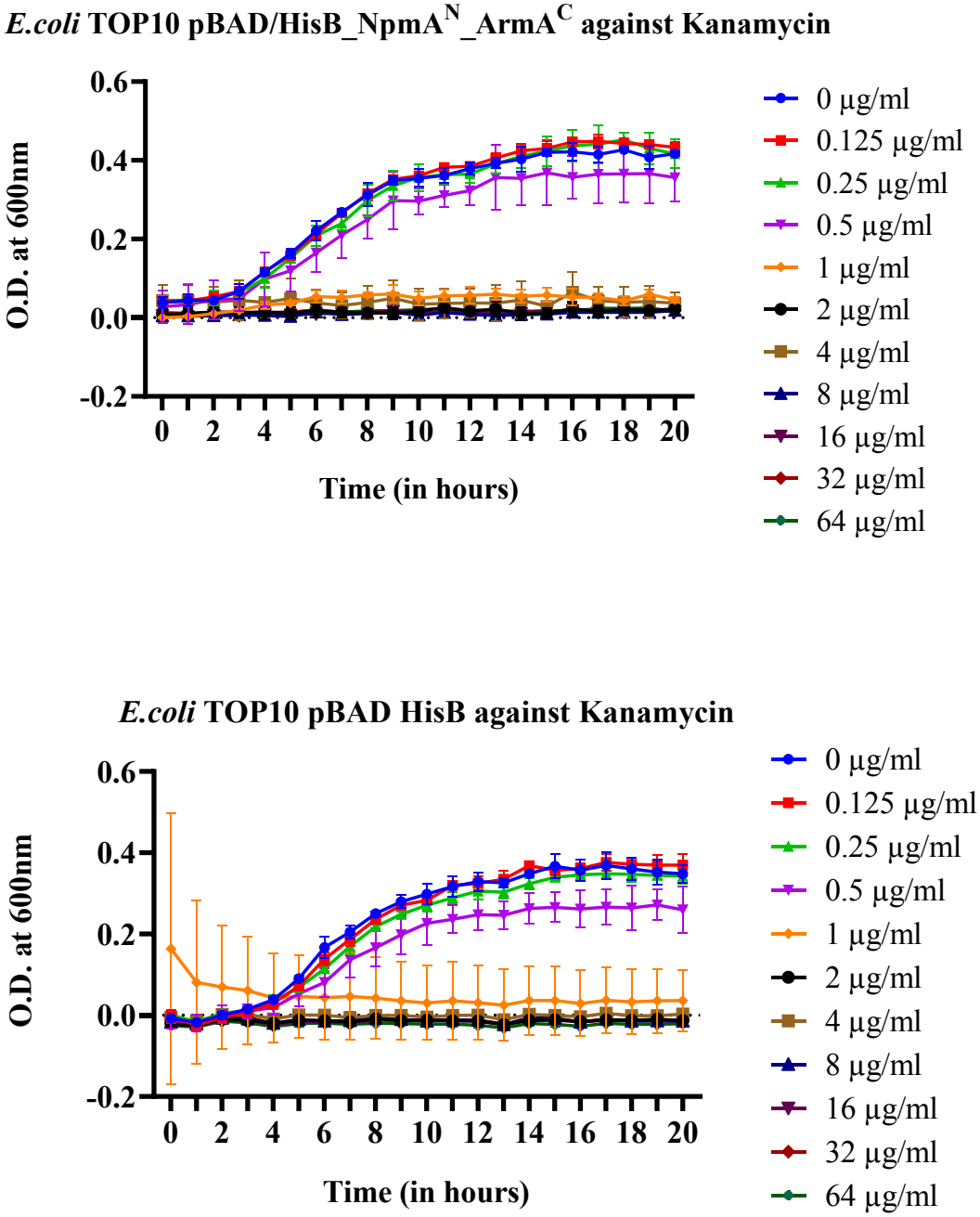
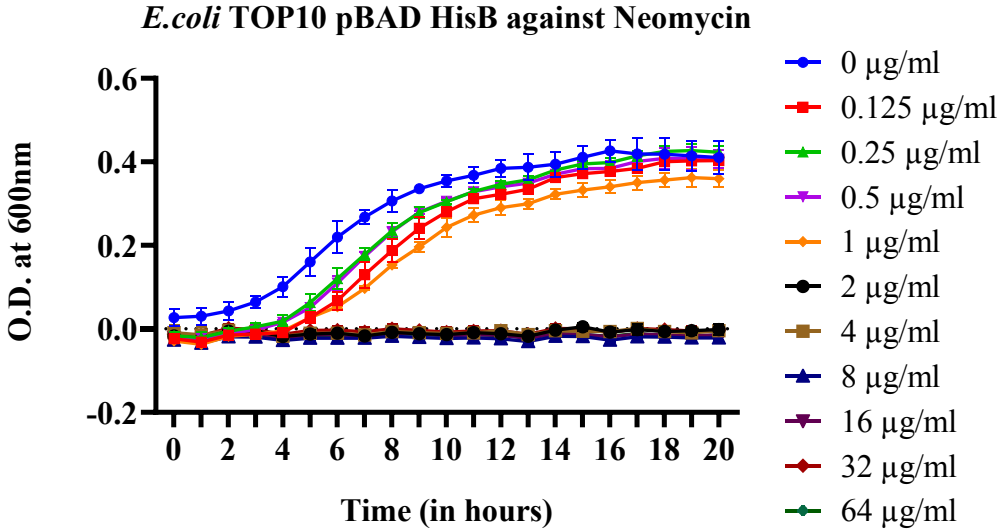


Figure 20 (a) - Comparison of the growth curves of transformed *E. coli* TOP10 with NpmA^N_ArmA^C cells under kanamycin with cells containing empty vector pBAD HisB



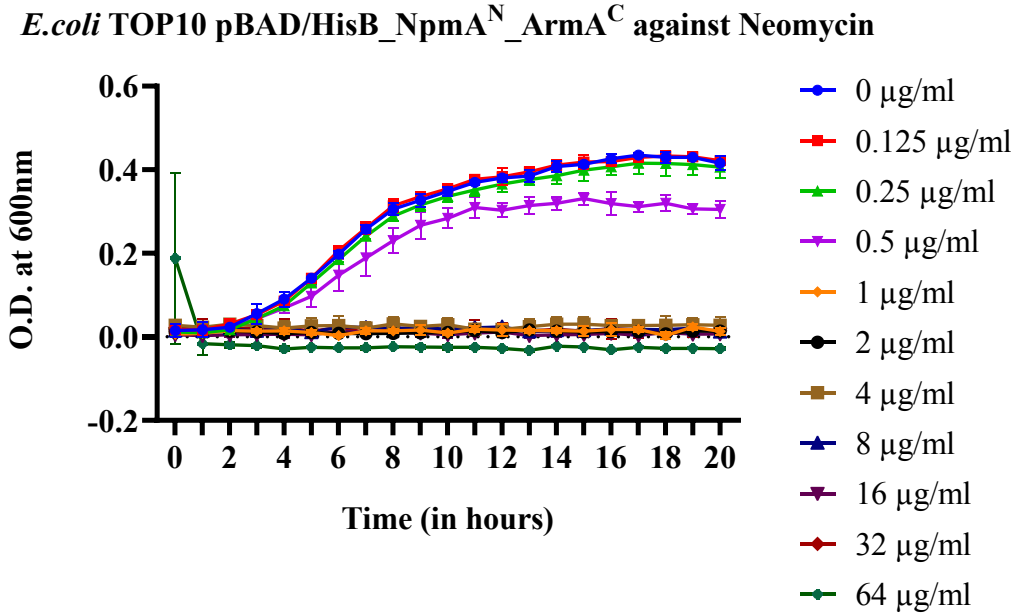


Figure 20(b) - Comparison of the growth curves of transformed *E. coli* TOP10 with NpmA^{N}_ArmA^{C} cells under neomycin with cells containing empty vector pBAD HisB

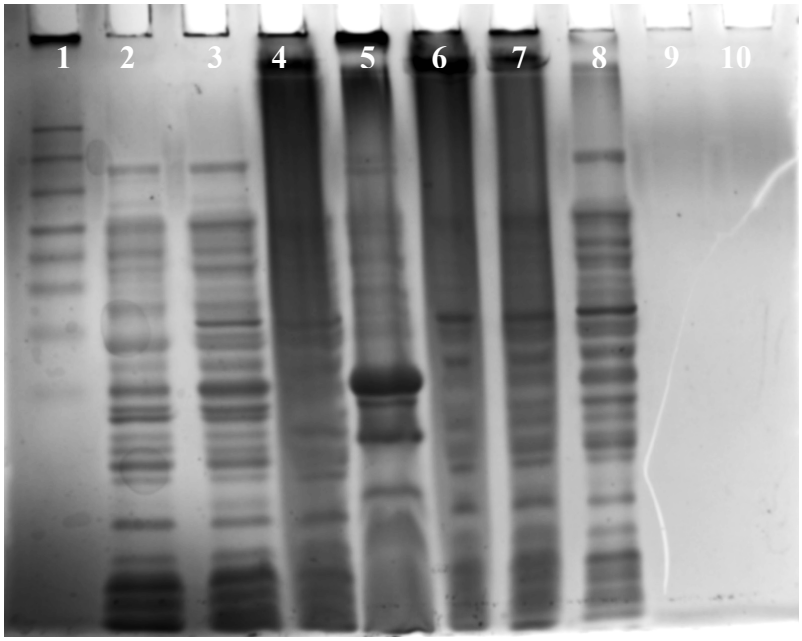
i. Ni-NTA affinity chromatography for Protein purification

Upon sonication, all the expressed protein should be seen in the cell lysate. The culture for $NpmA^N\_ArmA^C$ was induced with arabinose (20%) in order to express the desired protein. As seen in the western blot, the protein should be present in the soluble fraction after cell pellet has been removed.

Ni-NTA beads bind to the His tag present in the expressed protein and the column holds the bound protein until eluted with a higher volume of imidazole.

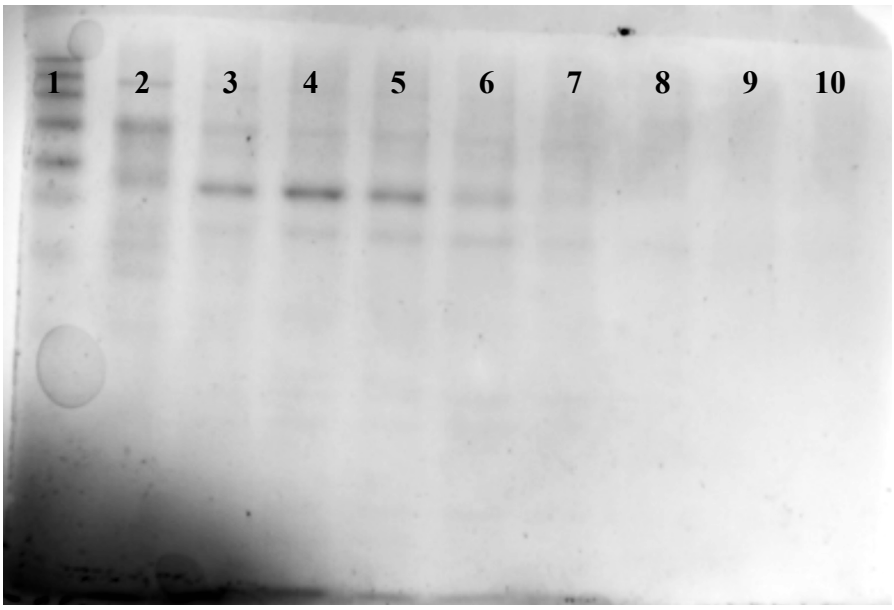
The size of the expressed protein is about 23.63 kDa which means on comparison in SDS PAGE, the protein should be seen in any of the elutes taken. The elutes however, did not have sufficient amount of expressed protein to be purified further.

Most of the protein of comparable size had been found in the insoluble fraction of the culture. This anomaly could be attributed to non-presence of expressed protein that was in the soluble form that could be purified further.



Lane 1 – Protein Ladder
Lane 2 – $NpmA^N\_ArmA^C$ un-induced fraction
Lane 3 - $NpmA^N\_ArmA^C$ induced fraction
Lane 4 - $NpmA^N\_ArmA^C$ soluble fraction
Lane 5 - $NpmA^N\_ArmA^C$ Insoluble fraction
Lane 6 – $NpmA^N\_ArmA^C$ Flow Through 1
Lane 7 - $NpmA^N\_ArmA^C$ Flow Through 2
Lane 8 - $NpmA^N\_ArmA^C$ Wash Buffer 1
Lane 9 - $NpmA^N\_ArmA^C$ Last wash buffer
Lane 10 - $NpmA^N\_ArmA^C$ Elute 10

Figure 21 - SDS PAGE of $NpmA^N\_ArmA^C$ pre-FT and FT



Lane 1 – Protein Ladder
Lane 2 – $NpmA^{N}\_ArmA^{C}$ Elute 1
Lane 3 - $NpmA^{N}\_ArmA^{C}$ Elute 2
Lane 4 - $NpmA^{N}\_ArmA^{C}$ Elute 3
Lane 5 - $NpmA^{N}\_ArmA^{C}$ Elute 4
Lane 6 – $NpmA^{N}\_ArmA^{C}$ Elute 5
Lane 7 - $NpmA^{N}\_ArmA^{C}$ Elute 6
Lane 8 - $NpmA^{N}\_ArmA^{C}$ Elute 7
Lane 9 - $NpmA^{N}\_ArmA^{C}$ Elute 8
Lane 10 - $NpmA^{N}\_ArmA^{C}$ Elute 9

Figure 22 - SDS PAGE of $NpmA^{N}\_ArmA^{C}$ Elutes after Ni-NTA chromatography

2. $NpmA^N\_RmtA^C$

This RMTase has been hypothesized as follows:

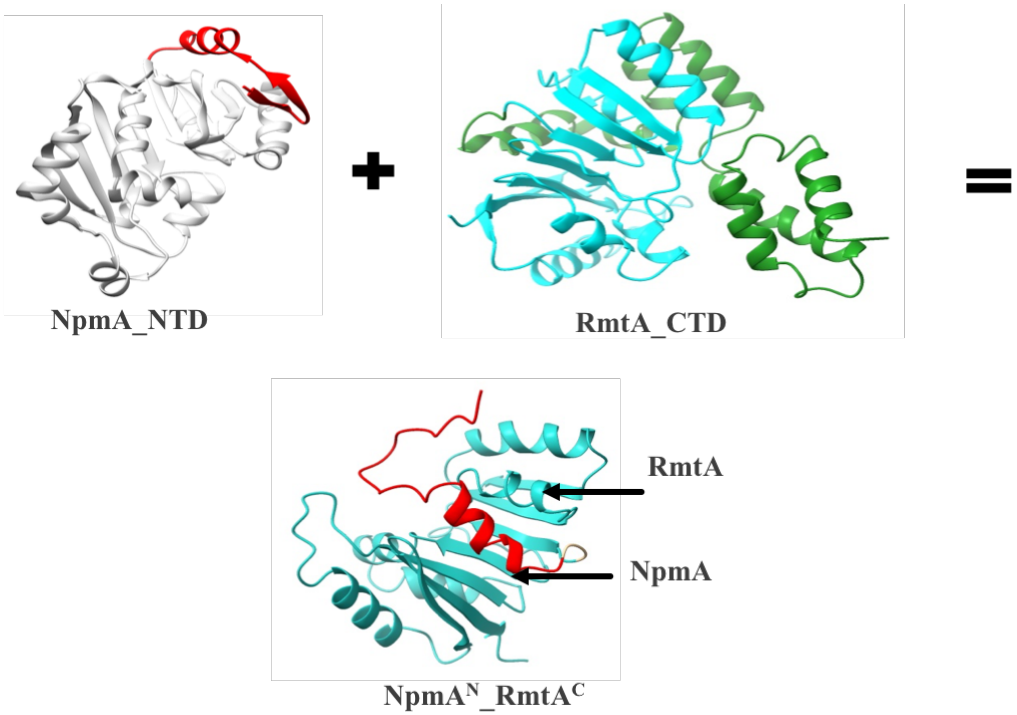
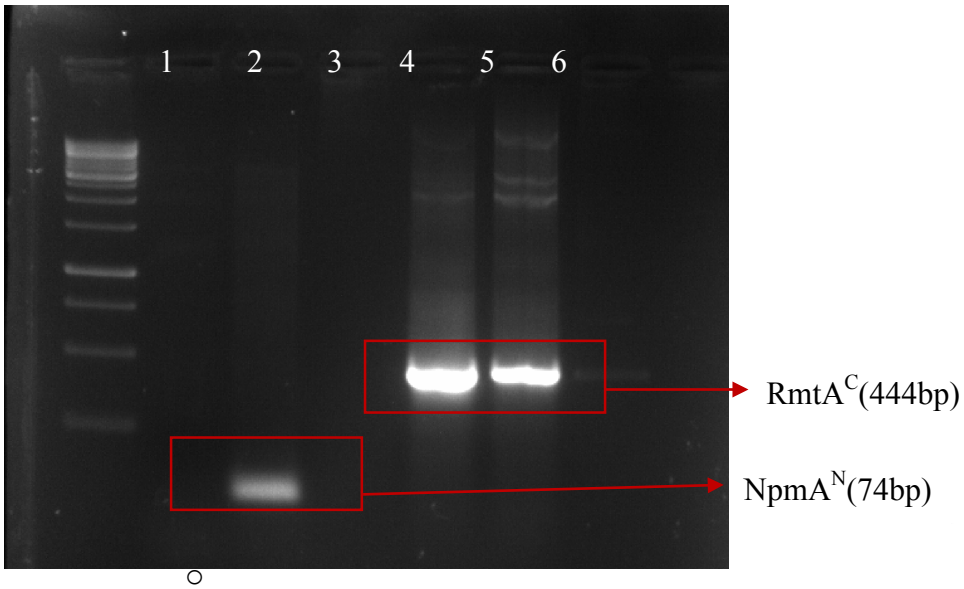


Figure 23 -Alphafold predicted structure of $NpmA^N\_RmtA^C$

a. Gene Amplification of Templates

PCR amplification of $NpmA^N$ and $RmtA^C$ was done successfully by using the required forward and reverse primers.



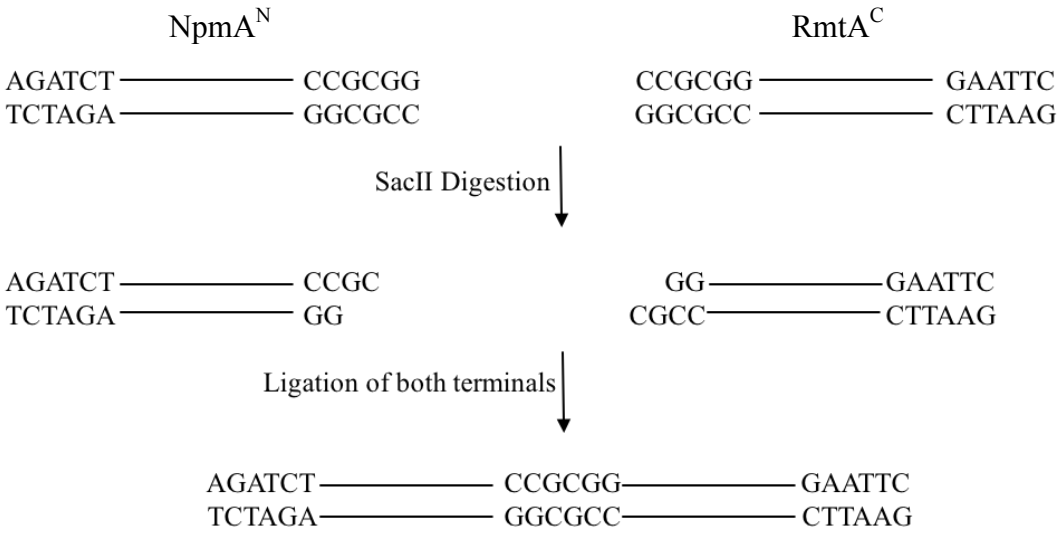
Lane 1 : 1 Kb ladder
Lane 3 : $NpmA^N$ Lanes 5-6 : $RmtA^C$

Figure 24 - PCR amplification of N terminal domain of NpmA and C terminal domain of RmtA

b. Restriction Digestion by SacII and Ligation of Terminals

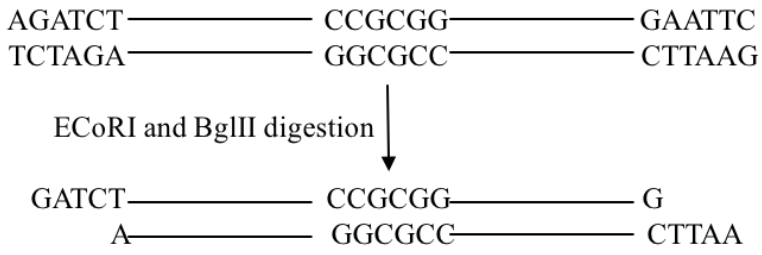
Both the inserts were digested using SacII-HF in the presence of 10x cutsmart buffer. This led to formation of sticky ends at the target site which was then used to ligate the inserts and form the required chimera.

The digestion was done for 2-3 hours at 37°C and Ligation for 1 hour at 37°C. The following steps were performed as:



c. Restriction Digestion by ECoRI and BglII

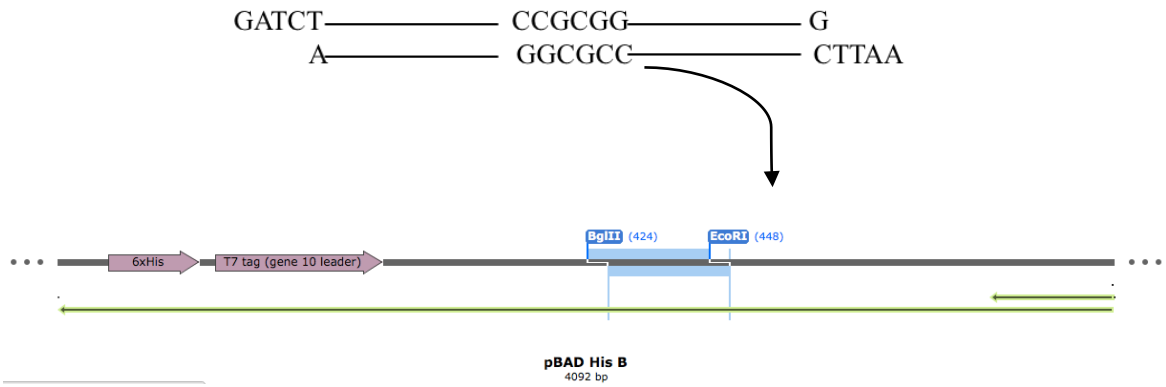
The so formed chimera was digested using ECoRI HF for 2 hours and then subsequently with BglII after adding NaCl to the mixture as BglII works in the presence of salt and cutsmart buffer is completely salt free. This led to formation of sticky ends for further ligation in vector. 37°C Both steps were performed for 2 hours each at 37°C.



d. Ligation in pBAD vector

pBAD vector which has a controlled expression is used in the experiment. The sticky ends formed by restriction digestion of pBAD vector by ECoRI and BglII are used to insert the chimera into the vector.

The vector is treated with rSAP (Shrimp Alkaline Phosphatase) before this ligation. This is done to remove the phosphorylated 5' and 3' ends. This prevents the religation of the linearized pBAD.



e. Transformation in *E. coli* TOP10 and plasmid isolation

Competent cells of TOP10 strain of *E. coli* were used for transformation. The pBAD vector, upon transformation, imparted ampicillin resistance to the TOP10 cells which would otherwise not be seen on an LA Amp plate. The presence of colonies on this plate show successful transformation.

These colonies were then screened through PCR amplification to further confirm the presence of chimera in the extrachromosomal plasmid of the transformed cells.

CHAPTER VI

SUMMARY

The Class I RMTases consist of an N-terminal Domain and a C-terminal Domain with a classical Rossmann fold. The 16S rRNA Methyl Transferases which use SAM and methylate the A site of translation by using 30S subunit as the substrate were studied during the project.

The two types of 16S RMTases, based upon the site of methylation, i.e. either G1405 or A1408 were looked at for determining the importance of the N-terminal Domain in binding to the substrate and whether the change in site of methylation also meant that the new RMTase was able to impart resistance against a broader group of aminoglycosides.

The 16S rRNA Methyl Transferases specifically NpmA, ArmA and RmtA were used to study the specificity of the N-terminal Domain of NpmA and the increase in tolerance against the target group of antibiotics, if any.

The new constructs which were designed using the N-terminal Domain of NpmA and the C-terminal Domain of ArmA/RmtA were introduced into the TOP10 cells and the expression was checked further by using protein expression estimation.

The following alphafold structures of the constructs were created using ChimeraX and were used as a reference for the experiments.

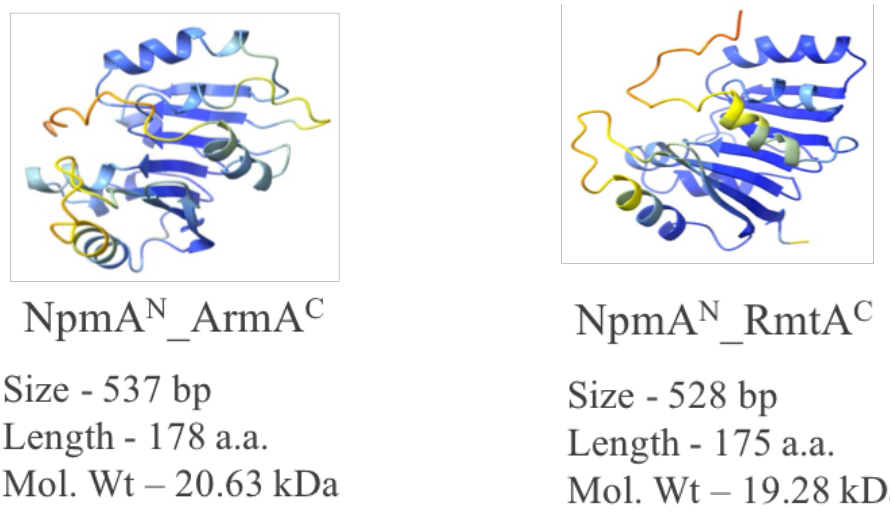


Figure 25- Alphafold predicted structures of – $NpmA^N$_$ArmA^C$ and $NpmA^N$_$RmtA^C$

Protein expression was checked for $NpmA^N$_$ArmA^C$ and the protein was observed in the soluble fraction during the western blot experiment for protein expression.

LIMITATIONS

After analyzing the results, it was seen that there was no significant difference in the tolerance of the TOP10 transformed strains as compared to the empty vector. This could be used to infer that N Terminal Domain might not be the only factor responsible for the resistance in A1408 RMTases.

The RMTase constructed is a protein which may not have folded correctly during the tertiary structure formation which would lead to improper recognition of the substrate which would lead to absence of resistance to 4,6 – DOS aminoglycosides even if the chimeric protein is present in the transformed strain of TOP10 cells.

For $NpmA^{N}$_$RmtA^{C}$, upon screening the transformed clones could not be confirmed which could be attributed to presence of false positive colonies, i.e. the colonies which were resistant enough to grow on ampicillin plates but did not express the chimeric protein.

For $NpmA^{N}$_$ArmA^{C}$, the results of NI-NTA chromatography were not in coherence with the results of western blot as expected.

FUTURE IMPLICATIONS

The study will further be conducted by using the same RMTases as the reference point, i.e NpmA and RmtA. In the future experiments, the C-terminal Domain of NpmA will be taken along with the N-terminal Domain of RmtA to construct the chimeric protein as – $RmtA^{N}$_$NpmA^{C}$ which will be used to study the importance of C-terminal Domain in binding to the 30S subunit at the A site of translation.

The chimeric protein, $NpmA^{N}$_$ArmA^{C}$ will also be processed further to check the protein expression. If the protein is found to be soluble, the folding will be analyzed by CD Spectroscopy and further purification will be done by crystallization.

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ABSTRACT Antimicrobial Resistance is one of the major causes of concern around the world and is projected to be evenmore prevalent in years to come. The study of this ability of bacteria to tolerate the presence of the existent antimicrobials by different mechanism, is important for understanding the cause of this problem. This study is focused on one such resistance mechanism, i.e. the alteration of target site of antimicrobials, specifically aminoglycosides that use 30S subunit of rRNA as a substrate for their antimicrobial action. The 16S RNA Methyl Transferases alter the A site of translation by methylation and provide resistance to the bacteria. These RMTases consist of

an N-terminal domain and a C-terminal domain 11

the N-terminal domain of NpmA to **the C-terminal** domains **of** 15

each. In this study, three different RMTases namely, NpmA, ArmA and RmtA are studied as two different chimeras, namely, NpmAN_ArmAC and NpmAN_RmtAC in the hope of understanding the specificity of the domains towards ribosome binding (and methylation). The chimeras were constructed by joining

ArmA or RmtA. The effect of change in binding site of the RMTase was seen by quantifying the change in the tolerance of wild type strain to the new transformed strain. The protein expression of the newly constructed RMTases were also checked by different techniques. Keywords: Antibiotic resistance, ESKAPE pathogens, Aminoglycoside, Aminoglycosideresistance, 16s rRNA Methyltransferases, Extrachromosomal DNA, Protein chimera, Cloning and Transformation, Aminoglycoside susceptibility assay, Bioscreen. CHAPTER I INTRODUCTION

Sahibakeen

The ability of microbes **to** survive or **grow in the presence of** 46

(Forwarded)
Bhupesh Taneja.

antibiotics designed to stop them or kill them is known as AMR (antimicrobial resistance). AMR emerges when microorganisms adapt over time and stop responding to medications, making it harder to treat illnesses, raising the death rate, and spreading disease. It is common for microorganisms to develop antibiotic resistance. However, the development and dissemination of the resistance may be accelerated by our activities. Use of antibiotics hastens thisnatural progression.

REFERENCES

- Al-Guranie, Dhifar Raa'd, and Sareaa Maseer Al-Mayahie. 2020. "Prevalence of E. Coli ST131 among Uropathogenic E. Coli Isolates from Iraqi Patients in Wasit Province, Iraq." *International Journal of Microbiology* 2020 (October): 8840561.
- Battesti, Aurélia, and Emmanuelle Bouveret. 2008. "Improvement of Bacterial Two-Hybrid Vectors for Detection of Fusion Proteins and Transfer to PBAD-Tandem Affinity Purification, Calmodulin Binding Peptide, or 6-Histidine Tag Vectors." PROTEOMICS 8 (22): 4768–71
- Bellucci, Maria Cristina, and Alessandro Volonterio. 2020. "Aminoglycosides: From Antibiotics to Building Blocks for the Synthesis and Development of Gene Delivery Vehicles." Antibiotics 9 (8): 504.
- Bhujbalrao, Ruchika, and Ruchi Anand. 2019. "Deciphering Determinants in Ribosomal Methyltransferases That Confer Antimicrobial Resistance." Journal of the American Chemical Society 141 (4): 1425–29.
- Block, Helena, Barbara Maertens, Anne Spriestersbach, Nicole Brinker, Jan Kubicek, Roland Fabis, Jörg Labahn, and Frank Schäfer. 2009. "Immobilized-Metal Affinity Chromatography (IMAC): A Review." In Methods in Enzymology, edited by Richard R. Burgess and Murray P. Deutscher, 463:439–73. Guide to Protein Purification, 2nd Edition. Academic Press.
- Bogaerts, Pierre, Marc Galimand, Caroline Bauraing, Ariane Deplano, Raymond Vanhoof, Ricardo De Mendonca, Hector Rodriguez-Villalobos, Marc Struelens, and Youri Glupczynski. 2007. "Emergence of ArmA and RmtB Aminoglycoside Resistance 16S RRNA Methylases in Belgium." Journal of Antimicrobial Chemotherapy 59 (3): 459–64.
- Bueno, Juan. 2015. "Antimicrobial Models in Nanotechnology: From the Selection to Application in the Control and Treatment of Infectious Diseases." In Nanotechnology in Diagnosis, Treatment and Prophylaxis of Infectious Diseases, edited by Mahendra Rai and Kateryna Kon, 19–38. Boston: Academic Press.
- Carter, Terrell D., and F. Wayne Outten. 2021. "Ni-NTA Affinity Chromatography to Characterize Protein–Protein Interactions During Fe-S Cluster Biogenesis." Methods in Molecular Biology (Clifton, N.J.) 2353: 125–36.
- Costello, Sarah E., Lalitagauri M. Deshpande, Andrew P. Davis, Rodrigo E. Mendes, and Mariana Castanheira. 2019. "Aminoglycoside-Modifying Enzyme and 16S Ribosomal RNA Methyltransferase Genes among a Global Collection of Gram-Negative Isolates." Journal of Global Antimicrobial Resistance 16 (March): 278–85.
- Cronan, John E. 2006. "A Family of Arabinose-Inducible Escherichia Coli Expression Vectors Having PBR322 Copy Control." Plasmid 55 (2): 152–57.

- Dadgostar, Porooshat. 2019. “Antimicrobial Resistance: Implications and Costs.” Infection and Drug Resistance 12 (March): 3903–10.
- Davies, Julian, and Gerard D. Wright. 1997. “Bacterial Resistance to Aminoglycoside Antibiotics.” Trends in Microbiology 5 (6): 234–40.
- Davies, Julian. 1971. “Bacterial Resistance to Aminoglycoside Antibiotics.” The Journal of Infectious Diseases 124 (Supplement_1): S7–10.
- De Oliveira, David M. P., Brian M. Forde, Timothy J. Kidd, Patrick N. A. Harris, Mark A. Schembri, Scott A. Beatson, David L. Paterson, and Mark J. Walker. 2020. “Antimicrobial Resistance in ESKAPE Pathogens.” Clinical Microbiology Reviews 33 (3): e00181-19.
- Deng, Yuting, Liangying He, Sheng Chen, Hongqing Zheng, Z. Zeng, Yahong Liu, Yan Sun, Junying Ma, Zhangliu Chen, and Jian-Hua Liu. 2011. “Plasmids Mediate Dissemination of RmtB-BlaCTX-M-9 Group Genes and RmtB-QepA in Enterobacteriaceae Isolates from Pets in China.” Antimicrobial Agents and Chemotherapy.
- “Aminoglycoside Resistance.” Infectious Disease Clinics of North America 30 (2): 523–37.
- “Aminoglycosides: Perspectives on Mechanisms of Action and Resistance and Strategies to Counter Resistance.” n.d. Accessed June 4, 2023.
- “Bacterial Growth Curve Indicating Various Phases.” n.d. ResearchGate. Accessed June 22, 2023.
- “Emergence and Spread of Antibiotic Resistance – Part 2.” n.d. ReAct. Accessed June 22, 2023.
- “Fitness Cost and Interference of Arm/Rmt Aminoglycoside Resistance with the RsmF Housekeeping Methyltransferases.” n.d. Accessed June 4, 2023.
- “Gene Expression from Plasmids Containing the AraBAD Promoter at Subsaturating Inducer Concentrations Represents Mixed Populations - PubMed.” n.d. Accessed June 22, 2023.
- “High Frequency of 16S Ribosomal RNA Methyltransferases among Klebsiella Pneumoniae Isolates: First Report of RmtA RmtD RmtE and RmtF Resistance Genes in Iran - سامانه نشریات دانشگاه تربیت مدرس - Infection Epidemiology and Microbiology.” n.d. Accessed June 10, 2023.
- “His-Tag | Definition & Data.” n.d. Cube Biotech. Accessed June 22, 2023.
- “Mechanisms of Bacterial Resistance to Antibiotics | JAMA Internal Medicine | JAMA Network.” n.d. Accessed June 4, 2023. https://jamanetwork.com/journals/jamainternalmedicine/article-abstract/615030.
- “Methyltransferase - an Overview | ScienceDirect Topics.” n.d. Accessed June 21, 2023. https://www.sciencedirect.com/topics/medicine-and-dentistry/methyltransferase.

- "Molecular Recognition and Modification of the 30S Ribosome by the Aminoglycoside-Resistance Methyltransferase NpmA." n.d. Accessed June 4, 2023. https://doi.org/10.1073/pnas.1402789111.
- "Researchers Develop a New Means of Killing Harmful Bacteria." 2015. MIT News | Massachusetts Institute of Technology. June 25, 2015.
- Denissen, Julia, Brandon Reyneke, Monique Waso-Reyneke, Benjamin Havenga, Tobias Barnard, Sehaam Khan, and Wesaal Khan. 2022. "Prevalence of ESKAPE Pathogens in the Environment: Antibiotic Resistance Status, Community-Acquired Infection and Risk to Human Health." International Journal of Hygiene and Environmental Health 244 (July): 114006. https://doi.org/10.1016/j.ijheh.2022.114006.
- Doi, Y., and Y. Arakawa. 2007. "16S Ribosomal RNA Methylation: Emerging Resistance Mechanism against Aminoglycosides." Clinical Infectious Diseases 45 (1): 88–94.
- Doi, Yohei, Doroti de Oliveira Garcia, Jennifer Adams, and David L. Paterson. 2007. "Coproduction of Novel 16S RRNA Methylase RmtD and Metallo-Beta-Lactamase SPM-1 in a Panresistant Pseudomonas Aeruginosa Isolate from Brazil." Antimicrobial Agents and Chemotherapy 51 (3): 852–56.
- Doi, Yohei, Jun-ichi Wachino, and Yoshichika Arakawa. 2008. "Nomenclature of Plasmid-Mediated 16S RRNA Methylases Responsible for Panaminoglycoside Resistance." Antimicrobial Agents and Chemotherapy 52 (July): 2287–88.
- European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). 2003. "Determination of Minimum Inhibitory Concentrations (MICs) of Antibacterial Agents by Broth Dilution." *Clinical Microbiology and Infection* 9 (8): ix–xv.
- Franken, Kees L. M. C., Hoebert S. Hiemstra, Krista E. van Meijgaarden, Yanri Subronto, J. den Hartigh, Tom H. M. Ottenhoff, and Jan W. Drijfhout. 2000. "Purification of His-Tagged Proteins by Immobilized Chelate Affinity Chromatography: The Benefits from the Use of Organic Solvent." Protein Expression and Purification 18 (1): 95–99. https://doi.org/10.1006/prep.1999.1162.
- Fritsche, Thomas R., Mariana Castanheira, George H. Miller, Ronald N. Jones, and Eliana S. Armstrong. 2008. "Detection of Methyltransferases Conferring High-Level Resistance to Aminoglycosides in Enterobacteriaceae from Europe, North America, and Latin America." Antimicrobial Agents and Chemotherapy 52 (5): 1843–45.
- Gaberc-Porekar, Vladka, and Viktor Menart. 2001. "Perspectives of Immobilized-Metal Affinity Chromatography." Journal of Biochemical and Biophysical Methods 49 (1): 335–60.

- Galimand, Marc, Patrice Courvalin, and Thierry Lambert. 2003. "Plasmid-Mediated High-Level Resistance to Aminoglycosides in Enterobacteriaceae Due to 16S RRNA Methylation." Antimicrobial Agents and Chemotherapy 47 (8): 2565–71. https://doi.org/10.1128/AAC.47.8.2565-2571.2003.
- Garrison, Aaron, and Robert Huigens. 2016. "Eradicating Bacterial Biofilms with Natural Products and Their Inspired Analogues That Operate Through Unique Mechanisms." Current Topics in Medicinal Chemistry 17 (December).
- Goulev, Youlian, Audrey Matifas, Vincent Heyer, Bernardo Reina-San-Martin, and Gilles Charvin. 2019. "An Expandable Toolbox for Combinatorial and Swift Generation of Expression Plasmids in Yeast." PLOS ONE 14 (August): e0220694.
- Guo, Xuejun, Belinda B. Dillon, Andrew N. Ginn, Agnieszka M. Wiklendt, Sally R. Partridge, and Jonathan R. Iredell. 2014. "Simple Multiplex Real-Time PCR for Rapid Detection of Common 16S RRNA Methyltransferase Genes." Diagnostic Microbiology and Infectious Disease 80 (1): 29–31. https://doi.org/10.1016/j.diagmicrobio.2014.05.023.
- Gür, Deniz, Ufuk Hasdemir, Aslı Çakar, İffet Çavuşoğlu, Tuğçe Çelik, and Burak Aksu. 2020. "Comparative in Vitro Activity of Plazomicin and Older Aminoglyosides against Enterobacterales Isolates; Prevalence of Aminoglycoside Modifying Enzymes and 16S RRNA Methyltransferases." Diagnostic Microbiology and Infectious Disease 97 (4): 115092.
- Gutierrez, Belen, Jose A. Escudero, Alvaro San Millan, Laura Hidalgo, Laura Carrilero, Cristina M. Ovejero, Alfonso Santos-Lopez, Daniel Thomas-Lopez, and Bruno Gonzalez-Zorn. 2012. "Fitness Cost and Interference of Arm/Rmt Aminoglycoside Resistance with the RsmF Housekeeping Methyltransferases." Antimicrobial Agents and Chemotherapy 56 (5): 2335–41.
- Guzman, L M, D Belin, M J Carson, and J Beckwith. 1995. "Tight Regulation, Modulation, and High-Level Expression by Vectors Containing the Arabinose PBAD Promoter." Journal of Bacteriology 177 (14): 4121–30. https://doi.org/10.1128/jb.177.14.4121-4130.1995.
- Guzmán, L., D. Belin, M. Carson, and J. Beckwith. 2013. "PBAD Promoter. Arabinose Expression by Vectors Containing the Tight Regulation, Modulation, and High-Level." In
- Hendriksen, Rene S., Valeria Bortolaia, Heather Tate, Gregory H. Tyson, Frank M. Aarestrup, and Patrick F. McDermott. 2019. "Using Genomics to Track Global Antimicrobial Resistance." Frontiers in Public Health 7. https://www.frontiersin.org/articles/10.3389/fpubh.2019.00242.
- Hochuli, E., W. Bannwarth, H. Döbeli, R. Gentz, and D. Stüber. 1988. "Genetic Approach to Facilitate Purification of Recombinant Proteins with a Novel Metal Chelate Adsorbent." Bio/Technology 6 (11): 1321–25.

- Holmes, D. J., D. Drocourt, G. Tiraby, and E. Cundliffe. 1991. “Cloning of an Aminoglycoside-Resistance-Encoding Gene, KamC, from Saccharopolyspora Hirsuta: Comparison with KamB from Streptomyces Tenebrarius.” Gene 102 (1): 19–26.
- Husain, Nilofer, Sonja Obranić, Lukasz Koscinski, J. Seetharaman, Fedora Babić, Janusz M. Bujnicki, Gordana Maravić-Vlahoviček, and J. Sivaraman. 2011. “Structural Basis for the Methylation of A1408 in 16S RRNA by a Panaminoglycoside Resistance Methyltransferase NpmA from a Clinical Isolate and Analysis of the NpmA Interactions with the 30S Ribosomal Subunit.” Nucleic Acids Research 39 (5): 1903–18. https://doi.org/10.1093/nar/gkq1033.
- Jerse, Ann E, and Richard F Rest. n.d. “Adhesion and Invasion by the Pa Th o g Enl"¢ Ne Isser Ia.”
- Johansson, B. G. 1972. “Agarose Gel Electrophoresis.” Scandinavian Journal of Clinical and Laboratory Investigation 29 (sup124): 7–19. https://doi.org/10.3109/00365517209102747.
- Kanazawa, Hiroki, Fumika Baba, Mai Koganei, and Jiro Kondo. 2017. “A Structural Basis for the Antibiotic Resistance Conferred by an N1-Methylation of A1408 in 16S RRNA.” Nucleic Acids Research 45 (21): 12529–35. https://doi.org/10.1093/nar/gkx882.
- Khlebnikov, Artem, Øystein Risa, Tove Skaug, Trent A. Carrier, and J. D. Keasling. 2000. “Regulatable Arabinose-Inducible Gene Expression System with Consistent Control in All Cells of a Culture.” Journal of Bacteriology 182 (24): 7029–34.
- Khodabakhsh, Fatemeh, Zohreh Dehghani, Mohammad Farid Zia, Mohammad Rabbani, and Hamid Mir Mohammad Sadeghi. 2013. “Cloning and Expression of Functional Reteplase in Escherichia Coli TOP10.” Avicenna Journal of Medical Biotechnology 5 (3): 168–75.
- Kowalska-Krochmal, Beata, and Ruth Dudek-Wicher. 2021. “The Minimum Inhibitory Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance.” Pathogens 10 (2): 165. https://doi.org/10.3390/pathogens10020165.
- Kurien, Biji T., and R. Hal Scofield. 2006. “Western Blotting.” Methods, Methods for Analyzing Cytokines, 38 (4): 283–93. https://doi.org/10.1016/j.ymeth.2005.11.007.
- Li, Xiaowei, Xin Sui, Yan Zhang, Yepeng Sun, Yan Zhao, Ying Zhai, and Qingyu Wang. n.d. “An Improved Calcium Chloride Method Preparation and Transformation of Competent Cells.”
- Liou, Grace F., Satoko Yoshizawa, Patrice Courvalin, and Marc Galimand. 2006. “Aminoglycoside Resistance by ArmA-Mediated Ribosomal 16S Methylation in Human Bacterial Pathogens.” Journal of Molecular Biology 359 (2): 358–64. https://doi.org/10.1016/j.jmb.2006.03.038.
- Lioy, Virginia S., Sylvie Goussard, Vincent Guerineau, Eun-Jeong Yoon, Patrice Courvalin, Marc Galimand, and Catherine Grillot-Courvalin. 2014. “Aminoglycoside Resistance 16S RRNA

Methyltransferases Block Endogenous Methylation, Affect Translation Efficiency and Fitness of the Host." RNA 20 (3): 382–91. https://doi.org/10.1261/rna.042572.113.

- Macmaster, Rachel, Natalia Zelinskaya, Miloje Savic, C. Robert Rankin, and Graeme L. Conn. 2010. "Structural Insights into the Function of Aminoglycoside-Resistance A1408 16S RRNA Methyltransferases from Antibiotic-Producing and Human Pathogenic Bacteria." Nucleic Acids Research 38 (21): 7791–99. https://doi.org/10.1093/nar/gkq627.
- Maeda, Yumi, Hiroshi Ueda, Jun Kazami, Genji Kawano, Eiji Suzuki, and Teruyuki Nagamune. 1997. "Engineering of Functional Chimeric Protein G–VargulaLuciferase." Analytical Biochemistry 249 (2): 147–52. https://doi.org/10.1006/abio.1997.2181.
- Mahmood, Tahrin, and Ping-Chang Yang. 2012. "Western Blot: Technique, Theory, and Trouble Shooting." North American Journal of Medical Sciences 4 (9): 429–34. https://doi.org/10.4103/1947-2714.100998.
- Maravić Vlahovicek, Gordana, Sonja Cubrilo, Karolina L. Tkaczuk, and Janusz M. Bujnicki. 2008. "Modeling and Experimental Analyses Reveal a Two-Domain Structure and Amino Acids Important for the Activity of Aminoglycoside Resistance Methyltransferase Sgm." Biochimica Et Biophysica Acta 1784 (4): 582–90. https://doi.org/10.1016/j.bbapap.2007.09.009.
- Mardanov, A., T. S. Strakhova, V. Smagin, and N. Ravin. 2007. "Tightly Regulated, High-Level Expression from Controlled Copy Number Vectors Based on the Replicon of Temperate Phage N15." Gene.
- McGettigan, Patricia, Peter Roderick, Abhay Kadam, and Allyson M. Pollock. 2017. "Access, Watch, and Reserve Antibiotics in India: Challenges for WHO Stewardship." The Lancet Global Health 5 (11): e1075–76. https://doi.org/10.1016/S2214-109X(17)30365-0.
- Mingeot-Leclercq, Marie-Paule, Youri Glupczynski, and Paul M. Tulkens. 1999. "Aminoglycosides: Activity and Resistance." Antimicrobial Agents and Chemotherapy 43 (4): 727–37. https://doi.org/10.1128/AAC.43.4.727.
- Morić, Ivana, Miloje Savić, Tatjana Ilić-Tomić, Sandra Vojnović, Sanja Bajkić, and Branka Vasiljević. 2010. "RRNA Methyltransferases and Their Role in Resistance to Antibiotics." Journal of Medical Biochemistry 29 (3): 165–74. https://doi.org/10.2478/v10011-010-0030-y.
- Morrison, D A. 1977. "Transformation in Escherichia Coli: Cryogenic Preservation of Competent Cells." Journal of Bacteriology 132 (1): 349–51.
- Mulani, Mansura S., Ekta E. Kamble, Shital N. Kumkar, Madhumita S. Tawre, and Karishma R. Pardesi. 2019. "Emerging Strategies to Combat ESKAPE Pathogens in the Era of Antimicrobial Resistance: A Review." Frontiers in Microbiology 10 (April): 539. https://doi.org/10.3389/fmicb.2019.00539.

- Navidinia, Masoumeh. n.d. “The Clinical Importance of Emerging ESKAPE Pathogens in Nosocomial Infections.” . . ISSN 7.
- Newman, Jason R, and Clay Fuqua. 1999. “Broad-Host-Range Expression Vectors That Carry the l-Arabinose-Inducible Escherichia Coli AraBAD Promoter and the AraC Regulator.” Gene 227 (2): 197–203. https://doi.org/10.1016/S0378-1119(98)00601-5.
- Nosrati, Meisam, Debayan Dey, Atousa Mehrani, Sarah E. Strassler, Natalia Zelinskaya, Eric D. Hoffer, Scott M. Stagg, Christine M. Dunham, and Graeme L. Conn. 2019. “Functionally Critical Residues in the Aminoglycoside Resistance-Associated Methyltransferase RmtC Play Distinct Roles in 30S Substrate Recognition.” Journal of Biological Chemistry 294 (46): 17642–53. https://doi.org/10.1074/jbc.RA119.011181.
- Osterman, I. A., O. A. Dontsova, and P. V. Sergiev. 2020. “RRNA Methylation and Antibiotic Resistance.” Biochemistry (Moscow) 85 (11): 1335–49.
- Patel, Dharti Keyur, Dhanya V. Menon, Darshan H. Patel, and Gayatri Dave. 2022. “Linkers: A Synergistic Way for the Synthesis of Chimeric Proteins.” Protein Expression and Purification 191 (March): 106012. https://doi.org/10.1016/j.pep.2021.106012.
- Pauwels, Ines, Ann Versporten, Nico Drapier, Erika Vlieghe, and Herman Goossens. 2021. “Hospital Antibiotic Prescribing Patterns in Adult Patients According to the WHO Access, Watch and Reserve Classification (AWaRe): Results from a Worldwide Point Prevalence Survey in 69 Countries.” Journal of Antimicrobial Chemotherapy 76 (6): 1614–24. https://doi.org/10.1093/jac/dkab050.
- Ph.D, Richard J. L. Paulton. 2010. “The Bacterial Growth Curve.” Journal of Biological Education, December.
- Picknett, T. M., and S. Brenner. 2001. “Operon.” In *Encyclopedia of Genetics*, edited by Sydney Brenner and Jefferey H. Miller, 1377. New York: Academic Press.
- Popescu, Saskia. 2019. “The Existential Threat of Antimicrobial Resistance.” Bulletin of the Atomic Scientists 75 (6): 286–89.
- Robichon, Carine, Jianying Luo, Thomas B. Causey, Jack S. Benner, and James C. Samuelson. 2011. “Engineering Escherichia Coli BL21(DE3) Derivative Strains To Minimize E. Coli Protein Contamination after Purification by Immobilized Metal Affinity Chromatography ▿.” Applied and Environmental Microbiology 77 (13): 4634–46. https://doi.org/10.1128/AEM.00119-11.
- Sandle, Tim. 2016. “Antibiotics and Preservatives.” In Pharmaceutical Microbiology, edited by Tim Sandle, 171–83. Oxford: Woodhead Publishing. https://doi.org/10.1016/B978-0-08-100022-9.00014-1.

- Santajit, Sirijan, and Nitaya Indrawattana. 2016. “Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens.” BioMed Research International 2016: 1–8. https://doi.org/10.1155/2016/2475067.
- Savic, M., J. Lovric, T. I. Tomic, B. Vasiljevic, and G. L. Conn. 2009. “Determination of the Target Nucleosides for Members of Two Families of 16S RRNA Methyltransferases That Confer Resistance to Partially Overlapping Groups of Aminoglycoside Antibiotics.” Nucleic Acids Research 37 (16): 5420–31. https://doi.org/10.1093/nar/gkp575.
- Savic, Miloje, Tatjana Ilic-Tomic, Rachel Macmaster, Branka Vasiljevic, and Graeme L. Conn. 2008. “Critical Residues for Cofactor Binding and Catalytic Activity in the Aminoglycoside Resistance Methyltransferase Sgm.” Journal of Bacteriology 190 (17): 5855–61. https://doi.org/10.1128/JB.00076-08.
- Schmitt, Emmanuelle, Marc Galimand, Michel Panvert, Patrice Courvalin, and Yves Mechulam. 2009. “Structural Bases for 16 S RRNA Methylation Catalyzed by ArmA and RmtB Methyltransferases.” Journal of Molecular Biology 388 (3): 570–82.
- Sharland, Mike, Celine Pulcini, Stephan Harbarth, Mei Zeng, Sumanth Gandra, Shrey Mathur, and Nicola Magrini. 2018. “Classifying Antibiotics in the WHO Essential Medicines List for Optimal Use—Be AWaRe.” The Lancet Infectious Diseases 18 (1): 18–20. https://doi.org/10.1016/S1473-3099(17)30724-7.
- Sharland, Mike, Sumanth Gandra, Benedikt Huttner, Lorenzo Moja, Celine Pulcini, Mei Zeng, Marc Mendelson, et al. 2019. “Encouraging AWaRe-Ness and Discouraging Inappropriate Antibiotic Use—the New 2019 Essential Medicines List Becomes a Global Antibiotic Stewardship Tool.” The Lancet Infectious Diseases 19 (12): 1278–80. https://doi.org/10.1016/S1473-3099(19)30532-8.
- Spriestersbach, Anne, Jan Kubicek, Frank Schäfer, Helena Block, and Barbara Maertens. 2015. “Purification of His-Tagged Proteins.” In Methods in Enzymology, edited by Jon R. Lorsch, 559:1–15. Laboratory Methods in Enzymology: Protein Part D. Academic Press.
- Tang, X, Y Nakata, H O Li, M Zhang, H Gao, A Fujita, O Sakatsume, T Ohta, and K Yokoyama. 1994. “The Optimization of Preparations of Competent Cells for Transformation of E. Coli.” Nucleic Acids Research 22 (14): 2857–58.
- Taylor, Sean C., and Anton Posch. 2014. “The Design of a Quantitative Western Blot Experiment.” BioMed Research International 2014 (March): e361590.
- Vinal, Kellie, and Graeme L. Conn. 2017. “Substrate Recognition and Modification by a Pathogen-Associated Aminoglycoside Resistance 16S RRNA Methyltransferase.” Antimicrobial Agents and Chemotherapy 61 (5): e00077-17.

- Wachino, Jun-ichi, Keigo Shibayama, Hiroshi Kurokawa, Kouji Kimura, Kunikazu Yamane, Satowa Suzuki, Naohiro Shibata, Yasuyoshi Ike, and Yoshichika Arakawa. 2007. "Novel Plasmid-Mediated 16S RRNA m 1 A1408 Methyltransferase, NpmA, Found in a Clinically Isolated Escherichia Coli Strain Resistant to Structurally Diverse Aminoglycosides." Antimicrobial Agents and Chemotherapy 51 (12): 4401–9. https://doi.org/10.1128/AAC.00926-07.
- Wachino, Jun-Ichi, Keigo Shibayama, Kouji Kimura, Kunikazu Yamane, Satowa Suzuki, and Yoshichika Arakawa. 2010. "RmtC Introduces G1405 Methylation in 16S RRNA and Confers High-Level Aminoglycoside Resistance on Gram-Positive Microorganisms." FEMS Microbiology Letters 311 (1): 56–60.
- Wachino, Jun-Ichi, Yohei Doi, and Yoshichika Arakawa. 2020. "Aminoglycoside Resistance: Updates with a Focus on Acquired 16S Ribosomal RNA Methyltransferases." Infectious Disease Clinics 34 (4): 887–902. https://doi.org/10.1016/j.idc.2020.06.002.
- Wang, Zhi-Gang, and Jian-Xin Wu. 2009. "DNA methyltransferases: classification, functions and research progress." *Yi Chuan = Hereditas* 31 (9): 903–12. https://doi.org/10.3724/sp.j.1005.2009.00903.
- Witek, Marta A., and Graeme L. Conn. 2014. "Expansion of the Aminoglycoside-Resistance 16S RRNA (m(1)A1408) Methyltransferase Family: Expression and Functional Characterization of Four Hypothetical Enzymes of Diverse Bacterial Origin." Biochimica Et Biophysica Acta 1844 (9): 1648–55. https://doi.org/10.1016/j.bbapap.2014.06.012.
- Wülfing, C., and A. Plückthun. 1993. "A Versatile and Highly Repressible Escherichia Coli Expression System Based on Invertible Promoters: Expression of a Gene Encoding a Toxic Product." Gene. https://www.semanticscholar.org/paper/A-versatile-and-highly-repressible-Escherichia-coli-W%C3%BClfing-Pl%C3%BCckthun/1e42bdb67ae21f6a4ca19df9fca0701279a3f1c6.
- Yang, Weiwei, and Fupin Hu. 2022. "Research Updates of Plasmid-Mediated Aminoglycoside Resistance 16S RRNA Methyltransferase." Antibiotics 11 (7): 906.
- Yang, Jiyong, Liyan Ye, Weiwei Wang, Yanping Luo, Youjiang Zhang, and Li Han. 2011. "Diverse Prevalence of 16S RRNA Methylase Genes ArmA and RmtB amongst Clinical Multidrug-Resistant Escherichia Coli and Klebsiella Pneumoniae Isolates." *International Journal of Antimicrobial Agents* 38 (4): 348–51.
- Yokoyama, Keiko, Yohei Doi, Kunikazu Yamane, Hiroshi Kurokawa, Naohiro Shibata, Keigo Shibayama, Tetsuya Yagi, Haru Kato, and Yoshichika Arakawa. 2003. "Acquisition of 16S RRNA Methylase Gene in Pseudomonas Aeruginosa." The Lancet 362 (9399): 1888–93. https://doi.org/10.1016/S0140-6736(03)14959-8.

- Zanichelli, Veronica, Michael Sharland, Bernadette Cappello, Lorenzo Moja, Haileyesus Getahun, Carmem Pessoa-Silva, Hatim Sati, et al. 2023. “The WHO AWaRe (Access, Watch, Reserve) Antibiotic Book and Prevention of Antimicrobial Resistance.” Bulletin of the World Health Organization 101 (4): 290–96.
- Zelinskaya, N., C. Rankin, R. Macmaster, M. Savic, and G. Conn. 2011. “Expression, Purification and Crystallization of Adenosine 1408 Aminoglycoside-Resistance RRNA Methyltransferases for Structural Studies.” Protein Expression and Purification. https://www.semanticscholar.org/paper/Expression%2C-purification-and-crystallization-of-for-Zelinskaya-Rankin/0c47e9cc05eaf7d1ee7f99382682e89842fba484.
- Zelinskaya, Natalia, Marta A. Witek, and Graeme L. Conn. 2015. “The Pathogen-Derived Aminoglycoside Resistance 16S RRNA Methyltransferase NpmA Possesses Dual M1A1408/M1G1408 Specificity.” Antimicrobial Agents and Chemotherapy 59 (12): 7862–65.
- Zhou, Y., H. Yu, Q. Guo, X. Xu, X. Ye, S. Wu, Y. Guo, and M. Wang. 2010. “Distribution of 16S RRNA Methylases among Different Species of Gram-Negative Bacilli with High-Level Resistance to Aminoglycosides.” European Journal of Clinical Microbiology & Infectious Diseases 29 (11): 1349–53. https://doi.org/10.1007/s10096-010-1004-1.
- Zwietering, M. H., I. Jongenburger, F. M. Rombouts, and K. Van ’T Riet. 1990. “Modeling of the Bacterial Growth Curve.” Applied and Environmental Microbiology 56 (6): 1875–81. https://doi.org/10.1128/aem.56.6.1875-1881.1990.