# EXPRESSION VECTOR CONSTRUCTION TO STUDY PROMOTER FUNCTION OF *Pseudomonas aeruginosa cob*A GENE: A β-GALACTOSIDASE BASED REPORTER ASSAY

**M. Sc. Thesis**

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**Submitted to**

**Central Department of Biotechnology**

**Tribhuvan University**

**Kirtipur, Kathmandu, Nepal**

**Submitted by**

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**Pooja Pathak**

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AMR | Antimicrobial resistance |
| AST | Antibiotic susceptibility |
| LB | Luria Bertani |
| DNA | Deoxyribonucleic acid |
| RNA | Ribonucleic acid |
| UTR | Untranslated region |
| SAM | S-adenosyl methionine |
| Tm | Melting temperature |
| CDC | Centers for disease control and prevention |
| MDR | Multidrug resistance |
| mM | Millimeter |
| µM | Micrometer |
| mRNA | Messenger Ribonucleic acid |
| TAE | Tris Acetate Ethylene Diamne Tetra Acetic Acid |
| STE | Sodium Tris EDTA |
| WHO | World Health organization |
| EDTA | Ethylene Diamne Tetra Acetic Acid |
| µg | Microgram |
| µl | Microliter |
| cDNA | Complementary deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide phosphate |

| | |
|---|---|
| EtBr | Ethidium bromide |
| Kb | Kilo base pairs |
| mg | Milligram |
| NCBI | National center for Biotechnology Information |
| PCR | Polymerase chain reaction |
| Rnase | Ribonuclease |
| RT | Room temperature |
| RT-PCR | Reverse trancriptase Polymerase chain reaction |
| SDS | Sodium dodecyl sulphate |
| β | Beta |
| ONPG | Ortho nitrophenyl β-galactoside |
| TSS | Transcription start site |
| SUMT | S-adenosyl uroporphyrinogen III methyltransferase |

# LIST OF TABLES

# LIST OF FIGURES

# Table of Contents

# ABSTRACT

The rapid emergence of global antibiotic resistance demands the immediate development of the antibiotics to mitigate this problem and essential gene target were employed as antimicrobial target that could potentially act as alternative approach for the potential drug targets. Since, SAM utilizing *cob*A gene is the essential gene for the pathogens like *Pseudomonas aeruginosa*, it was studied. However, the functional promoter region is still not known, to our knowledge, so for the study of the functional promoter region, *E.coli* expression system, (*cob*A) promoter-5'UTR-*lac*Z, was constructed by restriction digestion based cloning *lac*Z gene under the *cob*A native promoter and 5'UTR region.Thus, constructed expression vector was transformed into *E.coli* DH5α whereby the transformants were screened and confirmed by restriction digestion.The expression plasmids thus developed were subjected to the reverse transcription PCR to determine the *lac*Z gene mRNA expression from the constructs using *lac*Z gene specific primers. Furthermore, the functional promoter analysis was done by β-galactosidase assay in which the development of the yellow colour was determined spectrophotometrically. Moreover, protein quantification was also done to study the growth between the three expression vectors. Thus this cloning strategy is simple and effective to study the promoter function and region and the result showed that the 100 bp to 200 bp region upstream from TSS and 5'UTR is crucial and can be explored for the mechansim of action against pathogen.

Keywords: *cob*A, promoter, *lac*Z, β-galactosidase assay, cloning

# 1. INTRODUCTION

## 1.1 Background

Though the discovery and development of antibiotics in the 20$^{th}$ century was taken as the milestone in medical sciences for decreasing the fatalities from manageable infections, but the rapid emergence of resistant strains among the pathogens appear to be unavoidable since selective pressure for survival (Tello *et. al.,* 2012). Hence, antimicrobial resistance (AMR) is thought to be a global public health concern and according to CDC (2013), each year 2 million illness and 23,000 deaths are caused by drug resistance bacteria in the United States alone. Similarly an estimated 700,000 people die annually from infection with drug resistant microbes, a figure which is subjected to increase to about 10 million by 2050 (Hrvatin, 2017).

It is presumed that the emergence of drug resistance is one of the major problems increasing enormously at a rapid rate, indicating that even most recent approved drugs can become ineffective soon. Thus, the need of novel antimicrobial agent is greater than ever because of emergence of multidrug resistance in common pathogen, the rapid emergence of new infection (Lee et al., 2014) and the prevalence of resistance even in the last resort antibiotic colistin (Caniaux *et. al.,* 2017).

A set of synchronized plans and strategies for the judicious and of proper use of antimicrobial medications, referred as antimicrobial stewardship, launched by Centers for Disease Control and Prevention in 2013 with the aim to reduce antibiotic usage, to aware people for rational uses of the antibiotics, are critical to avoid AMR emergence. Moreover, WHO also has launched the program named as Global Antimicrobial Resistance Surveillance System (GLASS) in October, 2015 to establish a worldwide standard approach in moving forward by sharing data on antimicrobial resistances. Despite these efforts searching for new antimicrobials against the highly prioritized pathogens published by WHO in 2017 as critical and High priority (Lawe-Davies and Bennett, 2017) is ongoing and new antibiotics have not yet been discovered in numbers. The antibiotics, the oxazolidinones (Barbachyn and Ford, 2003) and the cyclic lipopeptides (Kern, 2006), Dalvance and Sivextro, or tedizolid phospate (Tim Sandle, 2015) have entered the market since 2000 but more could be required.

Taking these in considerations a novel approach to identify new antimicrobial compound with specific mode of action is thought to be sanctity and is of in urgent need. Contrary to the traditional systematic approaches for investigating new compound(s), an alternative approach, targeting Riboswitches, as they have been acknowledged as potential drug targets due to their ability to bind small molecules with high affinity and selectivity (Blount *et. al*., 2006, Diegan *et. al.,* 2011), could be an avenue to explore as

riboswitches represent such novel class of potential drug target structures selective to pathogens and have not yet been reported in human.

In recent years, pursuit of developing compound with antibacterial activity that act on RNA molecule has been seen and demonstrated by several review papers dealing with the discovery of small molecules that specifically bind to bacterial riboswitches and therefore could function as antibacterial drug candidate (Penchovsky and Stoilova 2012). Some studies have shown that one of the antibacterial properties of roseoflavinis due to specific binding to FMN riboswitch found in bacteria (Ott *et. al*., 2009). Similarly, TPP, SAM, purine, lysine (Blount and Breaker 2006), AdoCbl riboswitches can act as antibacterial target as they have essential function in pathogen. Thus, a molecule that not only acts upon the proteins of interest but also to probable riboswitch structures could be of high value to have multiple antimicrobial functions.

## 1.2 Current studies

The various efforts on antibiotics researches faces same problem of resistance due to the compounds having similar function mechanisms. For example, finafloxacin, approved for treating ear infection caused by *Pseudomonas aeruginosa*, is more effective than other fluroquinolones (Mckeage, 2015), however, is still susceptible to the same resistance mechanisms that affect other fluoroquinilones (Randell *et. al.,* 2017). Despite the new discovery of teixobactin killing bacteria without being prone to resistance, only targets Gram-positive bacteria (Ling *et. al.,* 2015). Thus, there is still a strong need for characterizing new classes of antibiotics with distinct mechanisms of action.

Thus, a broader approach is needed to address the problem. One of the approaches could be identification, validation and exploitation of a potential targets to discover and develop novel antibacterial agents that will be active against resistant species. Microbial genome analysis has shown a large number of potentially useful targets and has helped to access uncultivable microbes via screening gene products (Moellering, 2011).

Hence, bacterial essential pathway, critical for survival could be an alternative approach. Some have explored such avenues and in one of the works menaquinones (vitamin K) biosynthesis pathway has been taken as potential target for development of new antimicrobial agent for infection caused by pathogen that utilize menaquinones (Li *et. al.,* 2010). Similarly, fatty acid biosynthesis is also potential target for identifying new antimicrobial agent as it targets the fatty acid synthase (FASII), present in only bacteria (Flavin *et. al.,* 2010). In addition, chorismate biosynthesis pathway is essential for bacteria to grow but mammals do not possess this pathway and this could be a selective antibacterial target to develop drugs (Kumar *et. al.,* 2012).

Furthermore, understanding protein interactions happening during an infection provides option to target non-essential processes like bacterial adhesion, communication via quorum sensing, virulence factors and toxins, signalling (Monserrat-Martinez *et. al.,* 2019). Moreover, a new antibiotic compound, SCH-79797 and its derivative Irresistin-16 have been identified and they kill both gram-positive and gram-negative resistant bacteria. These antibiotics targets two independent cellular targets, folate metabolism and bacterial membrane integrity suggesting that the combining mechanism of actions may be an alternative approach to target bacterial pathogens (Martin *et. al.,* 2020). Nonetheless,there are various alternative antimicrobial appraocheslike bacteriophage therapy, antimicrobial peptide therapy (Ghosh *et. al.,* 2019).

In addition, riboswitches can be used as antibacterial drug targets and this might be possible due to the fact that riboswitches can recognize their ligand differently than proteins (Blount and Breaker, 2006). The examples of such compounds include pyrithiamine (PT), targeting TPP-riboswitches and L-aminoethylcysteine and dl-4-oxalysine acting on lysine riboswitches. Similarly, roseoflavin, synthesized by *Streptomyces*sps is a riboflavin analogue and has shown to inhibit the growth of gram-positive bacteria (Lunse *et. al.,* 2014).

Thus, the present study focuses on the vitamin B12 biosynthesis pathway as the antibacterial target as it is present in the bacteria only and not found in the mammals. In addition, to our knowledge, the gene (*cob*A) that have been selected to study have not yet been reported to be having riboswitch and not studied for antibacterial targets. Thus, for future research on probable riboswitch type regulation, the constructs have been designed in such a way that the mechanism of killing either by modulating promoter region or 5'- Upstream region (UTR) of the gene could be assayed through cloned reporter assay.

## 1.3 HYPOTHESIS

### 1.3.1 Null Hypothesis:

The cloned promoter region will not express the reporter gene cloned under the native promoter of *cob*A gene.

### 1.3.2 Alternate Hypothesis:

The cloned promoter region will express the reporter gene cloned under the native promoter of *cob*A gene for future analysis for modulation of promoter region and/or 5'-UTR.

# 1.4 OBJECTIVE

## 1.4.1 General objective

To evaluate optimal nucleotide length required upstream of Transcription Start Site (TSS) and 5'-UTR of *cob*A gene for the functional promoter activity for the development of novel drug target for emerging drug resistant.

## 1.4.2 Specific objectives

- To design specific primer for amplification of *cob*A gene promoter along with its 5'-UTR of *Pseudomonas aeruginosa* and *lac*Z gene of *Escherichia coli*
- To construct expression plasmid, pAG101, pAP201 and pAP501 by cloning 100 basepair (bp) 200 bp and 600 bp upstream region native promoter of *cob*A gene in the pUC19 vector
- To develop expression plasmid pAP103, pAP204 and pAP502 by cloning the constructed plasmids and sub-cloning of *lac*Z gene
- To analyze the mRNA expression of the constructed expression plasmid vector by Reverse Transcriptase- Polymerase Chain Reaction
- To study the functional assay by quantitative measurement of total protein by Lowry's Method
- To do comparative analysis of enzymatic activity between expression plasmids by β-galactosidase assay

# 1.5 RATIONALE

The decrease in the novel antibiotics development and the increase in emerging resistance among pathogens leading to develop "Super Bugs" are occurring synchronously. This results in the demand of new target and mechanism of action to combat the problem. The present study focuses on potential development of novel drug target by studying the cobA gene promoter through reporter assay as it is crucial for the bacteria and developing a paradigm towards drug discovery through 5'UTR as potential riboswitch structure.

# 1.6 SCOPE

The main focus of the study was to develop an efficient expression plasmids for the functional analysis of the promoter of *cob*A gene of *Pseudomonas aeruginosa*. The expression system developed through promoter cloning will be studied using beta-galactosidase assay. The optimized protocol will help to determine the required nucleotide region for promoter for gene expression.

# 2. LITERATURE REVIEW

## 2.1 Literature review on antimicrobial resistance

Antimicrobials are the therapeutic agents that are used against the broad range of infections, such as those caused by bacteria (antibiotics), viruses (antivirals), fungi (antifungals), and parasites (antimalarials). Antimicrobial resistance (AMR) is a resistance, which arises when the microorganism which cause infection (e.g. bacteria), to a medicine that would normally kill them becomes tolerant to these medicines and infections persists in the body (Jindal *et. al.,* 2015). Thus, this has led to the emergence of "Superbugs" such as methicillin resistant *Staphylococcus aureus* and extremely drug resistant Tuberculosis (WHO/Antimicrobials, 2018).

The antimicrobial resistance arises due to a natural evolutionary response to antimicrobial exposure but inappropriate use of antibiotics in healthcare, agriculture and environment is thought to have accelerated the process. Thus, the resistance has become one of the biggest threat to global health and food security (Holmes *et. al.,* 2016). Therefore, WHO (2019) has named antibiotic resistance as top five most important public health threats of the 21$^{st}$ century suggesting that the world is heading towards the post-antibiotic area.

This could be corelated by the fact that in the United States alone, at least 2.8 million people are infected with antibiotic resistant bacteria with death rate of more than 35,000 (CDC). In addition, recent estimates based on European antimicrobial resistance surveillance network (EARS-Net), more than 670,000 infections occur in European area and European Economic Area (EEA) due to the bacterial resistance and 33,000 people die each year due to these types of infections (ECDC report, 2018). An estimate also suggests that in Asia infection caused by resistant bacteria kill one child every five minutes. Similarly, WHO identified that South-East Asia (SEA) is at high risk of emergence and spread of AMR with highly transferable New Delhi metallo-β-lactamase-1(NDM-1) (WHO report, 2018). Patients in 10 hospitals across India with Multi drug resistance (MDR) were 1.57 times more likely to die as compared to similar susceptible infections. In addition, in Thailand 43% deaths in nine hospitals were attributed to healthcare-associated infections due to MDR (Yam *et. al.,* 2019).

On the basis of these scenario of antimicrobial resistance to 2050, unless action is taken, Jim O Neil (2004) suggested that the deaths due to AMR could reach to 10 million lives each year, imposing a loss of 100 trillion USD. On this basis, by 2050, the death toll could be one person every three seconds. (Figure 1)

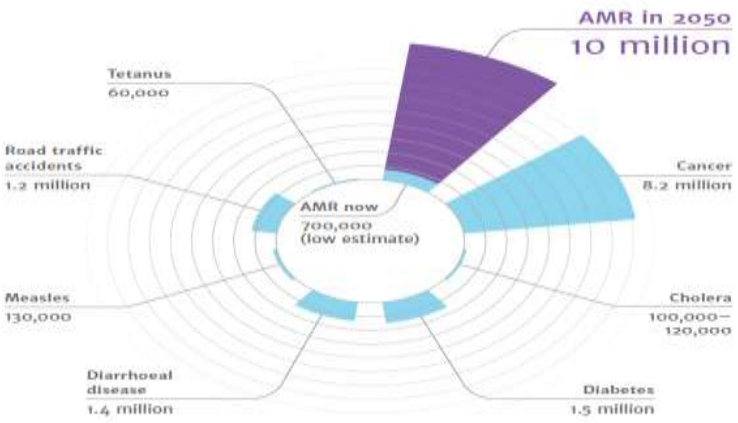



Figure (1): Death attributable to AMR every year compared to other cause of death (source: the review on antimicrobial resistance, Jim O Neil,2014).

## 2.2 Mechanism of antibiotic resistance

Intrinsic antibiotic resistance mechanisms are always present (natural) in the organism, only expressed as a response to exposure to the antibiotics. The most common mechanism involved are reduced permeability of the outer membrane and the activity of efflux pump. On the other hand, the acquired resistance mechanisms, are generally obtained by horizontal gene transfer or due to the mutation of its own chromosomal DNA. The plasmid mediated transmission of genes also one of the most common route for acquisition (reviewed in C Reygaert, 2018).

### 2.2.1 Mutations and horizontal gene transfer

Mutation is a spontaneous event which occurs regardless of the presence of the antibiotics. Bacteria have an average mutation rate of 1 for every $10^6$ to $10^9$ cell divisions and most of these mutations are harmful to the cells. Mutations that occur in antimicrobial resistance only occur in few types of genes such as those encoding drug targets, encoding drug transporters and regulators, and those encoding antibiotic modifying enzymes (Martinez, 2014).

Antibiotic resistance in a bacterium may also occur due to the transfer of antibiotic resistance genes. Transfer of antibiotic resistance genes between bacterial subpopulation via genetic exchange mechanism involves transformation with free DNA, transduction via bacteriophage or conjugation involving plasmids, collectively known as Horizontal gene transfer (HGT) (Peterson & Kaur, 2018).

The process of transformation involves acquisition of whole or piece of DNA into the genetic material of recipient cell and is best characterized in evolution of antibiotic

resistance strains of *Streptococcus* and *Neisseria*. For example, the persistence of penicillin resistance in *S. pneumoniae* may be due to high frequency of natural transformation in the organism (Hoffman-Roberts *et. al.,* 2005).

Transduction is a process in which the phage particles are packaged with bacterial DNA inside phage and transferred in to another bacterium. There are two types of transduction, generalized in which any segment of bacterial DNA can be packaged into the phage head and specialized in which the DNA adjacent to the phage insertion site is packaged (Van Hoek *et. al.,* 2011). It is believed to play a major role in evolution of resistance in *S. aureus*. The transfer of genes for penicillinase, metallo β-lactamse, and tetracycline resistance by transduction has been reported in *S. aureus* (Varga *et. al.,* 2016).

Plasmid- mediated conjugation as a gene transfer mechanism is more prevalent than either transformation or transduction. Mathematical modelling analysis has shown that conjugation may be 1,000 fold more common than transduction as a resistance gene transfer mechanism (Volkova *et. al.,* 2014). Some of the known examples for the spread of carbapenemase, $bla_{CTX\text{-}M}$ ESBL, and quinolone resistance genes are plasmid mediated conjugation among gram negative bacteria (Carattoli, 2013). However, on the gram positive bacteria other DNA elements known as conjugative transposons or integrative conjugative elements (ICEs) cause conjugation. ICE carry resistance genes, like in Tn916 family members that encode tetracycline resistance (A. P. Roberts & Mullany, 2011).

## 2.2.2 Mechanistic basis of mechanism

Bacteria have evolved various mechanisms of drug resistance against the drug molecule and can be achieved through multiple biochemical pathways. Bacteria have evolved several strategies of antibiotic resistance and mechansims are as follows (Dever and dermody, 1991).

i) Inactivation or alteration of antibiotics

ii) Modification of drug binding sites/targets

iii) Changes in cell permeability to antibiotics

### 2.2.2.1 Inactivation or alteration of antibiotics

One of the most successful bacterial strategies to deal with the availability of antibiotics is to produce the enzyme that inactivate the drug or to destroy the antibiotic itself. Antibiotic modification strategies, acquired in both gram positive and gram negative organisms, renders the antibiotic ineffective by the production of the three main enzymes: aminoglycoside modifying enzymes (AMEs), Chloramphenicol

acetyltranferase (CAT) and β-lactamses (Peterson & Kaur, 2018). The AMEs involve N-acetyl transferases (ACC), O-phospotransferases (APH), and O-adenyltransferases (ANT) that acetylate, phosporylate, or adenylate the aminogylcoside antibiotic (streptomycin, kanamycin, etc). Chemical modification of chloramphenicol, antibiotic that inhibits protein synthesis by binding with 50S ribosomal subunit, by CAT enzyme is by group transfer in both gram positive and gram negative organisms (Munita *et. al.,* 2016).

The enzymes β-lactamases destruct the β-lactam group (penicillin, cephalosporins, monobactems and carbapenems) of antibiotics by hydrolyzing the amide bond of the β-lactam ring making the antibiotic ineffective, in gram negative bacteria (Sirijan Santajit & Nitaya Indrawattana, 2016).

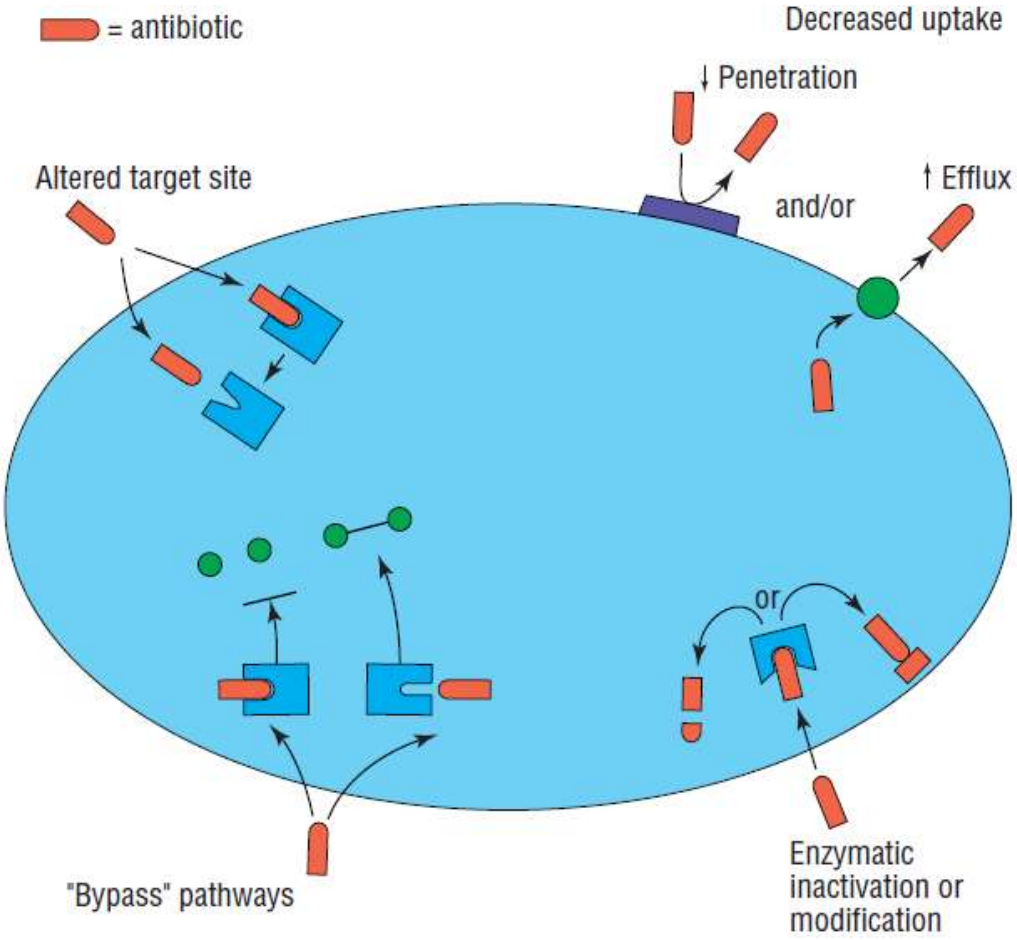



Figure (2): Biochemical mechanism of antibiotic resistance (Hawkey P M, 1988)

### 2.2.2.2 Modification of drug binding sites/targets

There are many targets that are modified by the bacteria to enable resistance to those drugs. The classical example of target modification is shown by MRSA strains where resistance to β-lactams is conferred by an exogenous Penicillin Binding Protein (PBP2a) in addition to the PBP. PBP2a, coded by the mecA gene, decrease the ability

to bind drug or inhibit the drug binding (C Reygaert, 2018). Similarly, Vancomycin resistance, a common problem in enterococci is due to the aqcuisition of Van gene cluster. Other target modification examples include point mutation or enzymatic alteration of the target. Enzymatic alteration of the target is studied in case of macrolide resistance conferred by erythromycin ribosomal methylation (erm) gene, which methylates adenine in the 23S rRNA (Munita *et. al.,* 2016).

#### 2.2.2.3 Changes in cell permeability to antibiotics

The presence of cell membrane permeability is one of the strategy of bacteria for the antibiotic resistance. The outer membrane present in the gram negative bacteria offers an intrinsic protection against the hydrophilic antibiotics such as β-lactams, tetracycline and fluoroquinilones. A reduction in the amount of *P. aeruginosa* porin protein OprD results in decreased drug influx in the cell, thus developing the resistance to imipenem. Similarly, loss of outer membrane protein (OMP) in *Acinetobacter baumani* and loss of outermembrane proteins, OmpK35 and OmpK36, in *Klebseilla pneumoneae* also exhibit resistance to β-lactams (Sirijan Santajit & Nitaya Indrawattana, 2016).

In addition, some pathogens developing the resistance have active efflux pumps, a proteinaceous transporters, which expel the drug. There are major five families: adenosine triphosphate binding cassette familiy (ABC), major facilitator superfamily (MFS), small multidrug resistance(SMR), multidrug and toxin extrusion (MATE), and the resistance nodulation division family (RND). In case of tetracycline resistance, Tet proteins belonging to the MFS family, carry out import or export of only one specific substrate (Peterson & Kaur, 2018).

## 2.3 Review of literature on current antibiotics and their mode of mechanism

The term antibiotic is coined from the word ‘antibiosis’ which means “against life”. Broadly defined as a substance, produced by one microorganism, or of biological origin which at low concentration can inhibit the growth or are lethal to other microorganisms (Russell, 2004). Since, the discovery of first antibiotic penicillin produced by soil inhabitating fungus *Penicillium notatum* by Alexander Fleming, more effective antimicrobials have been discovered and developed by modification of drug molecules (Ligon, 2004).

The antibiotics classes based on chemical or molecular structures include Beta-lactams, Macrolides, Tetracyclines, Quinolones, Aminoglycosides, Sulphonamides, Glycopeptides and Oxazolidinones (Ebimieowei & Ibemologi, 2016). The basic

mechanisms of antibiotics are as follows (Kapoor *et. al*., 2017) while the most common target of antimicrobial compounds are shown in the figure below.

- Inhibition of cell wall synthesis,
- Inhibition of protein synthesis,
- Cell membrane alteration,
- Inhibition of nucleic acid biosynthesis

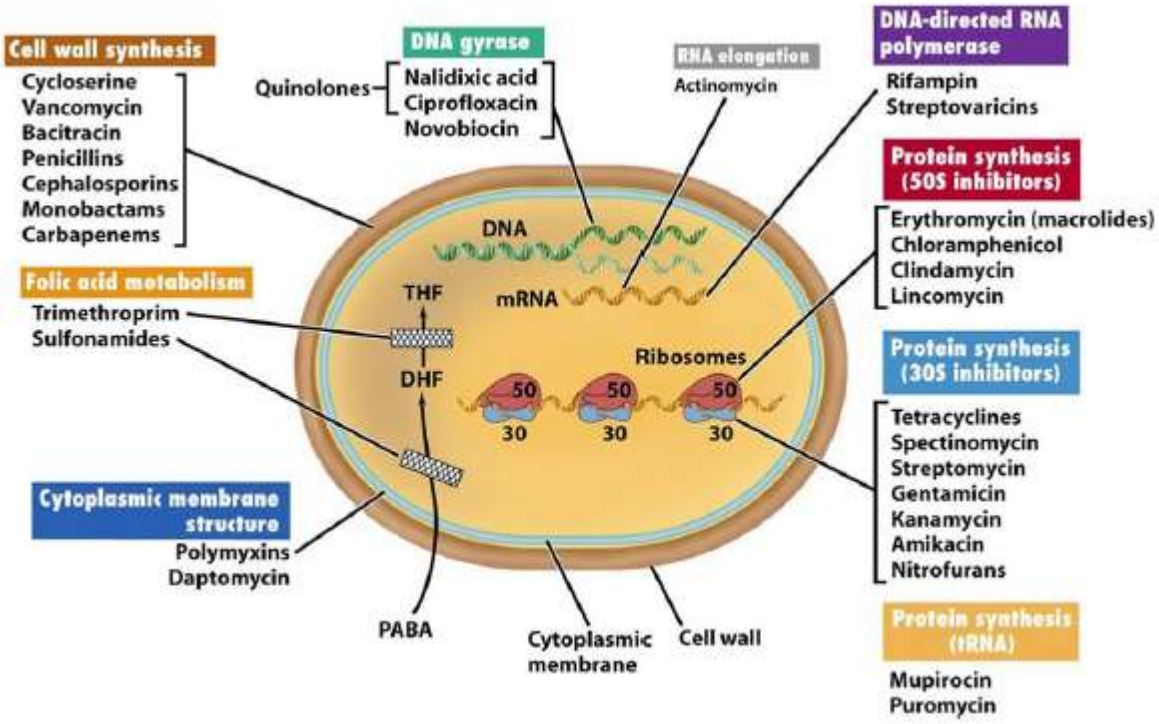



Figure (3): Antibiotic target sites (Ebimieowei & Ibemologi, 2016)

## 2.3.1 Beta-lactams

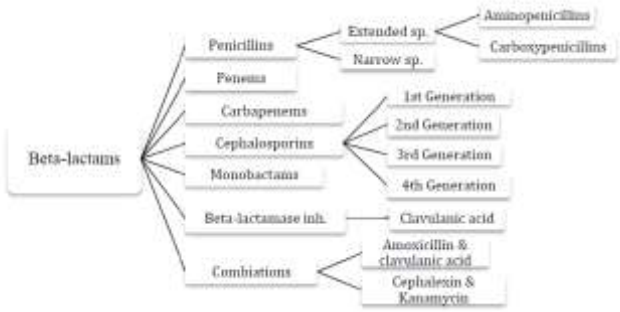



Figure (4): Beta-lactam class of antibiotics

As mentioned above, the first discovered antibiotic was β-lactam, i.e., penicillin. All β-lactam antibiotics have β-lactam ring in their molecular structure and the family include penicillins and derivatives, cephalosporins, monobactems and carbapenems. The β-lactam ring acts as a substrate for the enzyme transpeptidases, an enzyme termed for penicillin binding protein (PBP), which is responsible for cross linking peptides during peptidoglycan biosynthesis. β-lactams antibiotics disrupts the cell wall by competitively inhibiting PBP crosslinking of peptidoglycan (Dowling, Dwyer and Adley, 2014).

### 2.3.2 Aminoglycosides

Aminoglycosides are the antibiotics with broad spectrum of antibacterial activity. Streptomycin was the first antibiotic discovered in this class, first isolated in 1943 and has been greatly used against *Mycobacterium tuberculosis*. The most aminoglycosides have been isolated from *Streptomyces* spp, *Micromonospora* spp and *Bacillus* spp. Other members of aminoglycosides include gentamicin, neomycin, tobramycin, amikacin and kanamycin. Gentamicin is widely used for infections caused by gram negative rods like *Escherichia, Pseudomonas, Shigella* and *Salmonella* while Tobramycin, in particular, is used in treating Pseudomonas infections in cystic fibrosis patients (Ebimieowei & Ibemologi, 2016). Aminoglycosides are antimicrobials that kill the bacteria by inhibiting protein synthesis. They bind to the 16S rRNA of 30S ribosomal subunit which alter the integrity of the cell membrane (Vakulenko & Mobashery, 2003).

### 2.3.3 Sulphonamides

Sulphonamides are one of the oldest groups of antimicrobials still in use. The most commonly used sulphonamide is sulfamethoxazole. However, the combination with trimethoprim or with ormetoprim has been used to broaden the spectrum and to reduce selection of antibiotic resistance (M. C. Roberts, 2002). Sulphonamides are structurally similar to para-aminobenzoic acid (PABA), and act as a false substrate and compete with PABA for the enzyme dihydrofolate synthase and block the synthesis of dihydrofolic acid (DHFA) and trimethoprim inhibits the synthesis of tetrahydrofolic acid (THFA) and folate cofactor is inhibited. As a result of this, folate biosynthesis pathway is disrupted, required for thymine production, halting the bacterial growth and division (Capasso & Supuran, 2014).

### 2.3.4 Tetracycline

Tetracycline antibiotics were isolated from various species of *Streptomyces* in the late 1940s and is a broad- spectrum antimicrobials. Chlorotetracycline was the first member of this class and members of this class are grouped into different generations based on the method of the synthesis. Members like tetracycline, cholorotetracycline, oxytetracycline and demeclocycline are first generation. Members like doxycycline, lymecycline, minocycline are second generation while tigecycline is 3$^{rd}$ generation. Tetracycline shows antibacterial activity by binding to the 30S ribosome subunit of an organism. They interfere with the binding of aminoacyl-tRNA to the messenger RNA molecules, thus disrupting the bacterial protein synthesis (Chopra and Roberts, 2001).

### 2.3.5 Fluroquinolones

The fluroquinolones are synthetic broad-spectrum antibacterial drugs. This group of antibiotics have core quinolone structure and include member like ciprofloxacin, ofloxacin and levofloxacin. They interfere with the bacterial DNA metabolism by the inhibition of two enzymes, topoisomerase II (DNA gyrase) and Topoisomerase IV. Both of the enzymes have very similar structure. The DNA gyrase function is to introduce negative supercoils into the linear DNA wheareas DNA topoisomerase IV plays role in the splitting process of the DNA daughter chains. The mode of action of these antimicrobials is that the two quinolones molecules self-assemble and forms a dimer structure inside the gyrase induced DNA pocket and bind to the gyrase complex. Thus, by inhibiting DNA gyrase, repair enzymes are synthesized, which initiate uncoordinated DNA repair, leading to damage and cell death (Dowling, Dwyer and Adley, 2017).

## 2.4 Literature review on bacterial essential gene

Essential genes are those genes that are indispensable and vital for the survival of any living cell (Guo *et. al.,* 2015). They are required for basic cell activities and are considered as foundation of cellular life. Research on essential genes has shown theoretical as well as practical values in biology, industrial bioprocessing, and medicine. Moreover, because the deletion or inactivation of these genes show lethality to microorganisms, essential genes or protein encoded by them confers possible drug targets in the pharmaceutical industry (Mobegi *et. al.,* 2017; Peng *et. al.,* 2017).

Essential genes have been identified in number of different organisms and by various techniques. Essential genes have been determined in *Staphylococcus aureus* by an antisense RNA technique, in *Mycoplasma genitalium* by transposon mutagenesis, in *Haemophilus influenzae* by high density transposon mutagenesis, in *Vibrio cholerae* by

a mariner-based transposon, in yeast by genetic footprinting and in *M. genitalium* and *H. influenzae* by comparative genomics (Zhang, 2004; Hillyard & Redd, 2007). However, these experimental methods have huge economic and labor cost so the alternative approach has been resorted such as computational method which can predict essential gene more rapidly and with almost no economic cost. Subsequently, a study by Chen and Xu identify the essential gene in *Saccharomyces cerevisae* by using methods like artificial neural network and vector machine supply (Guo *et. al.,* 2015). Similarly homology modelling, metabolic reconstruction model, protein-protein interaction model are also reliable computational method (Mobegi *et. al.,* 2017).

Recent work done in *Helicobacter pylori* has shown that the trans-translation machinery is essential for its survival. It has been reported that ssrA, small RNA, and smpB, a protein cofactor are essential in *H. pylori* and therefore represent excellent target for novel antibiotics. Similarly, determination of essential cellular cycle genes, ccrM and menH, which regulate the bacterial cell cycle, has led to the development of potential broad spectrum antibiotics: borinic esters which are now in clinical trials (Juhas *et. al.,* 2012).

Therefore, gene essentiality study has practical importance and can also be used in industrial application. In a study done by Morimoto, Manabe and Mizoguchi, Genome reduction in host cell for the construction of minimal gene set lead to more efficient bioproduction. *Bacillus subtilis* and *E. coli* genome reduction showed beneficial traits such as *B. subtilis* strain MGB874 lacking 20.7 % of the genome produced significantly higher levels of heterologous enzymes alkaline cellulase and protease than the parent strain while *E. coli* lacking 22 % of the genomic DNA produced 2.4- fold more threonine than the wild type strain (Juhas *et. al.,* 2014; Morimoto *et. al.,* 2008).

Thus, to predict and identify the essential gene used in this study, various computational techniques and tools were used such as Clustal omega, MAUVE alignment, COBRA toolbox, Matlab and GNU linear programming (Suprim Tha Master's Thesis). Hence, Uroporphyrinogen III C-methyltransferase (*cob*A) was identified as essential gene and as its deletion shows fatality to test organism, it can be used as potential therapeutic target.

## 2.5 Literature review on Biosynthesis of adenosylcobalamin (Vitamin B12)

Vitamin B12, also known as cobalamin, is one of the most structurally complex cofactor synthesized by bacteria (Warren *et. al.,* 2002) and because of this highly complicated synthesis process makes its industrial production through chemical method a difficult task (Sattler *et. al.,* 1995). The molecule can be divided into three

parts: a central ring, an adenosyl moiety, and a nucleotide loop. These components in biological systems are assembled together enzymatically, at least 25 unique enzymes, to form a functional structure (Roth *et. al.,* 1996).

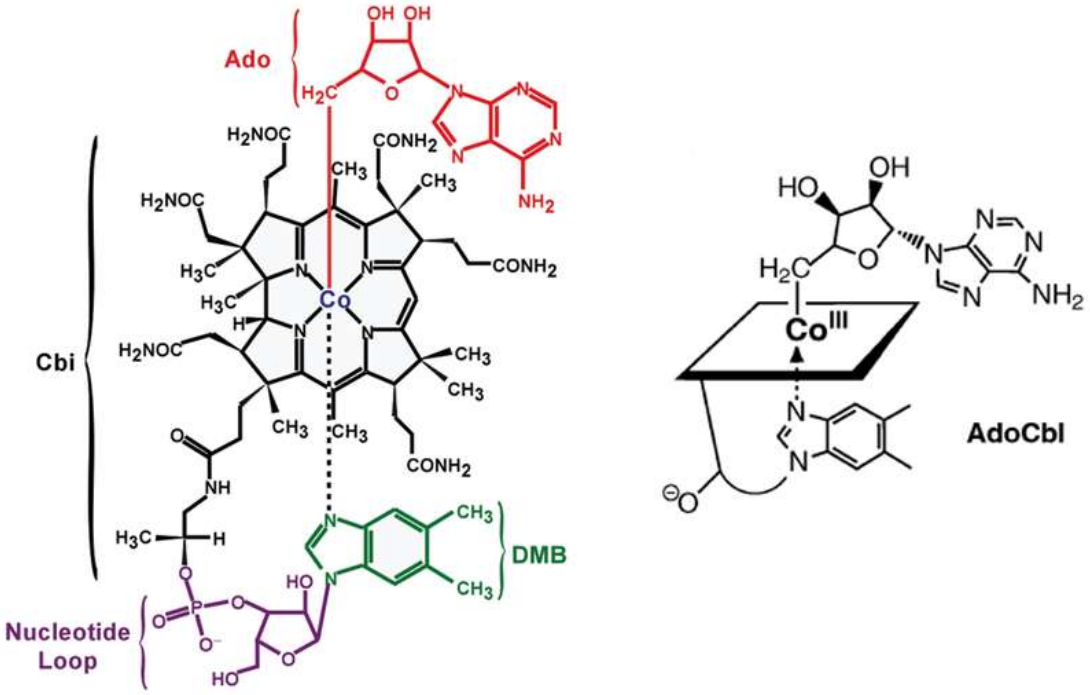



Figure (5): Structure of adenosylcobalamin (Vitamin B12) (Derviş, 2013)

Adenosylcobalamin has a 5' deoxyadenosyl moiety at its upper axial ligand and dimethylbenzimidazole (DMB) at its lower axial ligand. The central cobalt metal in the corrin ring is covalently bound with the methyl group of 5' ribose sugar and imidazole moiety of DMB. Furthermore, DMB is bound to the aminopropanol moiety via propionyl group extending from corrin ring (Roth *et. al.,* 1996). Animals, plants, fungi are not capable of producing cobalamin, making it the only vitamin exclusively produce by microorganisms.

Microbial *de novo* biosynthesis of vitamin B12 occurs through two different pathways i.e the aerobic pathway and the anaerobic pathway, in bacteria and archae, respectively, and some strains synthesize via salvage pathway (Fang *et. al.,* 2017). The cobalt insertion in corrin ring in the aerobic pathway, described in *Pseudomonas denitrificans*, occurs at later stage (Warren *et. al.,* 2002). The most studied anaerobic biosynthetic pathway involved in early insertion was described in *Salmonella typhymurium* and *Propionibacterium freudenreichii* (Roth *et. al.,* 1993).

Moreover, the corrin ring synthesis uses common precursor 5-aminolaevulinic acid (ALA). ALA is synthesized by one of the two routes, either by $C_4$ pathway where the ALA synthase catalyzes glycine and succinyl Co-A forming ALA, or by $C_5$ pathway where

the ALA is synthesized through the enzymatic reaction of glutamate. The biosynthesis of ALA is the first committed step in corrin ring formation, for the synthesis of tetrapyrole compound and is rate limiting (Raux *et. al*., 2000).

From ALA the biosynthesis of uroporphyrinogen III, a precursor of vitamin B12 corrin ring, involves mutiple steps. The first step is the condensation of two molecules of ALA to form monopyrrole porphobilinogen via porphobilinogen synthase, encoded by hemB gene, is the common step of all tetrapyrrole synthesis and uroporphyrinogen III is formed by polymeriztion and cyclization of four porphobilinogen molecules by the enzyme porphobilinogen deaminase and uroporphyrinogen synthase encoded by hemD.

The uroporphyrinogen is the major branch point in the pathway of tetrapyrrole containing compounds. Decarboxylation leads to the biosynthesis of hemes and chlorophylls. Similarly, methylation of uroporphyrinogen III at C-2 and C-7 results in the synthesis of precorrin-2, which is a common precursor of cobalamin, siroheme and coenzyme $F_{430}$. The transformation of uroporphyrinogen III to precorrin-2 is catalyzed by uroporphyrinogen III C-methyltransferase, which uses *S*-adenosyl L-methionine as methyl donor (Raux *et. al.,* 2000; Martens *et. al.,* 2002; Piao *et. al.,* 2004). This gene has been found to be critical for bacterial survival (Tha *et. al*., 2020) indicating its importance in vitamin B12 biosynthesis.

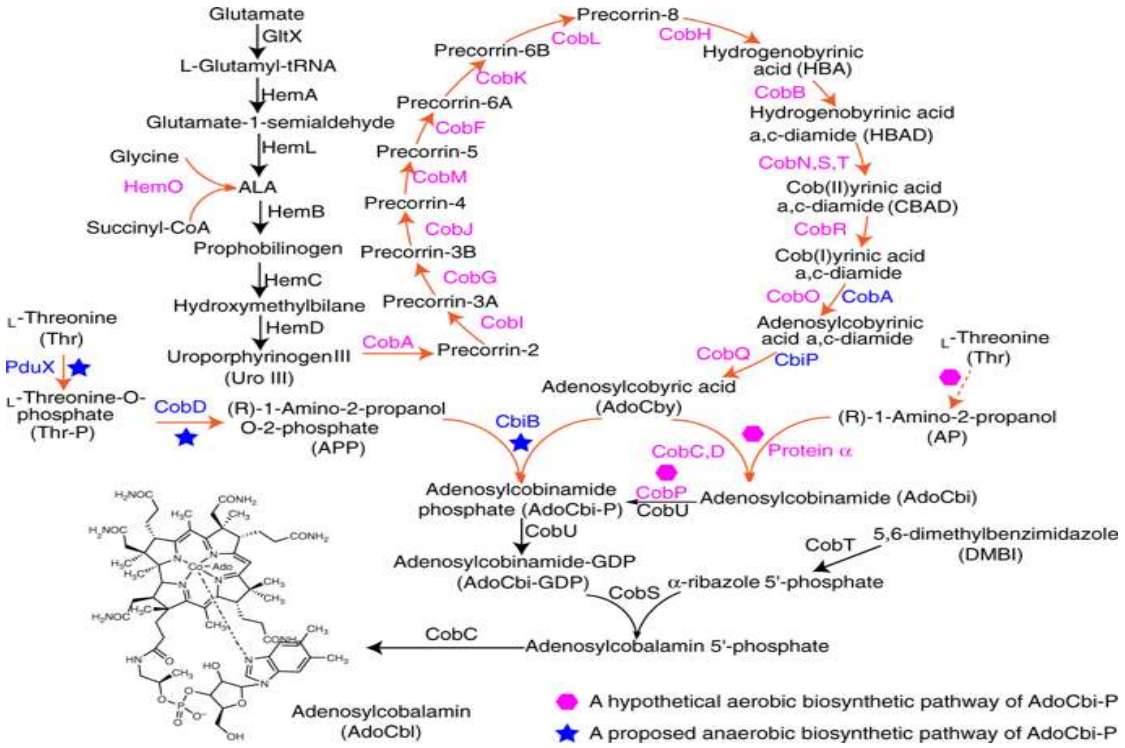



Figure (6): Biosynthetic pathway of adenosylcobalamin (adopted from Fang *et. al.,* 2018)

## 2.5.1 Uroporphyrinogen III C- methyl transferase (*cob*A)

Uroporphyrinogen III methyltransferase, also known as S-adenosyl uroporphyrinogen III methyltransferase (SUMT), a key enzyme in the vitamin B12 pathway, catalyzes the transformation of uroporphyrinogen III into precorrin-2 (dihydrosirohydochlorin) (Sattler *et. al.,* 1995, Martens *et. al.,* 2002). SUMT is encoded by *cob*A gene in *Pseudomonas denitrificans* (Crouzet *et. al.,* 1990) while it is also encoded by *cys*G gene in *E. coli* and *S. typhimurium* (Sattler *et. al.,* 1995). At least 22 *cob* genes involved in the biosynthesis of vitamin B12 has been isolated and their function has been identified (Roth *et. al.,* 1996).

Structure and functional analysis have suggested that most SUMT is a homodimer with around 280 amino acid residues of 28 kDa molecular weight. The SUMT subunit has kidney like shape consisting of two domains connected with each other by a linker. N-terminal domain is a five stranded parallel β-sheet with topology 3-2-4-1-5 associated with four α- helices. Similarly, C-terminal domain is a five stranded β-sheet with topology 1-2-5-3-4 wrapped by three α- helices (Vévodová *et. al.,* 2004). However, SUMT of *Bacillus megaterium* has monomeric form (Robin *et. al.,* 1991)

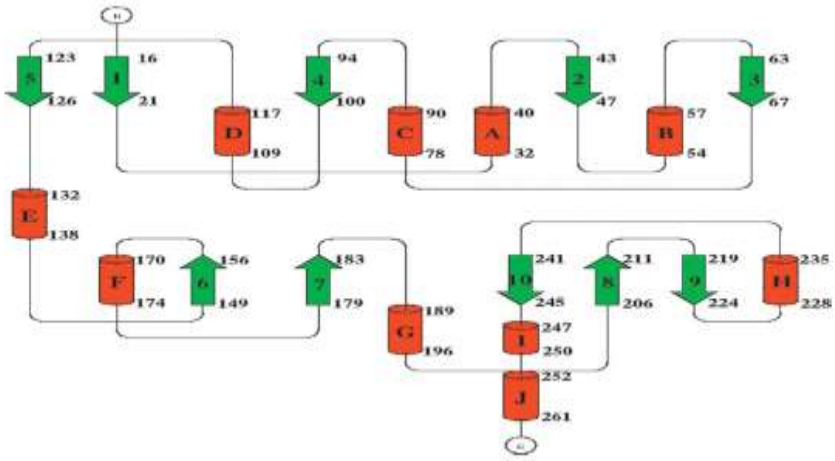



Figure (7): Topology diagram of SUMT with secondary structure element (adopted from vevodova *et. al.,* 2004)

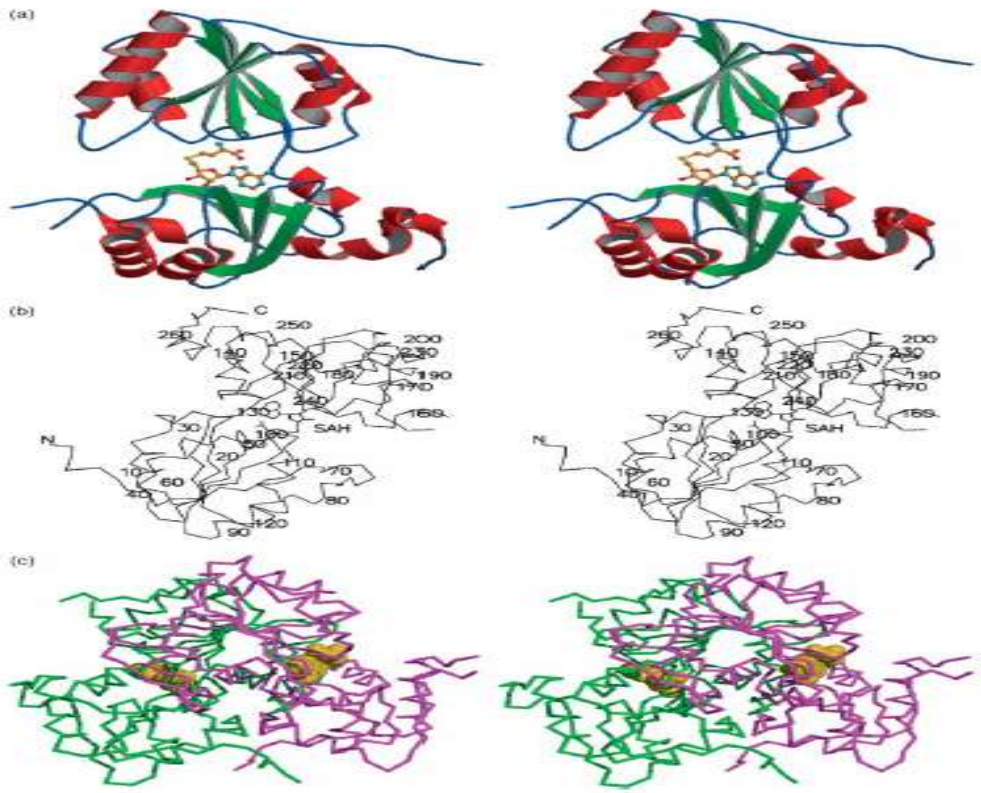



Figure (8): Overall structure of SUMT unit (adopted from vevodova *et. al.,* 2004)

## 2.6 Literature review on Riboswitch

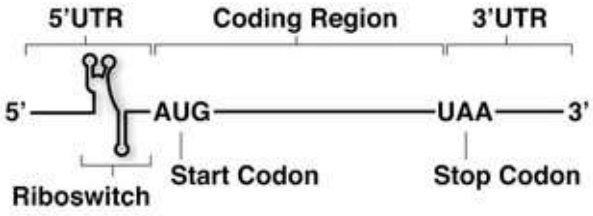



Figure (9): The mRNA Structure. Bacterial mRNA transcript having regulatory element, Riboswitch (Edwards and Batey, 2010).

Riboswitches are structured non coding RNA element, usually found in the 5' untranslated region of mRNA, where they regulate gene expression by forming alternative structure as a result of binding to small metabolites or ions as ligand (Tucker & Breaker, 2005). The typical riboswitches usually consist of two functional domains commonly termed as the aptamer domain and the expression platform. The aptamer domain adopts a three dimensional shape to act as ligand binding pocket, which selectively binds the target metabolite with high selectivity and unveil the appropriate regulatory response (Garst *et. al.,* 2011). The second domain, expression

platform, is usually downstream from the aptamer domain but in some cases the two domain may also overlap, leading to conformational changes, in response to ligand binding in aptamer region in regulating gene expression (Mandal & Breaker, 2004). Switching sequence, a overlap region between two domains, regulates the translation status of on and off states of the mRNA represented by either of the two mutually exclusive structures in the expression platform (Corbino *et. al.,* 2005).

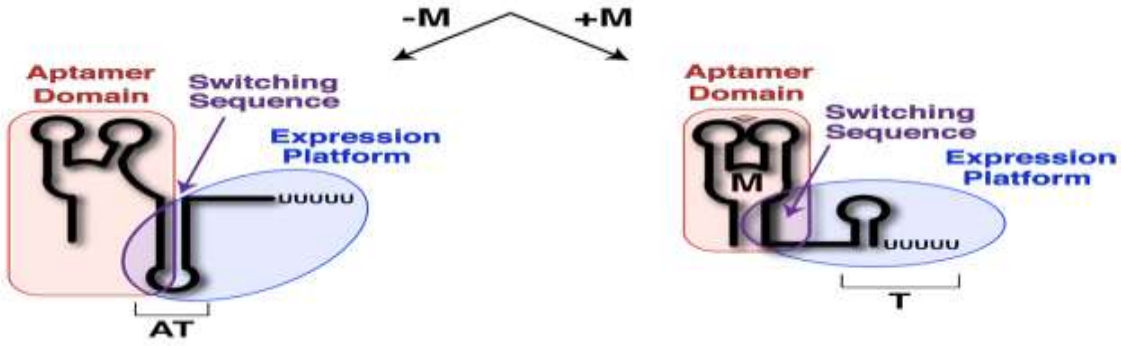



Figure (10): Structure of riboswitch domains.

A riboswitch can have different conformational forms to effect gene regulation depending whether ligand is bound or not. This diagram is an example showing transcriptional control by riboswitch. When metabolite is not bound (-M), the expression platform incorporates the switching sequences into an antiterminator stem-loop (AT) and the transcription proceeds through the coding region of the mRNA. When the metabolite is bound (+M), the switching sequence is incorporated into the aptamer domain, and the expression platform folds into a teminator stem loop (T), aborting the transcription (Edwards and Batey, 2010).

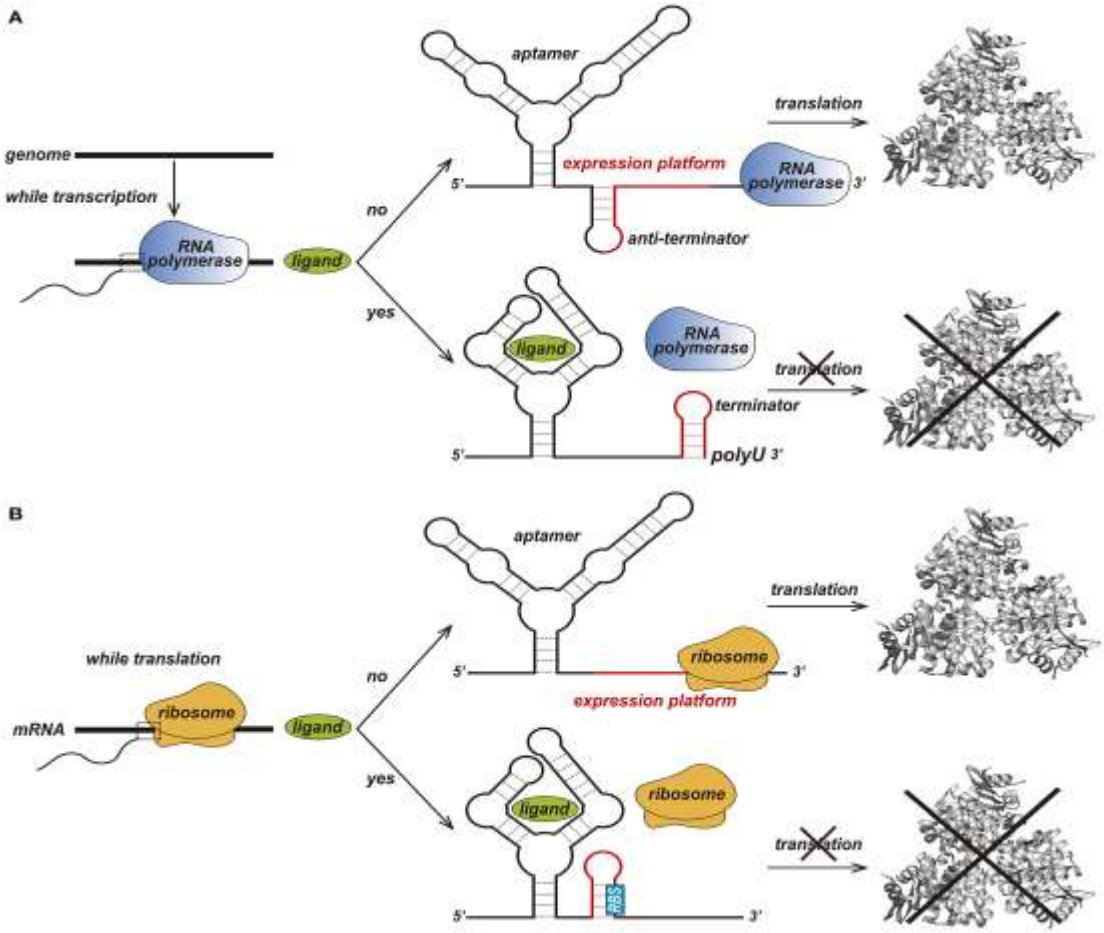



Figure (11): Two different forms of riboswitch regulatory mechanism (Antunes et. al., 2018).

Riboswitches are usually found in the prokaryotes and can detect the metabolites and control gene expression. They can regulate fundamental biochemical pathways either by prematurely terminating transcription or by preventing translation of hosts mRNA as shown in the figure above. However, some riboswitch like TPP riboswitch is found in eukaryotic mRNA (Winkler & Breaker, 2005).

Based on the type of ligand they bind, and their secondary structure, riboswitches are differentiated into families and classes. Some of the metabolite sensing riboswitch include cobalamin riboswitch which can sense adenosylcobalamin (adocbl), TPP riboswitch regulating thiamine biosynthesis, FMN riboswitch which binds flavin mononucleotide and regulates riboflavin biosynthesis. Similarly, other reported riboswitch class includes *S*-adenosylmethionine (SAM), guanine/adenine, glycine, the bacterial second messenger c-di-GMP. These are the most common riboswitch classes that exits. However there are also “orphan riboswitches” which lack assigned ligands (Breaker, 2011).

| Ligand class | Examples |
|---|---|
| Coenzymes and related compounds | Adenosylcobalamin<br>Flavin mononucleotide<br>Molybdenum/tungsten cofactors<br>*S*-adenosylmethionine<br>*S*-adenosylhomocysteine<br>Tetrahydrofolate<br>Thiamine pyrophosphate |
| Nucleotides and their derivatives | Adenine<br>Adenosine triphosphate<br>Cyclic diguanosyl-5′monophosphate<br>Cyclic diadenosyl-5′monophosphate<br>Deoxyguanosine<br>Guanine<br>Prequeuosine-1 |
| Amino acids | Glutamine<br>Glycine<br>Lysine |
| Sugars | Glucosamine-6-phosphate |
| Ions | Fluoride<br>Magnesium<br>Manganese<br>Nickel/cobalt |

Figure (12): Regulatory ligand for metabolite binding riboswitches (Sherwood & Henkin, 2016).

### 2.6.1 SAM Riboswitch

SAM, a universal methyl donor, is an important metabolite that serves as co-factor in different enzymatic reactions. This is synthesized from ATP and methionine by SAM synthetase. Enzymes known as methyltransferases uses SAM to transfer methyl group to substrates ranging from small molecules to proteins, RNAs and DNAs. During the methylation, SAM is converted to the neutral *S*-adenosylhomocysteine (SAH) (Sun *et. al.,* 2019). Because of the biological importance of SAM, the SAM riboswitches are among the most abundant riboswitches and are structurally diverse. Three superfamilies of SAM riboswitch have been identified with seven subclasses called SAM-I to Sam-VI and SAM-I/IV (Weickhmann *et. al.,* 2019).

The SAM-I riboswitch (or S-box) was the first SAM riboswitch family discovered from *Bacillus subtilis,* found in the 5'UTRs of sulfur metabolism genes and helps to control transcription of its target genes at the termination level. When SAM levels is low, transcription antiterminator stem loop formation is favoured while high levels of SAM forms alternative structure leading to the transcription termination. The variation of SAM-I, SAM-IV, was found in *Actinomycetales* and SAM-I/IV was identified in metagenome sequence. They have same binding site interactions while the scaffolding beneath the binding nucleotides differs (Price *et. al.,* 2014).

The SAM-II riboswitches are typically short sequences, identified as “metA” found predominantly in α-Proteobacteria. The SAM-II structure forms “H-type” pseudoknot upon SAM binding. Despite having SAM-II aptamer smaller than that of SAM-I aptamer, SAM binds with nearly same affinity. Though pseudoknot ends 2 NT upstream of the Shine-Dalgarno (SD) sequence, it is sufficient to occlude the ribosome binding site in “off” state (Corbino *et. al.,* 2005).

SAM-III ($S_{MK}$ box) also known as translational riboswitch, is a SAM- binding element found in the 5’UTR region of the metK gene (SAM synthetase) in *Lactobacillale s*sp. SAM-III has conserved secondary structure which has three helices and at the intersection is SAM binding site. SAM binding results in sequestration of SD sequence with an anti SD, thus occludes ribosome binding to the SD (Lu *et. al.,* 2008). The characteristic sequence and the secondary structure of SAM-III aptamers yield a tertiary structure and has binding pocket which are distinct compared to other SAM riboswitches (Poiata *et. al.,* 2009).

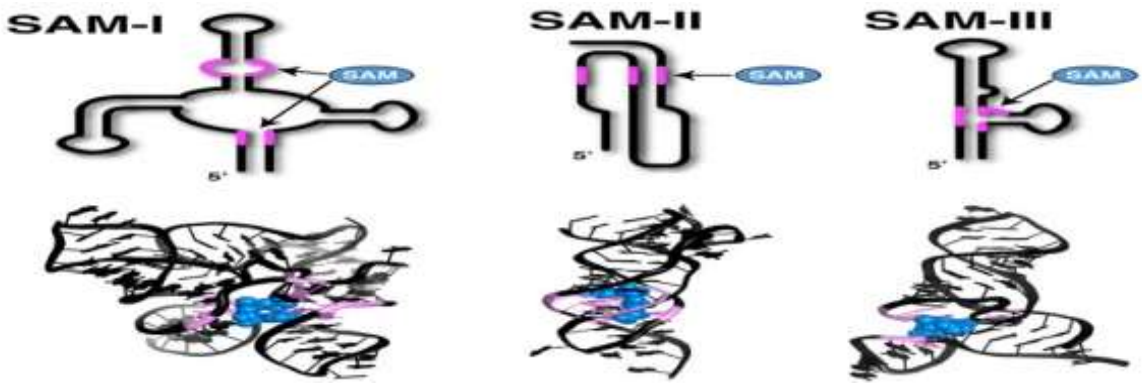



Figure (13): The SAM riboswitch family (Edwards and Batey, 2010)

## 2.7 Literature review on Gene Cloning

Gene cloning is an efficient genetic engineering technique which is used to produce exact copies of particular gene or DNA sequences. It allows us to create multiple clones and helps in the study of specific genes and gene expression. The genetic engineering technique utilizes the restriction enzyme to cut the target gene or gene of interest. The gene of interest is then inserted into the cloning vectors such as plasmids or bacteriophages. Plasmids with the foreign DNA inserted into them are called recombinant DNA as they contain new combination of genetic material. The recombinant DNA is then transferred into suitable host cells (“AgBiosafety at UNL-Biotech Basic Gene cloning”).

Basic steps involved in cloning

- Isolation of donor fragment or gene

- Selection of suitable restriction enzymes to digest both donor and vector DNA
- Incorporation of donor DNA fragment into the vector through ligation
- Transformation of recombinant vector into a suitable host cell
- Isolation of recombinant host cell

## 2.8 Literature review on Promoter

The major challenge in genomic era is the determination of when and how genes are turned "on and off". The first and key step in gene expression is promoter recognition by RNA polymerase enzyme and are vital component of expression vector because they control the binding of RNA polymerase to DNA. The Promoter is a region of DNA where transcription of a gene is initiated. The promoter sequences are cis-acting elements located upstream of the transcription start site (TSS) of open reading frame (ORF) (Howard and Benson, 2002).

Promoters are about 100-1000 base pair long and adjacent and typically upstream (5') of the sense strand of the transcribed gene (http://www.cs.tau.ac.il/~roded/courses/bnet-a06/lec11.pdf).

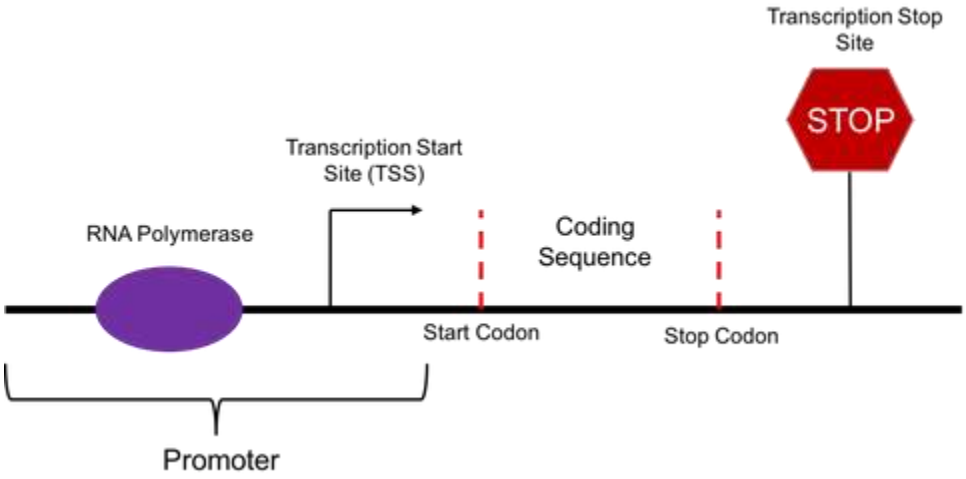



Figure (14): Schematic diagram of promoter region (https://www.addgene.org/mol-bio-reference/promoters/)

### 2.8.1 Bacterial promoters

Bacterial promoters contain at least three RNA polymerase recognition sequences. In bacteria RNA polymerase (RNAP) consists of five subunits (2α, β, β', ω) and sigma (σ) subunit factor. The sigma factor led RNAP sequence-specific binding at promoter (Borukov and Nudler, 2003). The substitution of one sigma factor by another lead to the transcription of different gene. Regardless of the σ factor, most of the promoter can be dissected into two functional sites, known as -10 and -35 region. The -10

element having consensus sequence TATAAT; essential for transcription initiation; -35 element having consensus sequence TTGACA, controls rate of transcription and above consensus sequence (or UP element) (Estrem *et. al.,* 1999). The mutation in the consensus sequences of the promoters can affect the expression level of the genes they control, without altering the gene products themselves (Lewin, 2008). There are also some promoters which contain one or more upstream promoter element (Ross *et. al.,* 1998).

The promoter motif is not strictly conserved within a set of promoters recognized by given σ factor and also differs according to the σ factor which recognizes them (e Silva *et. al.,* 2011).

The below table shows the different bacterial promoters used in the research (https://www.addgene.org/mol-bio-reference/promoters/)

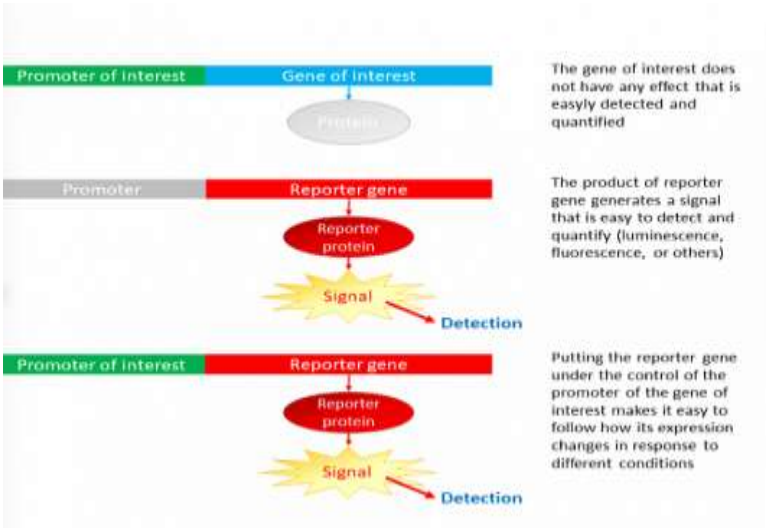
Table (1): Different Bacterial promoters used in the research

| Promoter | Expression | Description |
|---|---|---|
| T7 | Constitutive but requires T7 RNA polymerase | Promoter from T7 bacteriophage |
| Sp6 | Constitutive but requires Sp6 RNA polymerase | Promoter from Sp6 bacteriophage |
| lac | Constitutive in the absense of lac repressor (lacI or lacIq). Can be induced by IPTG or lactose | Promoter from Lac operon |
| araBad | Inducible by arabinose | Promoter of the arabinose metabolic operon |
| trp | Repressible by tryptophan | Promoter from *E. coli* tryptophan operon |
| Ptac | Regulated like the lac promoter | Hybrid promoter of lac and trp |

## 2.9 Literature review on reporter gene assay

Among various methods of assaying gene expression by high throughput screening, reporter gene assay is a sensitive and versatile method. Genetic reporters or reporter gene are used as an indicator for studying gene expression and cellular events coupled to gene expression. They are widely used in pharmaceutical and biomedical research and also in molecular biology and biochemistry (Assa T, 2008). The choice of reporter depends on the cell line used, the nature of the experiment (dynamics of gene expression versus transfection efficiency), and the adaptability of the assay for appropriate detection (Jiang *et. al.,* 2008).

Typically, Reporter genes are the genes which enables us in detection or measurement of the expression of the genes (An & Tolliday, 2009). The use of reporter genes fused to a gene of interest or regulatory sequences have been widely reported for studying gene expression and promoter activity in a diverse group of living organisms (Jugder *et. al.*, 2016). Reporter systems consist of a promoter and reporter gene. The choice of promoter controls the basal level of reporter gene activity. A good reporter gene can be identified easily and measured quantitatively when it is expressed (in the organism or cells of interest). Currently two types of reporter genes are commercially available and are classified as intracellular and extracellular reporter genes. Intracellular gene products are retained in the cells and include β- galactosidase, Luciferase, Chloramphenicol acyltransferase (CAT), Green fluorescent protein (GFP). While extracellular reporter products are secreted in the culture medium without disturbing the cells and include secreted placental alkaline phosphatase (SPAP), β-lactamase (New *et. al*., 2003; Mohammed *et. al*., 2015).




Source https://www.berthold.com/en/bioanalytic/knowledge/glossary/reporter-gene-assays/

### 2.9.1 Luc based reporter assay

As a reporter technology, bioluminescence has great potential by aiding in characterization of complexity of the living systems. Based on bioluminescence, *luc* reporter gene assay is developed and is used to analyze gene expression and promoter analysis in bacteria and eukaryotic cells. Out of all luciferases luciferase from fireflies (*Photinus pyralis*) and Renilla (*Renillareni formis*, a sea pansy) are generally used. This assay requires excess of luciferin and ATP (Assa T, 2008). The reaction generally occurs when a yellow-green light flourishes by luciferase using luciferin as substrate in the presence of ATP, Mg2+ and $O_2$ (Pardy, 1994). Nordeen study (Nordeen, 1988) describes *luc* reporter gene as ideal method for detection of low-level gene expression in both prokaryotes and eukaryotes. The expression is measured by analysing light intensity in in-vitro.

### 2.9.2 β-galactosidase based reporter assay

The *lac*Z gene of *Escherichia coli* is used in the quantification of transcriptional and translational activities that are associated with gene of interest in many different organisms. The *lac*Z gene, which encodes for β-galactosidase, is one of the most widely used reporter system in molecular biology (Zhang *et. al*., 1991). β-galactosidase is a tetrameric enzyme which catalyzes the breakdown of β-galactosides such as lactose. The chromogenic substrate o–nitrophenyl- β-D- galactopyranoside (ONPG) is used in a standard colorimetric enzyme assay that is read with a spectrophotometer. The product 0-nitrophenol, is yellow in solutions and absorbs light at 420nm. Other colorimetric based substrate is chorophenolred β-D-galactopyranoside (CPRG) while β-methyl umbelliferyl galactoside (MUG) and fluorescein digalactoside (FDG) are fluorescent- based assays (Schenborn & Groskreutz, 1999). Some of the plasmid carries only the alpha region of *lac*Z protein due to its large size. When alpha region of lacZ expresses an inactive protein, it can combine with β-galactosidase after complementing with *lac*Z protein that is devoid of alpha region (Juers *et. al*., 2012). This is possible when plasmid with alpha region of *lac*Z is transformed into bacteria with mutated alpha region. These bacterial cells will convert to β-galactosidase positive in the presence of plasmid.

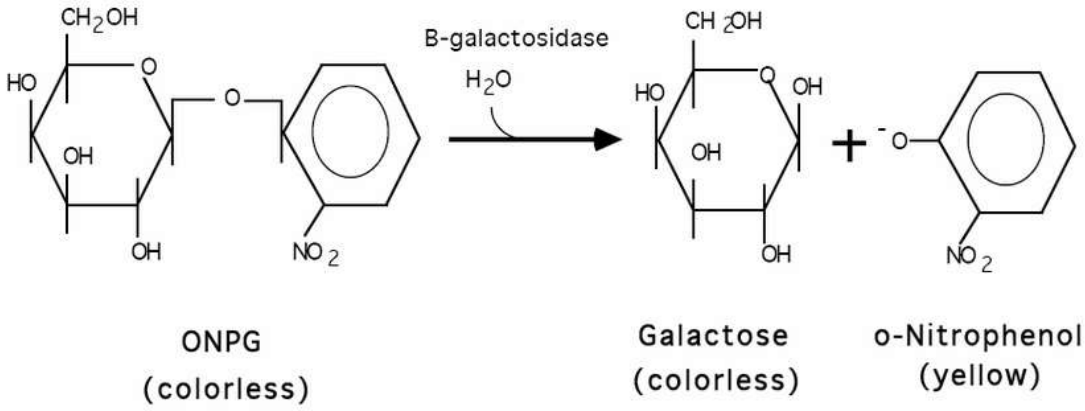



Figure (15): Chemical reaction of β-galactosidase assay (Rouf, 2007)

The *lac*Z gene from *E. coli* is a reporter gene used in determining promoter strength in both transiently and stably transfected cells because reporter protein expression corresponds with transcriptional activity of the promoter of the respective gene expression to be studied. For these type of study promoter sequences are cloned either upstream or downstream of the reporter gene and their effects are quantified (Jain & Magrath, 1991; Smale, 2010). However, an *E. Coli Lac*Z reporter fusion was also used to identify the bacterial protein secretion inhibitors (Alksne *et. al*., 2000). The promoter-*Lac*Z fusion was also used to study the transcriptional activity for sigma$^{54}$-dependent and sigma$^{54}$-independent promoters (Peng *et. al*., 2016). Similarly, a recombinant estrogen receptor- *lac*Z gene assay in yeast *Saccharomyces cerevisiae* was firstly used to study the estrogenic activity of the surfactants (Routledge & Sumpter, 1996). Thus, this reporter system could be explored to study the promoter strength or promoter region nucleotide required.

# 3. MATERIALS AND METHODS

## 3.1 Materials, reagents and chemicals used in the study

The sources of chemicals, restriction enzymes, master mix employed in this study are: restriction enzymes used were EcoRI HF (Thermo-scientific), SpeI (Thermo-scientific), BamHI (Takara Clone Tech) and PCR master mix (2X) from Zymogen company. T4-DNA ligase and its buffer from Takara Clone Tech, China. ONPG (Takara Clone tech). The DNA amplifying primers were purchased from Macrogen, Korea. TRIzol (Thermofischer) cDNA synthesis kit (Bio-rad). Other chemicals and reagents were obtained from commercial suppliers, Himedia, Merck and Thermo- Fischer company.

## 3.2 Bacteria and plasmid used in the study

*E. coli* DH5α was used as host bacteria for transformation and construct was prepared. Similarly, ATCC strain of *E. coli* 25923 and *P. aeruginosa* 27853 were used for *lacZ* gene amplification and *cob*A gene amplification. A pUC19 plasmid (Takara Clone Tech) with the size of 2686 bp was used as cloning vector with ampicillin resistance gene as a selection marker for transformed bacteria. The bacterial strains and plasmids employed in the study are listed in the table (2).

Table (2): Bacterial strains and plasmid used in the study

| **Strains and plasmids** | **Relevant properties** | **Source** |
|---|---|---|
| **Strains**<br>E. coli DH5α | F – Φ80 lacZ ΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1 | Lab stock |
| pUC19 | $AMP^R$, high copy number | Takara clone tech |
| pAG101 | pUC19 + CobAF100 promoter | Sita Ghimire thesis work |
| pAP201 | pUC19 + CobAF200 promoter | This work |
| pAP501 | pUC19 + CobAF600 promoter | This work |

| | | |
|---|---|---|
| pAP103 | pUC19 + CobAF200 promoter + *lac*Z gene | Sita Ghimire thesis work and my project work |
| pAP204 | pUC19 + CobAF200 promoter + *lac*Z gene | This work |
| pAP502 | pUC19 + CobAF200 promoter + *lac*Z gene | This work |

## 3.3 Primer designing and primer set (Suprim Tha)

Primer for PCR amplification for the promoter of *cob*A gene specific set of *Pseudomonas aeruginosa* (PA96 genome) were designed based on the nucleotide sequences in the NCBI using different freely available tools named as OligoCalc, M-fold, Oligoanalyzer. Using OligoCalc and Oligoanalyzer tool, the self-complementarity, homodimer, heterodimer, GC%, Tm and other parameters were analyzed. The DNA folding structure was analyzed using M-fold web server. The restriction site of EcoRI (GAATTC) was created in forward primers whereas BamHI (GGATTC) and SpeI (ACTAGT) were designed in the reverse primer respectively.

Similarly, the *lac*Z gene specific set of Open Reading Frame (ORF) primer (forward and reverse) was designed manually using *E. coli* CFT073 with restriction sites SpeI and BamHI respectively.

Table (3): Primer sets used in the study

| Primer | Sequence | Mer | Tm | Amplicon (bp) |
|---|---|---|---|---|
| CobAF100 | 5'- GAA CT***G AAT TC***C GCC TCG GCA AGC AAC T -3' | 29 | 73.3 | 2522 |
| CobAF2O0 | 5'-CAA TA***G AAT TC***G GCG AAC GCA TCC TCG GC -3' | 29 | 73.3 | 2645 |
| CobAF600 | 5'-GCC GAG ***GAATTC*** GAT GAA ATC AAC CCG CTG -3' | 30 | 73.5 | 3058 |
| CobAR | 5'-CAT T***GG ATC C***GT AAC CAT T***ACTAGT***TC TCC TCA GGC ATT CG -3' | 41 | 77.9 | - |
| *LacZF* | 5'- CGG C ***AC TAG T***AT GAC TAT GAT | 30 | 69.4 | 3075 |

| | | | | |
|---|---|---|---|---|
| | TAC GGA TTC -3' | | | |
| *LacZR* | 5'-ATA C***GG ATC C***TT ATT TTT GAC ACC AGA CCA AC -3' | 32 | 70 | - |

## 3.4 Construction of expression plasmid pAG101, pAP201 and pAP501

DNA isolated from *Pseudomonas aeruginosa* 27853 was used for PCR amplification of *cob*A gene F100, F200 and F600 promoter and DNA of *E. coli* for amplification of *lac*Z gene respectively. To construct an expression plasmid pAP103, pAP204 and pAP502, first *cob*A promoter was cloned in vector pUC19 and designated as pAG101, pAP201 and pAP501 which was further modified by sub-cloning of *lac*Z gene under control of *cob*A native promoter and designated as pAP103, pAP204 and pAP502 respectively.

### 3.4.1 Isolation of pUC19 plasmid

The pUC19 plasmid was recovered from *E.coli* DH5α strain by alkaline lysis method.

#### 3.4.1.1 Bacterial culture and cell harvest

*E.coli* DH5α strain was revived by streaking on Luria Bertani (LB) agar plate with 100 µg/ml ampicillin and incubated overnight at 37ºC. A single isolated colony was inoculated in 5ml of LB/ampicillin (50 µg/ml) broth and incubated at 37ºC for 18 hours at 200 rpm. Then, 1.5 ml of the overnight culture was transferred to sterilized eppendorf tube and centrifuged at 13,000 rpm for 1 minute. The supernatant was discarded and the cells were re-suspended in 500 µl of STE buffer and mixed by vortexing. The tube was then centrifuged at 13000 rpm for 1 minute and supernatant was discarded.

#### 3.4.1.2 Cell lysis alkaline lysis method

The cell pellet after washing with STE solution was resuspended in 200 µl of alkaline lysis solution I and mixed by vortexing for five seconds. To this solution, 200 µl of freshly prepared alkaline solution II was added and gently mixed by inversion of tube for five times. The bacterial solution then becomes clear as the cell lysis occurs. To this mixture, 200 µl of cold alkaline lysis solution III was added and mixed by inverting the tube for several times and incubated in ice for 5 minutes. During this step, a cloudy precipitate becomes visible due to precipitation of proteins. The mixture was then centrifuged at 13,000 rpm for 10 minutes at $4^{o}$C. Then the supernatant solution consisting plasmid DNA was transferred to a fresh eppendorf tube.

### 3.4.1.3 Plasmid recovery

To the supernatant 1 µl of RNAse (25 mg/µl) was added, mixed well and incubated for 30 minutes at 37 $^{o}$ C. One ml of ice cold isopropanol was added to the supernatant and was left in ice for 30 minutes to allow precipitation. The mixture was centrifuged at 13,000 rpm for 10 minutes and supernatant was discarded. The pellet was then washed with 70% ethanol and centrifuged at 13,000 rpm for 5 minutes. The supernatant was then discarded and was the pellet was left to air dry. Then pellet was dissolved in 20 µl of nuclease free water (NFW) and used for visualization by 0.8% agarose gel electrophoresis at 60 volt for 60 minutes. Concentration of isolated plasmid was quantified by nanodrop reading.

### 3.4.2 Genomic DNA extraction

Bacterial DNA was extracted from the TIANGEN DNA secure kit protocol (http://www.tiangen.com/asset/imsupload/up0688479001433140730.pdf) but with slight modification. Three ml of bacterial culture suspension was pipetted in a centrifuge tube and centrifuged for one minute at 10,000 rpm. The supernatant was discarded and to the pellet, 200 µl of Buffer GA (for gram negative bacteria) was added and mixed thoroughly by vortexing. For gram positive bacteria, that step was replaced by lysozyme treatment. For that, 180 µl of enzymatic lysis buffer [(20 mM Tris-cl, pH 8; 2mM sodium EDTA; Triton X-100; lysozyme (final concentration of 20 mg/ml)] was added. Then it was incubated for 30 minutes at 37°C. Lysozyme was prepared with buffer, otherwise it would not be active. Four µl of RNAse (100 mg/ml) was mixed by vortexing for 15 seconds and incubated for 30 minutes at 37°C. After incubation, 2 µl of proteinase K was added, mixed by vortexing and incubated for 25 minutes at 37°C. 220 µl of buffer GB was added to the sample, vortexed for 15 seconds and incubated at 70°C for 10 minutes to yield a homogenous solution. It was then briefly centrifuged to remove the drops from inside the centrifuge cap. 200 µl of absolute ethanol was added to the sample and mixed by vortexing for 10 seconds followed by brief centrifugation. The mixture was pipetted into the spin column $CB_3$ (2ml collection tube) and centrifuged at 12,000 rpm for 30 seconds.

Flow through was discarded and spin column was placed into the collection tube. After that, 500 µl of buffer GD (absolute ethanol had been added) was added to the spin column $CB_3$ and centrifuged at 12,000 rpm for 30 seconds. After flow through was discarded and spin column was replaced into the collection tube, 600 µl of buffer PW (absolute ethanol had been added) was added to the spin column $CB_3$ and centrifuged at 12,000 rpm for 30 seconds. Flow through was discarded and spin column was placed into the collection tube and step was repeated. After that, centrifugation was done at

12,000 rpm for 2 minutes to dry the membrane completely. The spin column $CB_3$ was placed in a new centrifuge tube and 50 µl of TE buffer was added to the center of the membrane. It was then allowed to incubate at room temperature for 10 minutes and centrifuged at 12,000 rpm for 2 minutes. The final eluted supernatant was taken as extracted genomic DNA and the concentration was measured using nanodrop.

### 3.4.3 *cob*A promoters and *lac*Z gene PCR amplification

The genomic DNA samples isolated from of *P. aeruginosa* 27853 and ATCC strain *E. coli* 25923 was used as template DNA for PCR amplification of *cob*A gene promoters (for forward 100, 200, and 600) and *lac*Z gene respectively. PCR amplification was performed by preparing the reaction mixtures as follow and PCR condition was optimized. PCR was performed by using thermal cycler (Cleaver scientific). Then 5 µl PCR products were subjected to 1% agarose gel electrophoresis along with 1 kb ladder DNA (thermo scientific) in the separate well.

**Table 4: Reaction mixtures for *cob*A and *lac*Z gene amplification**

| Components (stock concentration) | Volume (working concentration) |
|---|---|
| Template DNA | 3µl |
| Forward primer (10 µM) | 2.5 µl (1 µM) |
| Reverse primer (10 µM) | 2.5 µl(1 µM) |
| DMSO | 0.8µl (3.2%) |
| Master mix(2X) | 12.5 µl (1X) |
| NFW | 3.7µl (to adjust final volume) |
| Total | 25 µl |

**Table 5: Reaction condition for CobAF100 promoter (2522 bp)**

| Cycle | Steps | Temperature | Time |
|---|---|---|---|
| 1 cycle | Initial denaturation | 95°C | 5 minutes |
| 30 cycle | Denaturation | 95°C | 1 minute |
| | Anealing | 60.6°C | 1 minute |
| | Extension | 72°C | 2 minutes 32 seconds |
| 1 cycle | Final extension | 72°C | 10 minutes |
| | On hold | 4°C | infinite |

**Table 6: Reaction condition for CobAF200 promoter (2645 bp)**

| Cycle | Steps | Temperature | Time |
|---|---|---|---|
| 1 cycle | Initial denaturation | 95°C | 5 minutes |
| 30 cycle | Denaturation | 95°C | 1 minute |
| | Anealing | 61.2°C | 1 minute |
| | Extension | 72°C | 2 minutes 39 seconds |
| 1 cycle | Final extension | 72°C | 10 minutes |
| | On hold | 4°C | infinite |

**Table 7: Reaction condition for CobAF600 promoter (3058 bp)**

| Cycle | Steps | Temperature | Time |
|---|---|---|---|
| 1 cycle | Initial denaturation | 95°C | 5 minutes |
| 30 cycle | Denaturation | 95°C | 1 minute |
| | Anealing | 62.9°C | 1 minute |
| | Extension | 72°C | 3 minutes 35 seconds |
| 1 cycle | Final extension | 72°C | 10 minutes |
| | On hold | 4°C | Infinite |

**Table 8: Reaction condition for *lac*Z gene (3045 bp)**

| Cycle | Steps | Temperature | Time |
|---|---|---|---|
| 1 cycle | Initial denaturation | 95°C | 5 minutes |
| 30 cycle | Denaturation | 95°C | 1 minute |
| | Anealing | 46°C | 1 minute |
| | Extension | 72°C | 3 minutes 45 seconds |
| 1 cycle | Final extension | 72°C | 10 minutes |
| | | | |
| | On hold | 4°C | Infinite |

### 3.4.4 PCR product purification

After confirmation of correct amplicon size from gel electrophoresis, PCR product was purified by FORGENE KIT METHOD. Firstly, the volume of PCR reaction solution was estimated. To recover the DNA fragments between 2000 bp and 10,000 bp, four times the buffer BD to that of PCR product was added, one times buffer BD-S was added and mixed. However, if the DNA fragments size is less than 2000 bp, only the buffer BD (after addition of isopropanol) is added. Then the solution was transferred to the spin column (DNA only column) and centrifuged at 12,000 rpm for 1 minute was done after holding the mixture for 1 minute at room temperature. The flow through was discarded and to the spin column, 700µl of buffer WB1 was added, held for 1 minute and centrifuged at 12,000 rpm for 1 minute. The flow through was discarded, the step was repeated once and was centrifuged at 12,000 rpm for 2 minutes to spin down excess buffer from the membrane. The spin column was then placed to the new centrifuge tube and 30-50 µl buffer EB (Nuclease free water) was added to the silica gel membrane. It was then kept at room temperature for 2 minute and centrifuged at 12,000 rpm for 1 minute. The final eluted supernatant was taken as PCR product and was used for restriction digestion.

### 3.4.5 Preparation of *E.coli* DH5α competent cells (calcium chloride method)

First, the bacterial strain was revived by streaking on LB agar plate and incubated overnight at 37°C. After incubation, a single isolated colony was inoculated in 5 ml LB broth and incubated at 37°C for 18 hours. Then 500 µl of overnight culture was inoculated in 50 ml of LB broth in 100 ml conical flask and incubated at 37°C at 200 rpm till the $O.D._{600}$ reached 0.4. The bacterial culture was then aliquoted in two chilled falcon tubes and chilled in ice for 10 minutes. Then centrifugation was done at 4000 rpm for 10 minutes to harvest the cells and the supernatant was discarded. Pellet was washed with autoclaved chilled distilled water. The pellet was then re-suspended in 30 ml solution of mixture of chilled 80mM magnesium chloride and 20mM calcium chloride. Suspension was then centrifuged at 4000 rpm for 10 minutes and pellet was re-suspended in 1 ml 100 mM calcium chloride. 200 µl suspensions were then aliquoted each in fresh eppendorf tube and used for transformation by heat shock or stored at -40°C in 20% glycerol until use.

#### 3.4.5.1 Heat shock

200 µl of competent cell was taken in a fresh eppendorf tube and 1 µl plasmid (pUC19) was mixed with it. The mixture was chilled in ice for 30 minutes. The tube was then placed in pre-heated water bath for heat shock at 42°C for 90 seconds. Immediately, the tube was then transferred to the ice slurry and kept for 5 minutes. After that 1 ml of LB

media was added in the tube and incubated at 37°C with 200 rpm agitation for 1 hour. The tube was then centrifuged at 10,000 rpm for 1 minute and supernatant was discarded. The pellet was suspended in 100 µl of fresh LB medium and plated on LB agar plate supplemented with 100 µg/ml ampicillin. The plates were then incubated overnight at 37°C.

### 3.4.6 Restriction digestion for pUC19 vector

Plasmid pUC19 as vector was used to clone the *cob*A promoter and restriction digested with enzymes EcoRI and BamHI as given below.

Restriction digestion of vector

| | |
|---|---|
| Vector | 30 µl |
| EcoRI (2U/ µl) | 1 µl |
| BamHI (3U/ µl) | 1 µl |
| Buffer K (10X) | 5 µl |
| NFW | 13 µl |
| Total | 50 µl |

### 3.4.7 Restriction digestion of insert (CobAF100, CobAF200, CobAF600)

| | |
|---|---|
| Purified *cob*A gene | 20 µl |
| EcoRI (2U/ µl) | 0.5 µl |
| BamHI (3U/ µl) | 0.5 µl |
| Buffer K (10X) | 3 µl |
| NFW | 6 µl |
| Total | 30 µl |

The reaction mixture was incubated at 37°C for 3 hours in water bath and then to 65°C for 20 minutes in order to inactivate the enzyme and stored at -20°C.

For the recovery of the digested vector and insert, both digested vector and insert were subjected to the 0.8% low melting agarose along with 1 Kb ladder. It was then allowed to run for 60 minutes at 70V. The gel was visualized under UV trans-illuminator (254nm) and the bands of insert and vector of desired size were cut out and stored in fresh centrifuge tube for further experiments.

### Gel elution for insert and vector purification from low melting agarose gel

Bands of insert and vector seen on required size were cut out with scalpel and placed in fresh centrifuge tube. Weights of the gel slices were taken and it was used for elution of insert and vector using the kit.

### 3.4.8 Restriction digested product purification from QIA quick® Gel Extraction Kit

Restriction digested insert and vector DNA were extracted from QIA quick® Gel Extraction Kit (Cat No. 28704). After the weight of gel piece was calculated, 3 volume of buffer QG was added to 1 volume of gel (100 mg nearly equals to 100 µl). The mixture was heated at 50°C for 10 minutes. During the time, tube was vortexed every 2-3 minutes to help dissolve gel. Then, 1 gel volume of isopropanol was mixed and sample was applied to spin column having collection tube and centrifuged at 13,000 rpm for 1 minute. The flow was discarded and column was placed to the same tube. Buffer PE (750 µl) was added to the spin column and left at room temperature for 5 minutes. It was then centrifuged at 13,000 rpm for 1 minute and column was placed to the same tube after discarding the flow through. The spin column was the re centrifuged at 13,000 rpm for 1 minute and the remaining buffer was discarded from the collection tube. Spin column was moved to the new centrifuge tube and 30 µl of NFW was added to the middle of QIA quick membrane. It was then incubated at RT for 5 minutes and centrifuged for 1 minute at 13,000 rpm. The final eluted supernatant was taken as purified digested product and used for ligation reaction.

### 3.4.9 Gel electrophoresis for quantification of insert (CobAF100, CobAF200, CobAF600) and vector (pUC19)

The product eluted after the purification was then visualized in 1% agarose gel electrophoresis along with 1 Kb DNA ladder for calibration and quantification. Quantification is based on the intensity of the band with respect to the ladder bands.

The concentration of the digested vector and the insert was estimated based on the intensity of the band observed.

### 3.4.9.1 Ligation

DNA insert and plasmid vector i.e., CobAF100, CobAF200, CobAF600 gene and pUC19 vector prepared from double digestion were subjected to the ligation activated by T4 DNA ligase enzyme. Ligation mixture was prepared as follows

**Ligation mixture for CobAF100 and pUC19**

| | |
|---|---|
| T4 ligation buffer (10X) | 2 µl (1X) |
| Insert (CobAF100 ) | 9 µl |
| Vector (pUC19) | 3 µl |
| T4 DNA Ligase | 0.5 µl |
| NFW | 5.5 µl (To adjust final volume) |
| Total | 20 µl |

**Ligation mixture for CobAF200 and pUC19**

| | |
|---|---|
| T4 ligation buffer (10X) | 2 µl (1X) |
| Insert (CobAF200 ) | 12 µl |
| Vector (pUC19) | 3 µl |
| T4 DNA Ligase | 0.5 µl |
| NFW | 2.5 µl (To adjust final volume) |
| Total | 20 µl |

**Ligation mixture for CobAF600 and pUC19**

| | |
|---|---|
| T4 ligation buffer (10X) | 2 µl (1X) |
| Insert (CobAF600) | 4 µl |
| Vector (pUC19) | 8 µl |
| T4 DNA Ligase | 0.5 µl |
| NFW | 5.5 µl (To adjust final volume) |
| Total | 20 µl |

### 3.4.9.2 Transformation after ligation

Ligation mixture was incubated overnight at 16°C. After overnight incubation, the mixture was used for transformation into competent *E. coli* DH5α host bacteria by heat shock method. Briefly, 10 µl of ligated product was added to 200 µl of *E. coli* DH5α competent cells. The transformation was performed in duplicate sets.

### 3.4.9.3 Screening of transformants

### Plasmid extraction

Ampicillin resistant transformed colonies grown on the plate were selected and inoculated to 10 ml of LB/ampicillin (50 µg/ml) broth and incubated at 37°C with shaking at 200 rpm. The plasmids were then isolated by alkaline lysis method and it was then further proceed for digestion.

### Restriction digestion

The isolated plasmid was then digested with the restriction enzyme EcoRI to confirm the cloning of *cob*A gene promoter (CobAF100, CobAF200, and CobAF600) to pUC19 vector. The restriction digestion mixture was prepared as follows and incubated at 37°C for 3 hours to allow digestion. The reaction mixture was subjected to 0.8% agarose gel electrophoresis and required size (around 5000 bp) was compared with 1 kb ladder DNA run along with the product on the adjacent wells.

**Restriction digestion with EcoRI**

| | |
|---|---|
| Plasmid | 5 µl |
| EcoRI(2U/µl) | 0.3 µl |
| BSA Buffer (10X) | 1 µl (1X) |
| NFW | 3.7 µl |
| Total | 10 µl |

Then the confirmed cloned transformants was designated as plasmid pAG101, pAP201 and pA501 for the CobAF100, CobAF200 and CobAF600 promoter with the vector respectively. The glycerol stock was also prepared for the respective plasmid and stored at -20°C after proper labeling.

### 3.4.9.4 Confirmation of *cob*A gene promoter cloning by PCR

Cloned plasmid isolated with target gene was confirmed by PCR using the primer set for respective CobAF100, CobAF200 and CobAF600 and CobR. The plasmid was used as a template DNA for the PCR. It was also confirmed as cloned after the required amplicon size was observed. The construct was used as vector plasmid for the *lac*Z gene cloning.

## 3.5 Subcloning of *lac*Z gene into the plasmid construct pAG101, pAP201 and pAP501 to construct expression plasmid pAP103, pAP204 and pAP502

The expression plasmid was constructed by subcloning of *lac*Z gene to construct pAG101, pAP201 and pAP501. The *lac*Z gene which code for β-galactosidase is cloned under these construct and this sub-cloning is carried out by restriction digestion, in which the vector is restriction digested with SpeI and BamHI.

**Restriction digestion of *lacZ* gene and the construct**

| | | | |
|---|---|---|---|
| Insert (*lac*Z) | 30 µl | Vector | 25 µl |
| SpeI (10U/ µl) | 0.5 µl | SpeI (10U/ µl) | 0.5 µl |
| BamHI (3U/ µl) | 0.5 µl | BamHI | 0.5 µl |
| Buffer K (10X) | 5 µl | Buffer K (10X) | 4 µl |
| NFW | 14 µl | NFW | 10 µl |
| Total | 50 µl | Total | 40 µl |

Reaction mixtures were then incubated for 3 hours at 37°C for digestion. Then the tubes were kept at 65°C for 30 minutes to stop the digestion reaction. The mixtures were then subjected to 0.8% low melting agarose gel electrophoresis and the product was then eluted out from gel extraction kit as mentioned above and DNA concentration was also quantified.

## Ligation reaction

Insert DNA (*lac*Z) and the plasmid vector i.e., pAG101, pAP201 and pAP501 prepared from double digestion were subjected to the ligation activated by T4 DNA ligase enzyme. Ligation mixture was prepared as follow

| **Ligation reaction mixture for pAG101 and *lac*Z gene** | | **Ligation reaction for pAP201 and *lac*Zgene** | |
|---|---|---|---|
| Insert | 9 µl | Insert | 6 µl |
| Vector(pAG101) | 3 µl | Vector(pAP201) | 3 µl |
| Ligation buffer | 2 µl | Ligation buffer | 2 µl |
| DNA ligase | 0.5 µl | DNA ligase | 0.3 µl |
| NFW | 5.5 µl | NFW | 8.7 µl |
| Total | 20 µl | Total | 20 µl |

**Ligation reaction for pAP501 and *lac*Z gene**

| | |
|---|---|
| Insert | 9 µl |
| Vector | 4.5 µl |
| Ligation buffer | 2 µl |
| DNA ligase | 0.5 µl |
| NFW | 4 µl |
| Total | 20 µl |

Ligation mixture was incubated overnight at 16°C. After overnight incubation, the mixture was used for transformation into competent *E. coli* DH5α host bacteria by heat shock method. Briefly, 10µl of ligated product was added to 200µl of *E. coli* DH5α competent cells. The transformation was performed in duplicate sets.

## 3.5.1 Confirmation of transformants

### Plasmid extraction

Ampicillin resistant transformed colonies grown on the plate were selected and inoculated to 10 ml of LB/ampicillin (50 µg/ml) broth and incubated at 37°C with shaking at 200 rpm. The plasmids were then isolated by alkaline lysis method and it was then further proceeded for digestion.

### Restriction digestion

The isolated plasmid was then digested with the restriction enzyme SpeI to confirm the cloning of *lac*Z gene to plasmid vector pAG101, pAP201 and pA501. The restriction digestion mixture was prepared as follows and incubated at 37°C for 3 hours to allow digestion. The reaction mixture was subjected to 0.8% agarose gel electrophoresis and required size (around 8000 bp) was compared with 1 kb ladder DNA run along with the product on the adjacent wells.

**Restriction digestion with SpeI**

| | |
|---|---|
| Plasmid | 5 µl |
| SpeI(10U/µl) | 0.3 µl |
| BSA Buffer (10X) | 1 µl (1X) |
| NFW | 3.7 µl |
| Total | 10 µl |

Then the confirmed cloned transformants was designated as plasmid pAG103, pAP204 and pA502. The glycerol stock was also prepared for the respective plasmid and stored at -20°C after proper labeling.

**Confirmation of *lac*Z gene cloning by PCR**

Cloned plasmid isolated with target gene was confirmed by PCR using the primer set for respective lacZ forward and lacZ reverse primer. The plasmid pAG103, pAP204 and pA502 were used as a template DNA for the PCR. It was also confirmed as cloned after the required amplicon size was observed. The construct was used as vector plasmid for the *lac*Z gene cloning.

## 3.6 Transcription analysis

### 3.6.1 RNA preparation

RNA isolation was carried out using TRIzol method. The RNA isolation was done for the expression plasmid pAP103, pAP204, pAP502 and IPTG induced pUC19. All the apparatuses were rinsed in 0.1N NAOH and in RNAse free water. Working bench was also rinsed with RNAse ZAP to remove contaminant RNA. For the isolation of RNA single colony was transferred to LB broth containing ampicillin antibiotic and incubated at 37°C for 6 hours. After that, 1.5 ml of culture broth was centrifuged at 12,000 rpm for 2

minutes and the supernatant was discarded. To the pellet 1 ml of TRIzol was added and homogenized by pipetting. It was then kept at room temperature for 5 minutes. Then 200 µl of chloroform was added to the mixture and was followed by ice incubation for 15 minutes. It was then centrifuged at 12,000 rpm at 4°C for 15 minutes. The mixture then separates into red organic phase containing protein, in interphase containing DNA and colorless aqueous phase containing RNA. The aqueous phase containing RNA was transferred to fresh tube and the RNA was precipitated by adding 500 µl of isopropanol. The tube was then incubated on ice for 10 minutes and centrifuged at 12,000 rpm at 4°C for 10 minutes. The supernatant was removed, RNA pellet was washed with 1 ml of 75% ethanol by flicking, then centrifuged at 7,500 rpm for 10 minutes at 4°C. The supernatant was removed, air dried and dissolved in 40 µl of RNAse free water, mixed by pipetting and stored at -80°C.

### 3.6.2 cDNA synthesis by Reverse-transcriptase PCR

The first strand cDNA synthesis from isolated RNA was performed by using 5X iScript$^{TM}$ cDNA synthesis kit, Bio-Rad. The reaction mixture was prepared on the basis of the table below.

Table (9): Reaction mixture for cDNA synthesis

| Component | Volume per reaction, µl |
|---|---|
| 5X iScript reaction mix | 2 µl |
| iScript reverse transcriptase | 0.5 µl |
| Nuclease free water | 2.5 µl |
| RNA template | 5 µl |
| Total | 10 µl |

Thus, the prepared cDNA synthesis reaction mixture was subjected to PCR in following condition.

Table (10): Reaction condition for cDNA synthesis

| | | |
|---|---|---|
| Priming | 25$^{o}$C | 5 minutes |
| Reverse transcription | 46$^{o}$C | 20 minutes |
| RT inactivation | 95$^{o}$C | 1 minute |
| Optimal step | 4$^{o}$C | Hold |

The synthesized single stranded cDNA was stored at -20$^{o}$C till its use.

### 3.6.3 PCR amplification of cDNA

The double stranded DNA from single stranded cDNA was achieved by PCR using a set of *lac*Z gene specific primer and cDNA. The PCR mixture was prepared as

Table (11): Second strand synthesis using cDNA template

| **Reagents** | **Volume (µl)** |
|---|---|
| 2X Master Mix | 5 µl |
| *lac*Z forward | 1.5 µl |
| *lac*Z reverse | 1.5 µl |
| cDNA | 1.5 µl |
| Nuclease free water | 0.5 µl |
| Total | 10 µl |

## 3.7 Beta-galactosidase assay

β-galactosidase assay was done by protocol described by J.H. Miller in "Experiments in Molecular Genetics" with slight modification. The protocol determines the amount of β-gal with the O-nitrophenyl-β-galactoside (ONPG).

The expression vector culture and the pUC19 vector were incubated overnight at 37$^{o}$C with the ampicillin (50 µg/ml). The overnight culture was diluted in 1:100 ratios in fresh medium. The culture was then allowed to grow for 3 hours at 37$^{o}$C at 200 rpm. The solutions required for assay (described in appendix) was prepared. After 3 hours,

Isopropyl β-d-1-thiogalactopyranoside (IPTG) (1mM) was added to the pUC19 culture for the induction of inducible for *lac* promoter. The cells were then allowed to grow for 2 hours and then the cells were kept at ice for 20 minutes, to stop the growth. The 200 µl sample was taken in a test tube to which 1.8 ml of assay buffer and 30 µl of chloroform was added. The assay buffer contains SDS and chloroform which helps in the cell lysis for the extraction of intracellular cytosolic components for the activity. The mixture was then subjected to vortexing for 10 seconds and kept in water bath at 28$^{o}$C for 5 minutes. The reaction was then started by adding 200 µl of ONPG solution (4mg/ml) to the solution and reaction time was noted. The solution was then kept for 30 minutes at 28$^{o}$C and 1 ml of stop solution was added to the solution to stop the reaction.

The assay was quantified spectrophotometrically. The optical density at 420 nm, 550 nm and 600 nm was taken and the units of activity was calculated using the below formula (https://openwetware.org/wiki/Beta-Galactosidase_Assay_(A_better_Miller))

1 Miller Unit = $1000 * (Abs_{420} – (1.75*Abs_{550}))/ (t*v*Abs_{600})$

Where

$Abs_{420}$ = absorbance of the yellow O-nitrophenol

$Abs_{550}$ = absorbance of scatter from cell debris, which when multiplies by 1.75 approximates the scatter observed at 420nm

t = reaction time in minutes

v = volume of culture assayed in millimeter

$Abs_{600}$ = cell density of the culture

## 3.8 Protein quantification

Protein quantification was done by spectrophotometric analysis by phenol-folin reagent (Lowry's method) using Bovine serum albumin (BSA) as standard. Standard solution of different concentration was prepared. The reagent was prepared (appendix) and the measurement was done.

### 3.8.1 Standard preparation

Stock solution of standard protein, BSA, 2 mg/ml was prepared and different concentration of standard solution was then prepared from the stock solution described in the following table (12).

Table (12): Standard solution of different concentration

| S.N | Volume of BSA (µl) | Volume of distilled water (µl) | Concentration (µg/ml) |
|---|---|---|---|
| 1 | 20 | 980 | 20 |
| 2 | 40 | 960 | 40 |
| 3 | 60 | 940 | 60 |
| 4 | 80 | 920 | 80 |
| 5 | 100 | 900 | 100 |
| 6 | 200 | 800 | 200 |
| 7 | 300 | 700 | 300 |
| 8 | 400 | 600 | 400 |
| 9 | 500 | 500 | 500 |

To 100 µl of the standard solution of different concentration and test sample, 4.5 ml of alkaline $CuSo_4$ reagent was added and was incubated at room temperature for 10 minutes. Then 0.5 ml of folin's phenol reagent was added and the mixture was mixed rapidly and allowed to incubate at room temperature for 30 minutes. The spectrophotometric reading was taken at 660 nm and blank was set with distilled water. From the standard graph the amount of protein in the given unknown test sample was calculated.

# 4. RESULTS AND DISCUSSION

In previous work antimicrobial extracts from screened *Streptomyces* spswith modified culture medium had shown inhibitory effects on the multidrug resistance bacteria (Sita Ghimire thesis, 2019). Despite knowing that the extracts have bactericidal effect, the mode of action of those extracts against these bacteria was unknown. The media was modified based on computational works where indole and kinase inhibitors were screened as competitive inhibitors for *Salmonella typhimurium* CobA protein. This suggested that the raw materials used could have been probably modified in possible structure that could potentially mimic the computational works. Since CobA protein uses *S*-adenosyl methionine (SAM) (Warren *et. al.,* 1990) and SAM is known to exhibit riboswitch effect (Batey, 2011) it was presumed that c*ob*A mRNA could possess riboswitch structure and ligand effect could be studied because it has long 5'-UTR mRNA (data not shown; https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP007224.1).

In recent publication, *cob*A gene of *Propionicbacterium* strain UF1 has vitamin $B_{12}$ riboswitch (Li *et. al.,* 2020). Although, this is in contrast to our speculation of SAM riboswitch but our assumption of probable riboswitch structure at 5'-UTR region has been validated. Despite the fact that bacteria have vitamin $B_{12}$ symporters but *cob*A gene has been found to be essential (Tha *et. al.,* 2020) suggesting it is critical target to develop drug. Thus, hypothesis was that the drug developed through modification of media could act at protein and mRNA level.

This leads us to develop the hypothesis that the vitamin B12 synthesis may be a new target as mode of action by antimicrobials. Vitamin $B_{12}$is an important cofactor which is synthesized by the bacteria but are not produced by humans (Piao *et. al.,* 2004) so it can be a possible target without significant impact to human. The *cob*A gene codes for SAM-dependent Uroporphyrinogen III methyl transferase (EC=2.1.1.107), the key enzyme of cobalamin (vitamin B12) biosynthesis, which catalyzes the transformation of uroporphyrinogen III into precorrin-2. It is the first enzyme committed to the corrin ring contraction in cobalamin biosynthesis (Vevodova *et. al.,* 2004).

*cob*A gene is SAM utilizing gene and has long 5' untranslated region (UTR), WHO prioritized pathogen *Pseudomonas aeruginosa* was taken as test organism. Since, the 5' UTR is the sequences directly upstream of the translation start codon and has important regulatory function (Gu *et. al.,* 2014), targeting this region could lead to instability in peptide synthesis. Thus, the promoter region along with mRNA 5'-UTR with ribosome binding site (RBS) were chosen and cloning strategy was developed. To our knowledge, there is no report on upstream nucleotide requirement for functional promoter activity in transcribing *cob*A gene of *P. aeruginosa* and three sets of different primers were

designed to amplify different sizes of amplicon upstream of transcription start site (TSS) and 5' UTR to elucidate upstream nucleotide requirement. The primer was designed for *Pseudomonas aeruginosa* isolate PA96 (https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP007224.1) *cob*A gene as forward primers that was either 100 bp or 200 bp or 600 bp upstream of the TATA box ( -10 from TSS) and *lac*Z gene was cloned downstream the promoter region at start codon site. The functional β-galactosidase activity of LacZ protein as reporter assay would allow determining the gene expression under the cloned promoter region.

## 4.1 Cloning of different length 5'UTR upstream region of *cob*A gene promoter (CobAF100, CobAF200 and CobAF600) in pUC19 plasmid to construct pAG101, pAP201 and pAP501

### 4.1.1 PCR amplification of different sized upstream promoter region with 5'-UTR of *P. aeruginosa* 27583

Three different 5'-upstream nucleotide region from TSS were selected for forward primer that had EcoRI restriction recognition site at 5'-end of the primer. The reverse primer was designed in such a way that it had BamHI at 3'-end flanked by 3 nucleotides on both sides followed by SpeI site 5'-nucleotide upstream of ribosome binding site (RBS) to amplify native RBS of the *cob*A gene along with start codon ATG in complimentary sequence. This included native 5' UTR region downstream of promoter region. The PCR product, on comparing with 1 kb Thermo Scientific DNA ladder, gave a clear band corresponding to around 2522 bp, 2645 bp and 3058 bp amplicons, respectively, (Figure 16) after 1% agarose gel electrophoresis. This DNA band was similar to nucleotide size as calculated, suggesting that the amplification was done successfully.

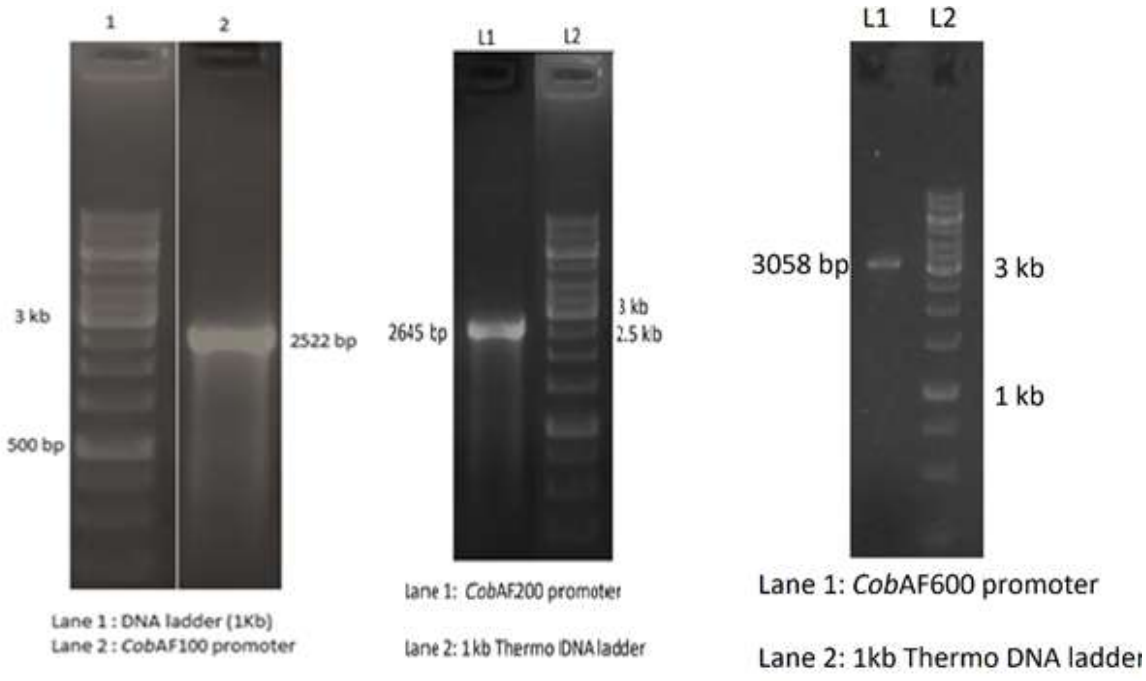



Figure (16): The PCR products of different sized upstream region of promoter after electrophoresis in 1 % agarose gel. The first figure (left) shows the PCR amplicon for the 100 bp upstream region of promoter with size 2522 bp. Similarly in the second figure (middle) shows the 2645 bp amplicon for the 200 bp upstream region of promoter and the third figure (left) shows the 3058 bp amplicon for the 600 bp upstream region of promoter.

### 4.1.2 Isolation of pUC19 plasmid and gel quantification

A large number of cloning vectors are available and the choice of vector selection depends on number of factors, such as the size of the insert, copy number and cloning method. One of the most commonly used and standard cloning vectors is plasmids (Casali N and Preston A, 2003).

As the vector varies in the copy number, host specificity, restriction enzymes recognition sites in the multiple cloning sites (MCS), the purpose of the study and the need of constitutive or inducible promoter are determining factors for choosing suitable vector for the cloning strategy. For our study, pUC19 was selected as cloning vector because of its high copy number with pBR322 based, ColE1 or pMB1 (mutant), origin of replication and is useful as it produces greater yield of recombinant plasmid for subsequent manipulation (Casali N and Preston A, 2003).The selected vector has α-complement unit of *lac*Z gene but it gets lost when the gene of interest is cloned in the MCS for helping in for final construct development as β-galactosidase reporter assay. In addition, *E. coli* DH5α is suitable host to transform this protein for mentioned reporter assay.

The plasmid was isolated using alkaline extraction method as described (materials and methods) and the plasmid was confirmed by single restriction digestion by BamHI enzyme as the restriction enzyme recognition site for this enzyme is present in the vector. The isolated plasmid after digestion with the restriction enzyme BamHI was confirmed by electrophoresis and visualization in 0.8% agarose gel. The digested product was then run along with undigested plasmid during gel electrophoresis. The single band of digested plasmidcorresponding to 2.6 kb upon comparing with the standard known Thermo Scientific DNA ladder (Figure 17) confirmed that it is pUC19.

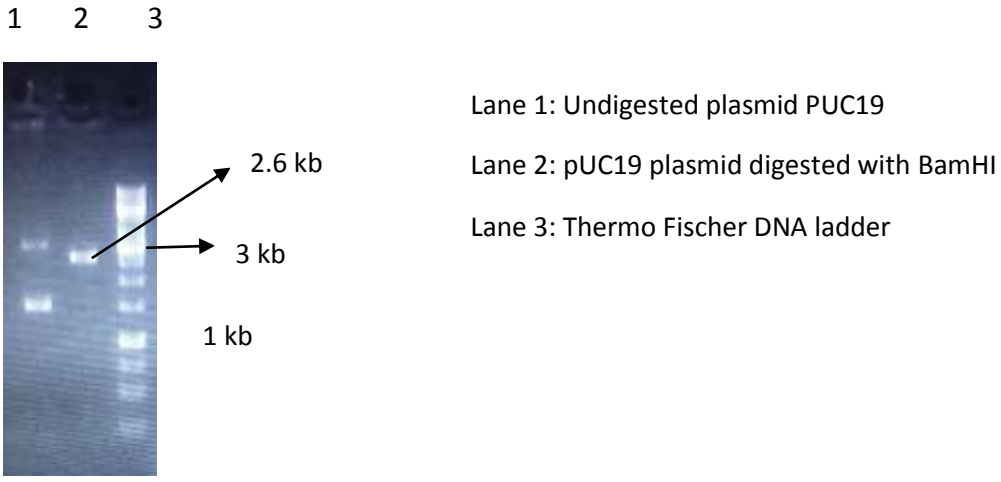



Figure (17): The gel electrophoresis for undigested plasmid and restriction digested plasmid DNA (2.6 kb) along with the DNA ladder

### 4.1.3 Restriction digestion of insert CobAF100, CobAF200 and CobAF600 and pUC19 plasmid

Restriction endonuclease is key enzyme in molecular cloning which has specific restriction recognition sites for its action. The restriction enzyme generates fragment either with blunt end or with sticky ends which could be sub-cloned in cloning or expression vector (Nathans and Smith, 1975). In the present study, the choice of restriction enzyme was done on the basis of the gene under study and the available vector used for cloning. The restriction enzyme which does not show any restriction sites in the nucleotide region of the expected PCR amplicon. The enzyme EcoRI and BamHI was chosen as both were compatible to use for our vector and the unique restriction enzyme site SpeI was created in the revere primer of the *cob*A gene promoter. It was done to aid in the sub-cloning of the *lac*Z gene in the expression vector constructed that helps in the confirmation of the expression analysis to study promoter function.

The PCR product for the CobAF100 and CobAF200 and CobAF600 with their respective amplicon sizes were purified and double digested with restriction enzymes EcoRI and BamHI. The restriction digested product, CobAF100, CobAF200 and CobAF600 promoter

and pUC19 vector digested with EcoRI and BamHI, were electrophoresed and their respective quantities were calculated by visualization in 1% agarose gel. The sequences between the restriction sites used for the digestion in the vector was only 21 bp, similarly the amplicon length that was cut after the digestion was 9 bp, so, the small fragment of digested products was not visualized after agarose gel electrophoresis. The gel extraction helps to prevent the re-ligation of the digested fragments in the vector and it also helps to prevent the effect of unused dNTPs, restriction enzymes and buffer used in the restriction digestion. The concentration of inserts and vector were visualized (Figure 18) and subjected to ligation.

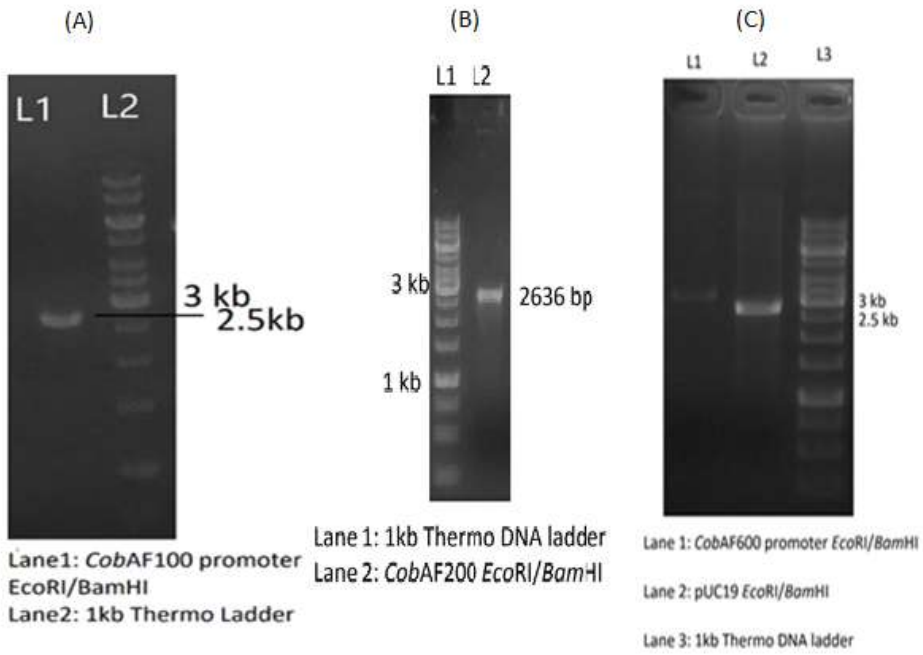



Figure (18): Restriction digested products with enzymes EcoRI and BamHI after electrophoresis in 1% agarose gel for their quantification. (A) Restriction digested product of plasmid pUC19 with 2665 bp. (B) Restriction digested product of 100 bp length promoter with size 2515 bp. (C) Restriction digested product of 200 bp length promoter with size 2636 bp. D. Restriction digested product of 600 bp length promoter with size 3049 bp.

### 4.1.4 Ligation and Transformation

*E.coli* is the most preferable host for expression of heterogenous and non-heterogenous proteins by recombinant DNA technology due to its simplicity, inexpensive cost and rapid high-density cultivation, well known genetics and large number of compatible molecular tools available (Fakruddin *et. al.,* 2013). *E.coli* is also chosen as host organism for *in vivo* DNA assembly as it has various strength including faster growth rates, higher

plasmid yields and greater transformation efficiency. *E.coli* DH5α competent cell is a versatile strain used for general cloning and sub-cloning. It is *E.coli* K-12 derivative used to maintain and amplify plasmid DNA and is one of the most common laboratory *E.coli* strain. *E.coli* DH5α as competent cells is used in DNA assembly and cloning procedures where the transformation of the constructed plasmid is done (Kostylev *et. al.,* 2015), as it has distinct characteristics due to its multiple mutations.

The purified vector and inserts (CobAF100, CobAF200, and CobAF600) were ligated by using T4 DNA ligase enzyme. Then inserts and vector DNA were ligated based on the formula described in materials and methods. After incubation, the ligation mixture was subjected to transformation in competent *E. coli* DH5α using heat shock method. The overnight incubated ampicillin resistant transformants were selected after plating on LBA-Amp (100µg/ml) plate (Figure19) as newly cloned vector plasmid will have ampicillin resistant gene marker intact.

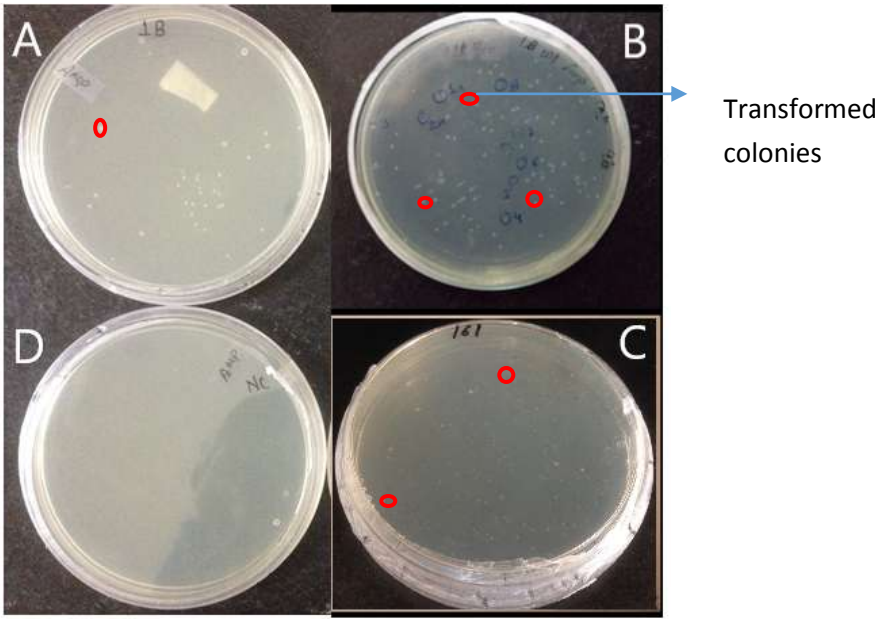



Figure (19): Plate of *E.coli* DH5αt ransformants in LBA plates with ampicillin. (A) Plate with ligation mixture (pUC19+CobAF100), (B) Plate with ligation mixture (pUC19+CobAF200), (C) Plate with ligation mixture (pUC19+CobAF600), and (D) Negative control plate. The transformed colonies were selected as shown in the figure B.

### 4.1.5 Confirmation of cloning by restriction digestion of positive transformants

Cloning of inserts into pUC19 vector was confirmed by restriction map analysis of isolated plasmids from transformants. Plasmid DNA isolated from positive clones was

subjected to restriction digestion with enzyme EcoRI. The linearized plasmid showed 5.2 kb DNA band for CobAF100 promoter, 5.3 kb DNA band for CobAF200 promoter and 5.7 kb DNA band for CobAF600 promoter. The different band size is due to the difference of the base pair length for inserts as the inserts are within differences of 100 bp, 200 bp and 600 bp from the transcription initiation site (the amplicon consists 2403 bp long 5'-UTR mRNA). Thus, the band size is as a result of corresponding cumulative base pair of amplicon size and pUC19. This indicated that the promoter along with 5'-UTR mRNA region was successfully cloned in the vector and the newly developed plasmid was designated as plasmid pAG101, pAP201, and pAP501 for CobAF100, CobAF200, and CobAF600, respectively (Figure 20). Alongside the restriction digestion confirmation, the cloning was also confirmed with PCR using the primers used for respective gene to be cloned. The overall schematic strategy of development of the plasmids is illustrated in Figure 21.

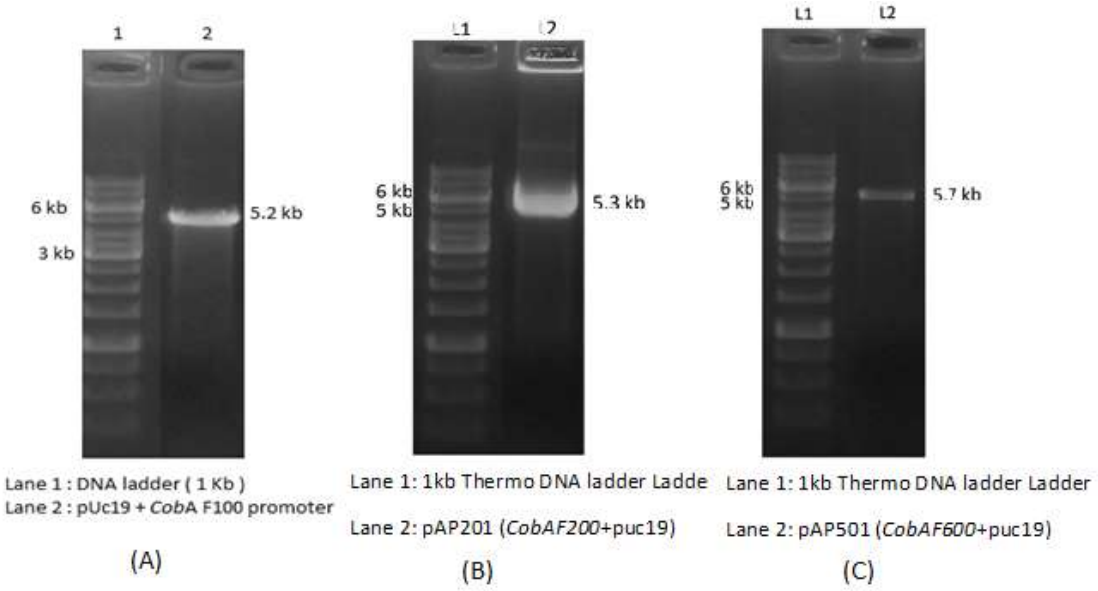



Figure (20): Agarose gel electrophoresis (0.8%) for restriction digestion of plasmids to confirm cloning of inserts in pUC19 vector. (A)Restriction digestion of plasmid pAG101 with size 5.2 kb, (B) Restriction digestion of plasmid pAP201 with size 5.3 kb, and (C) Restriction digestion of plasmid pAP501 with size 5.7 kb

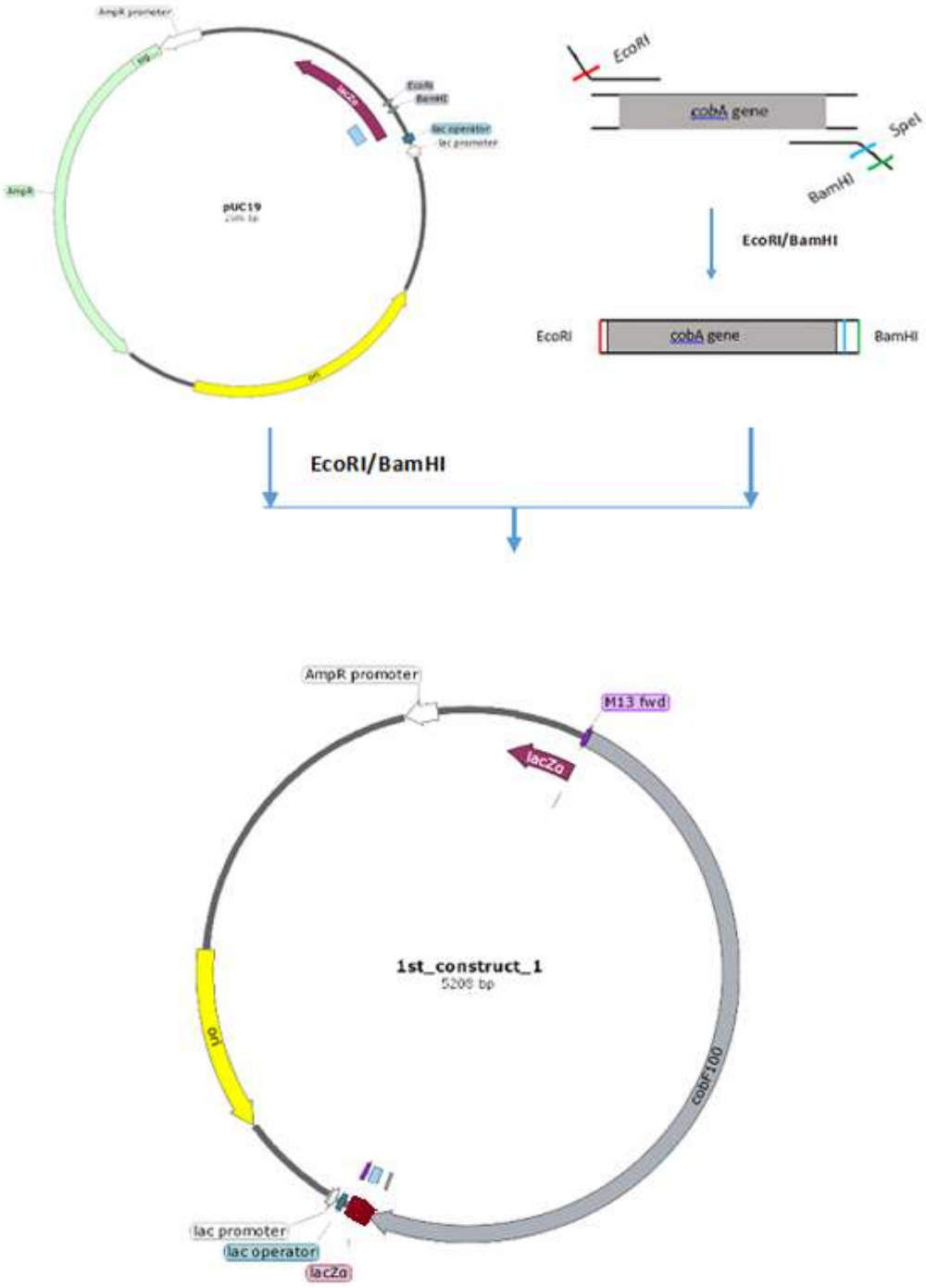



Figure (21): Schematic diagram of cloning of promoter along with its 5'UTR in plasmid vector pUC19 to construct the expression plasmid pAG101

## 4.2 Construction of pAP103, pAP204 and pAP502 plasmid for the expression of *lacZ* gene under *P. aeruginosa cob*A gene promoter

### 4.2.1 PCR amplification of *lacZ* gene

Reporter genes are linked to other sequences to make the reporter protein or the reporter protein itself is fused to another protein known as fusion protein. Most of the reporter genes are accommodated downstream of the promoter region but that is closed towards the gene which is under study for simultaneous expression of these genes (Debnath *et.al.,* 2010). In our study, *lac*Z gene was used as reporter gene to study the promoter activity and the gene expression regulation.

The PCR product gave a clear band corresponding to 3075 bp amplicon on comparing with 1 kb Thermo Scientific DNA ladder (Figure 22) after 1% agarose gel electrophoresis. This DNA band was similar to nucleotide size as calculated for the gene, suggesting that the amplification was done successfully.

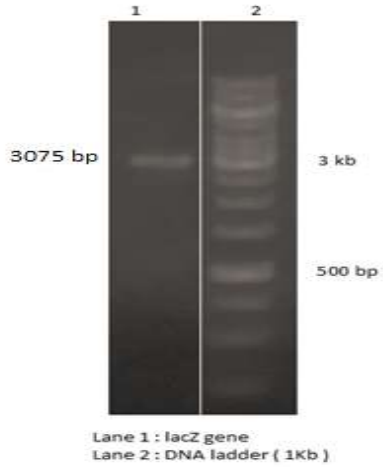



Figure (22): The PCR product amplification of *lac*Z gene with 3075 bp amplicon size shown in 1% agarose gel electrophoresis

### 4.2.2 Restriction digestion of plasmids pAG101, pAP201, pAP501 and *lacZ* gene

For construction of expression plasmid pAP103, pAP204 and pAP502, the plasmid pAG101, pAP201, pAP501 was prepared for subcloning of *lac*Z gene by double digestion with SpeI (restriction recognition site created during PCR and subsequent cloning) and BamHI. Similarly, PCR amplified *lac*Z gene was double digested with SpeI and BamHI. The linearized vector corresponding to 5.2 kb, 5.3 kb and 5.7 kb DNA fragment and 3067 bp of insert were gel purified. It was then empirically quantified by visualization in 0.8% agarose gel (Figure 23, 24 and 25). The vector and insert was subjected to ligation and

after ligation, using heat shock method, the ligation reaction mixture was used to transform competent *E. coli* DH5α prepared by calcium chloride method. The transformants were then selected in selective media containing 100 µg/ml antibiotics.

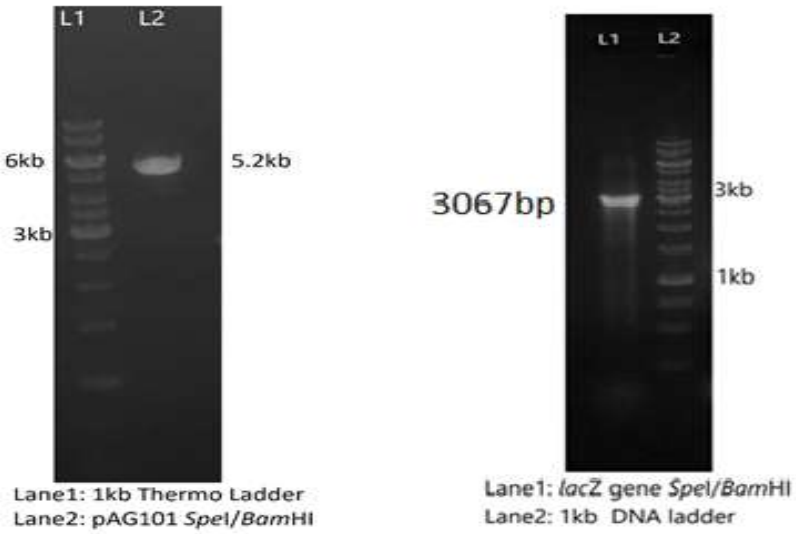



Figure (23): Restriction digested products with enzymes BamHI and SpeI in 1% agarose gel electrophoresis for their quantification. Restriction digested product of plasmid pAG101 with 5.2 kb (left) and Restriction digested product of *lac*Z gene with size 3067 bp (right).

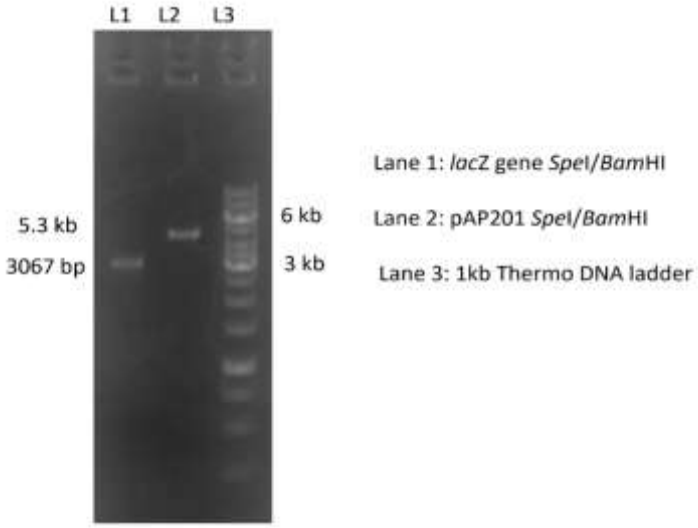



Figure (24): Restriction digestion of plasmid pAP201 and *lac*Z gene with restriction enzymes SpeI and BamHI. Lane (1) shows the restriction digested product of *lac*Z gene wheareas lane (2) shows the restriction digested products of plasmid pAP201.

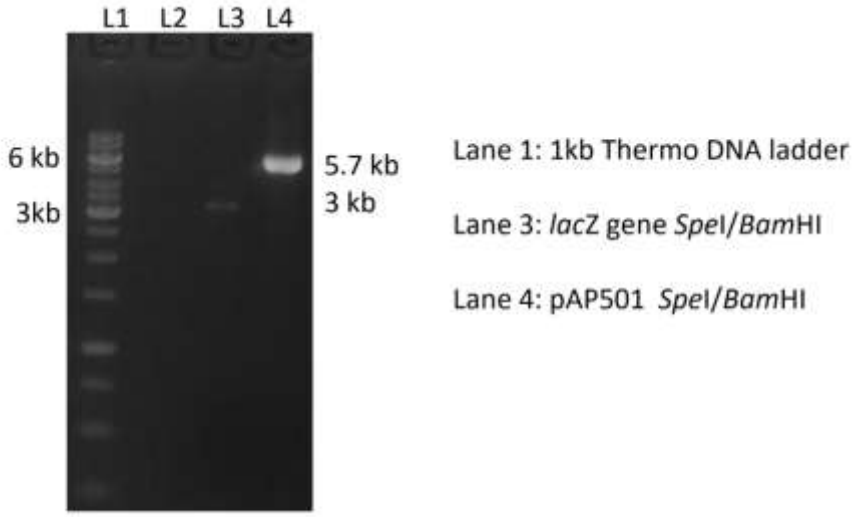



Figure (25): Restriction digestion of plasmid pAP501 and *lac*Z gene with restriction enzymes SpeIand BamHI. Lane (3) shows the restriction digested product of *lac*Z gene wheareas lane (4) shows the restriction digested products of plasmid pAP501

### 4.2.3 Confirmation of cloning of *lacZ* gene in plasmids

Plasmid DNA was isolated from positive clones surviving in ampicillin plate and was confirmed by restriction digestion for insertion of *lac*Z gene in pAG101, pAP204 and pAP502. The linearized plasmids showed 8.2 kb, 8.3 kb and 8.7 kb DNA band respectively (Figure 26) with *Spe*I digestion corresponding to cumulative base pair of pAG101, pAP201, and pAP501 with PCR amplified *lac*Z gene. The restriction digestion analysis indicated that the *lac*Z gene was successfully sub-cloned in plasmids and these newly developed plasmids were designated as plasmid pAP103, pAP204 and pAP502, respectively. Alongside the restriction digestion confirmation, the cloning was also confirmed with PCR using the primers used for respective gene to be cloned. The overall schematic strategy of development of expression vector is illustrated in Figure 27.

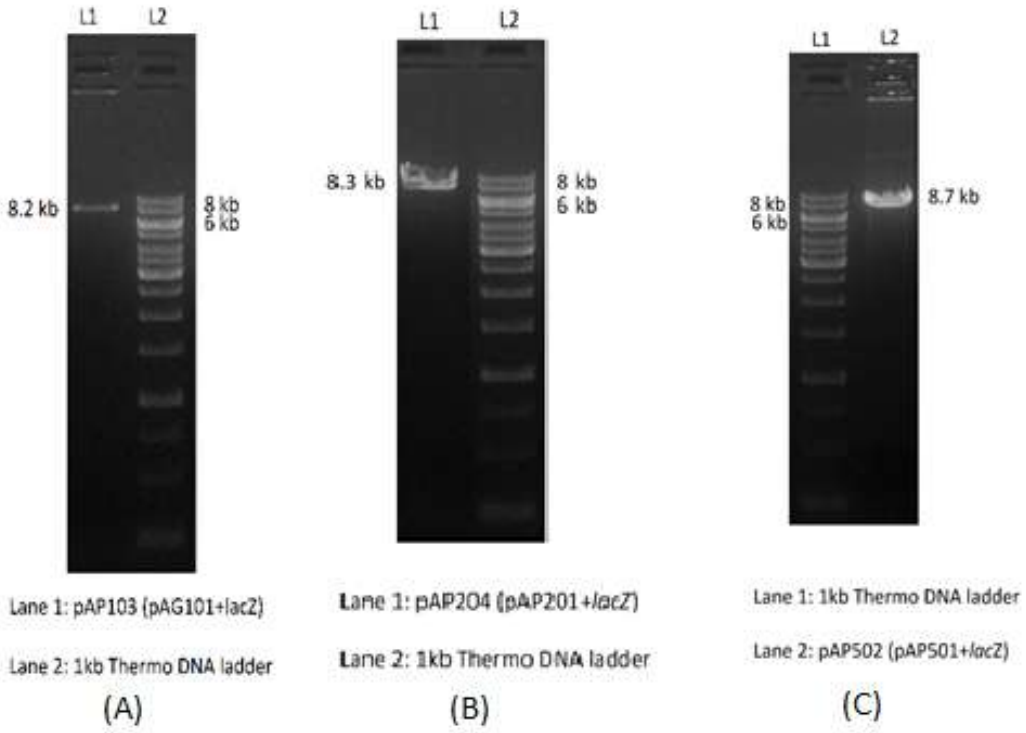



Figure (26): Confirmation of *lac*Z gene cloned in the vector and restriction digestion of positive transformants with enzyme SpeI. The band size for the positive clone in figure (A), (B) and (C) were 8.2 kb, 8.3kb, 8.7 kb for the plasmids pAP103, pAP204, pAP502 respectivley with *lac*Z gene.

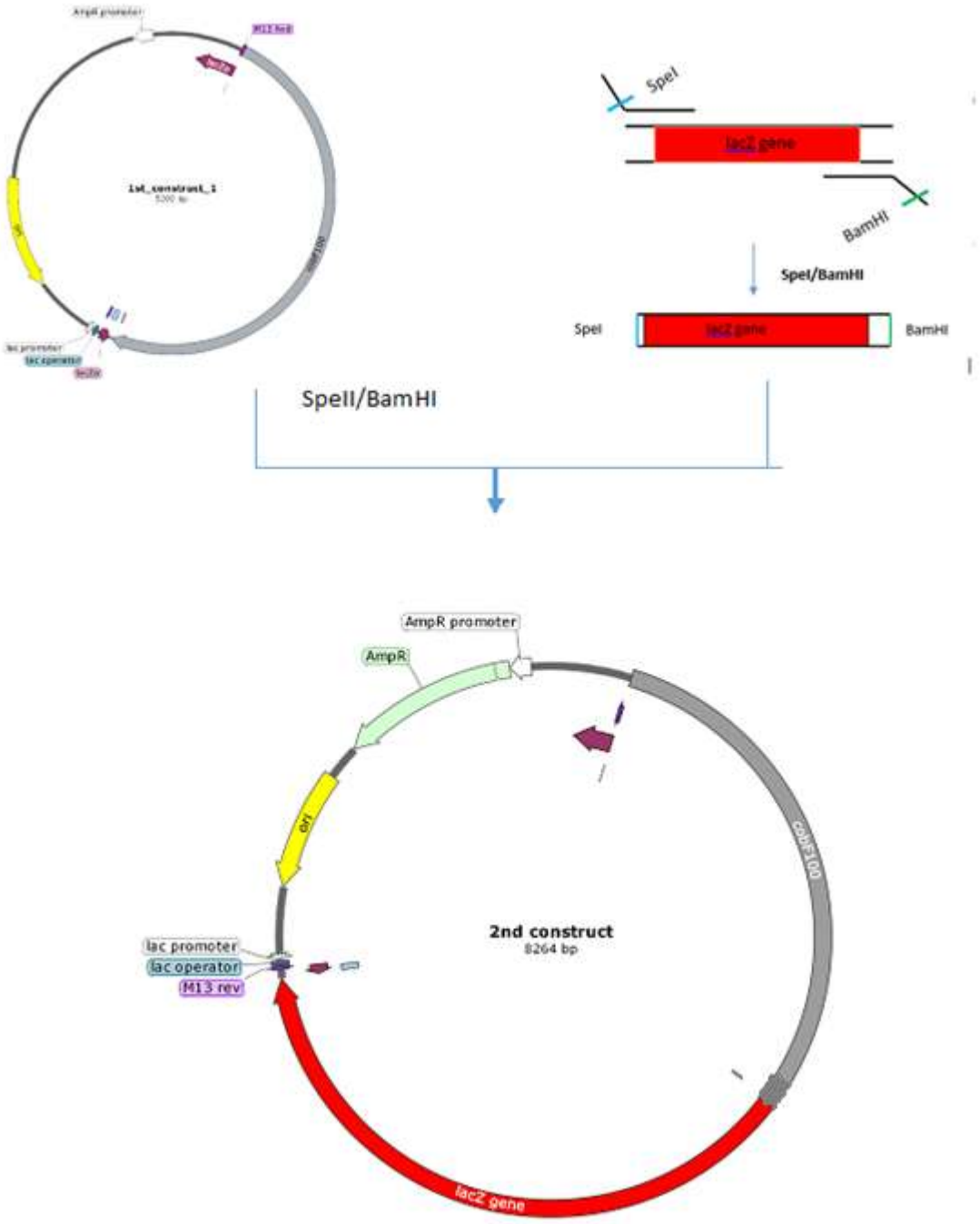



Figure (27): Schematic diagram of cloning of *lac*Z gene in pAG101 (1st construct) to construct the expression plasmid pAP103 (2nd construct).

## 4.3 Gene expression analysis

### 4.3.1 Expression analysis of cloned *lacZ* mRNA by Reverse Transcriptase (RT) – PCR

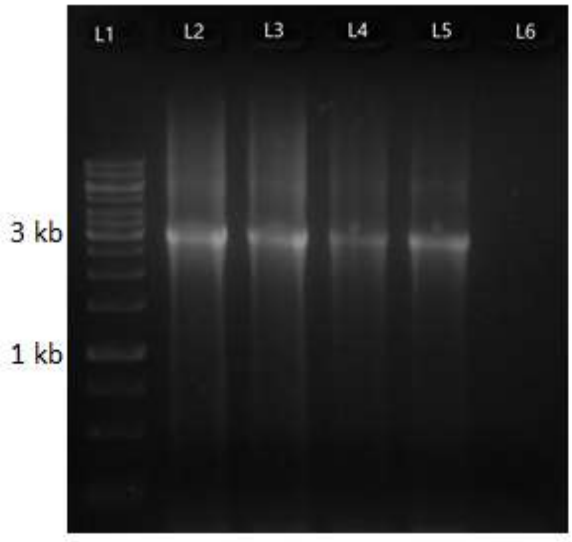



Figure (28): PCR amplification of *lac*Z gene using cDNA as a template in 1% agarose gel electrophoresis. Lane 1: 1 kb Thermo DNA ladder, Lane 2: *lac*Z gene under CobAF100 promoter, Lane 3: *lac*Z gene under CobAF200, Lane 4: *lac*Z gene under CobAF600 promoter, Lane 5: IPTG induced *lac*Z gene in pUC19; and Lane 6: LacZ alpha complementation lacking DH5α as Negative control for mRNA expression

Expression of the gene is simply genetic information present in DNA into protein. According to central dogma of molecular biology, information can pass from nucleic acid to nucleic acid or nucleic acid to protein but it is not possible from protein to nucleic acid or protein to protein (Watson *et. al.,* 2008). In first step of gene expression, transcription of genetic information embedded in DNA into mRNA is required. Strong evidence of gene expression can be determined by tracing mRNA levels in the cell (Zoglowek *et. al.,* 2014). Whether the cloned promoter is functional or not was analyzed by detecting the *lac*Z gene specific mRNA in the developed constructs by RT-PCR after transforming and cellular growth. Since *cob*A gene expression is related to cobalamine biosynthesis (Escalante-Semerena *et. al.,* 1990), the promoter would be functional for *de novo* biosynthesis which would also allow the cloned promoter to respond to the cellular signals.

In the present study, *lacZ* gene mRNA expression is used to study the expression of the gene under the study. *lac*Z gene was cloned under the *cob*A gene along with its three

different putative promoter having different length. In order to confirm that the present model works for the gene expression, mRNA expression was determined for *lac*Z gene cloned in pAP103, pAP204, pAP502. For mRNA expression analysis, total RNA was prepared from the cell harbouring expression plasmids and total RNA was used as the template for cDNA synthesis by RT-PCR . RNA was isolated from the expression plasmids (constructs) by Trizol method as described in materials and methods. Then, cDNA was synthesized by using 5X iScript RT-PCR kit, as per the manufacturer's instruction. Then, using thus generated cDNA as template PCR was carried out using primer pair that was used for amplification of *lac*Z gene from genomic DNA. Upon electrophoretic analysis of PCR product respective band of DNA (3075bp) was found in the test sample (Figure 28). Similarly, RT-PCR of RNA isolated from host strain without expression plasmid was done as the negative control to determine that the RT-PCR product is from cloned gene in expression plasmid. As a positive control RT-PCR of pUC19 with IPTG induced *lac*Z gene (has lac promoter) was done to confirm the expression is of *lac*Z gene. From this mRNA expression analysis, it is validated that the gene is expressed under the promoter present in the study.

## 4.4 Total Protein Estimation

In order to study any protein function in whole cell extracts, total protein of the cells with heterologous expression system has to be calculated. This allows to quantify the protein level in the cell extract and which could then be used to check the total protein expressed from cloned plasmid based on the activity of that particular cloned protein. To check the activity of the cloned LacZ protein the total protein level in the different constructs were conducted.

### 4.4.1 Calibration curve of standard sample

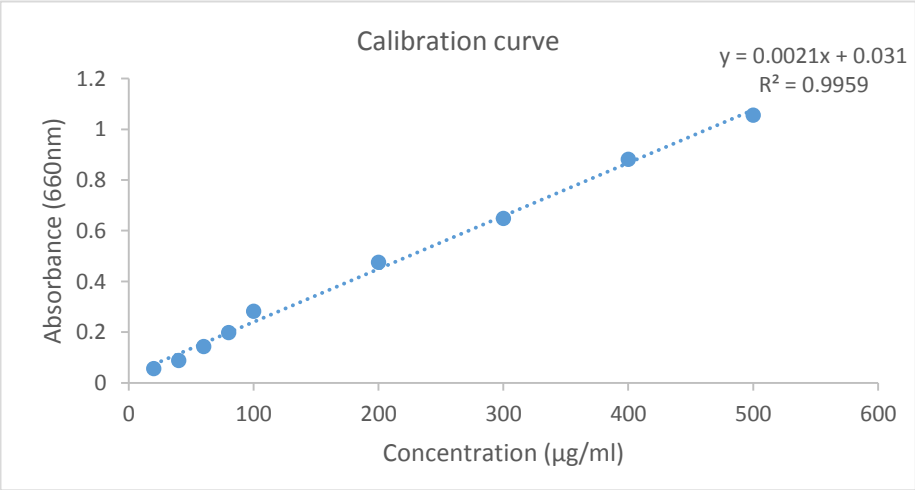



Figure (29): Calibration curve of standard sample (BSA)

Using bovine serum albumin (BSA) the calibration curve was plotted to calculate the total protein from the cultured transformants. From the standard solution prepared above, following calibration curve was prepared by measuring absorbance of standard solutions at 660 nm using Lowry's method. Using the graph as standard the constants 0.0021 and 0.031 were calculated (Figure 29) to determine the X axis (concentration) based on the absorbance at 600 nm in Lowry's method for calculation of concentration of protein in unknown sample was.

### 4.4.2 Total Protein Estimation of different transformants.

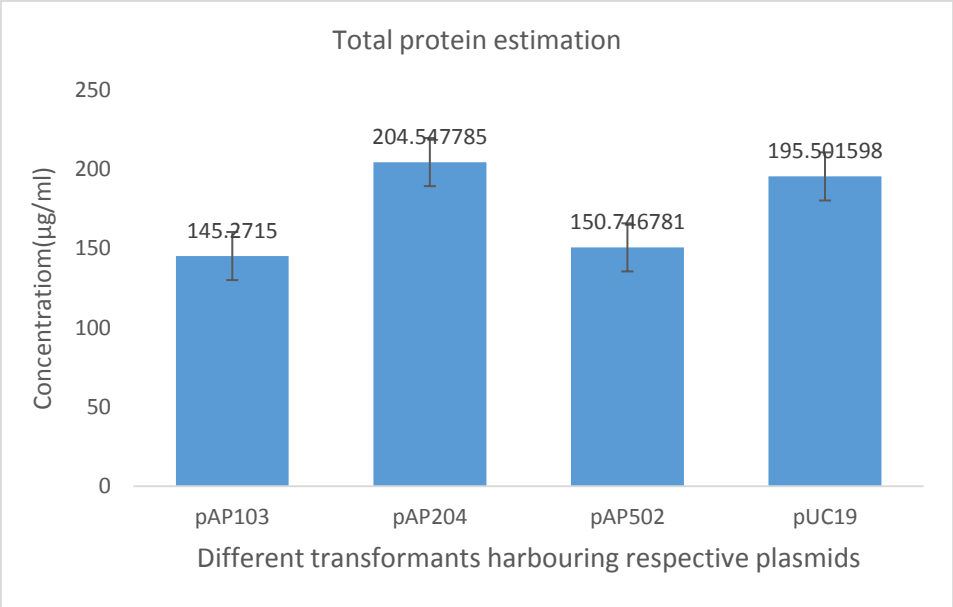



Figure (30): Graphical representation of total protein concentration in the sample

From the above bar-diagram, it showed that the cell having expression plasmid consisting upstream 200 bp from transcription start site (TSS) and 5'UTR of *cob*A gene has slightly higher protein production in comparison to control (cell with pUC19). As the genes are cloned in the vector under the native promoter of *cob*A gene with different promoter length upstream from TSS, increased protein production might be due to functional expression of the *cob*A gene, encoding uroporphyrinogen III methyl transferase. The enzyme helps in the vitamin B12 production, which in return helps in the membrane formation. As a result of this, more cell production might have been increased resulting in the higher protein production.

## 4.5 Enzyme Activity Test (Reporter assay)

The reporter gene vector analysis system is the most commonly used tool to study the regulation of gene expression. So, to evaluate the promoter activity, the pUC19-promoter-*lac*Z was constructed. In general, the reporter assay uses some kind of signalling that could be detected easily than the protein purification and kinetic studies.

In LacZ protein-based reporter assay, the enzyme activity is measured and it is correlated with the amount of protein translated (Slouch J.M, 2013). Thus, the present cloned promoter, 5'-UTR of *cob*A gene would express LacZ protein which would then exhibit β-galactosidase activity that can be spectrophotometrically measured and enzyme activity can be calculated (Sambrook, J *et. al.,* 1989). Depending upon the enzyme activity amount of protein can be thought to be higher or lower to explain whether the promoter region cloned has any role in *cob*A gene expression.

The study of expression plasmid consisting upstream 100 bp, 200 bp and 600 bp from transcription start sites and 5'-UTR of *cob*A gene for their functional strength can be done through reporter assay. β-galactosidase activity uses *lac*Z gene as reporter assay which determines the functionality through the activity of *lac*Z gene. The reporter gene determines the promoter strength as the reporter protein expression corresponds with the transcriptional activity of the promoter of the respective gene expression to be studied. For these type of the study promoter sequences are cloned either upstream or downstream of the reporter gene and their effects are quantified (Jain & Magrath, 1991; Smale, 2010). In the present study, promoters to be studied are cloned upstream of the *lac*Z gene. However, the study is done to determine the functional promoter region required for the efficient transcription of mRNA under the promoter.

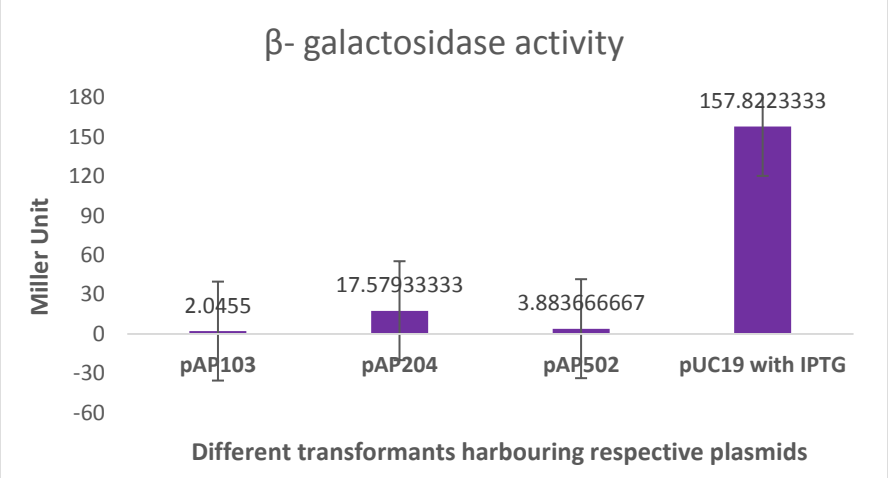



Figure (31): β-galactosidase activity of different transformants harbouring respective plasmids

In the above chart, β-galactosidase activity of the expression plasmids pAP103 is low in comparision to other expression plasmids. The results show that the promoter is regulated by some metabolites as corrin biosynthesis is tightly regulated at operon level and feedback inhibition by different metabolites (Fang *et. al.,* 2017). It may be also due to the expression of the gene but the mRNA does not get translated.

β-galactosidase activity of the expression plasmids pAP204 shows higher β-galactosidase activity among the other two expression plasmids while is low in comparision to the IPTG induced pUC19. This suggests that the *lac*Z could be functional and the slight activity may be due to the fact that gene expressed is regulated and as vitamin B12 synthesis is tightly regulated involving more than 30 genes (Roth *et. al.,* 1993) it can be presumed that *cob*A gene is tightly regulated at promoter level, too.

Similarly, the study on expression plasmids pAP502 also shows low activity indicating that the upstream nucleotide required for the efficient transcription of mRNA under *cob*A promoter lies between 100-200 base pairs upstream of transcription initiation site. Thus, not only TATA box and -35 but additional upstream nucleotides are required. This supports our hypothesis that upstream nucleotides are critical for gene expression.

# 5. SUMMARY

The rapid emergence of global antibiotic resistance demands the immediate development of the antibiotics to mitigate this problem. Essential gene targets were employed as antimicrobial target that could potentially act as alternative approach for the potential drug targets. In this study, *E.coli* expression system, promoter-5'UTR-*cob*A-*lac*Z, was constructed by cloning *lac*Z gene under the *cob*A native promoter and 5'UTR region. Thus, constructed expression vector was transformed into *E.coli* DH5α whereby the transformants were screened and confirmed by restriction digestion. The reverse transcription PCR was performed to determine the *lac*Z gene mRNA expression from the constructs using *lac*Z gene specific primers. Furthermore, the functional promoter analysis was done by β-galactosidase assay in which the development of the yellow colour was determined spectrophotometrically. Moreover, protein quantification was also done to study the growth between the three expression vectors.

Gene expression cloned plasmids were constructed using designed different sets of primers. The 1$^{st}$ set of the primer amplified 100 bp upstream of TSS and 5'UTR region was constructed to which *lac*Z gene was subcloned. The construct was then studied for the β-galactosidase activity and the transcriptional analysis. Similarly, remaining two sets where the forward primer is 200 bp and 600 bp upstream of TSS and 5'UTR along with *lac*Z gene was constructed and studied for the β-galactosidase activity and its transcriptional analysis was done. From the transcriptional analysis it is observed that the constructs have transcription but the reporter assay shows that difference in the activity. The β-galactosidase activity of the three constructs shows that the 200 bp upstream of TSS and 5'UTR has high activity in comparison to the 100 bp and 600 bp. From these results, it is clear that although the transcripts were formed, the better transcriptional activity was shown by the 200 bp promoter. The protein quantification showed that the expression vector with 200 bp upstream promoter has high protein content than that of the control used in the study.

# 6. CONCLUSION

The main purpose of the study is to elucidate the promoter function. Present study revealed with reporter assay that the β-galactosidase activity was highest in the 200 bp region. This leads to identify the nucleotide region required for functional promoter upstream of TSS and 5'UTR for *cob*A gene of *P. aeruginosa* is 200 base pair. The protein quantification study also showed that 200 bp region is crucial as it produces more protein than expression vector with 100 bp and 600 bp. This suggests that the 200 bp upstream from TSS and 5'UTR can be used to study different activities and can be explored for the mechanism of action against pathogen.

# 7. RECOMMENDATION

Thus, it is recommended that for future research on CobA protein with the native promoter it would be wise to use 200 bp upstream of transcription start site. In addition, any drug that could be developed could also look for suppression of the promoter region to prevent transcription. Moreover, the long stretch of 5'-UTR of *P. aerugenosa cob*A mRNA should be explored for secondary structure and any ligand that can induce riboswitch like structure would be a good drug candidate.

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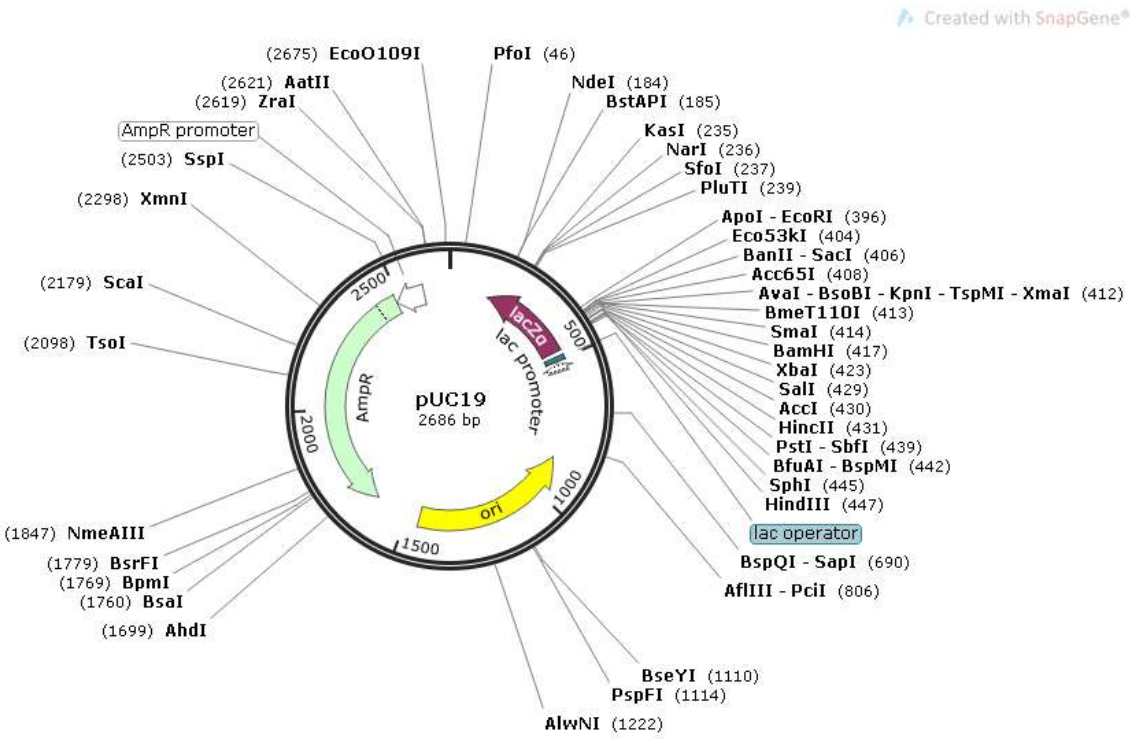
# APPENDICES

### Web site visited

| | |
|---|---|
| UniProt | http://www.ebi.ac.uk/uniprot/ |
| NCBI | https://www.ncbi.nlm.nih.gov/ |
| ExPASy | https://www.expasy.org/ |
| Addgene | https://www.addgene.org/ |
| NEB | https://international.neb.com/ |
| TaKaRa | https://www.takarabio.com/ |

### Vector used in the study




## Web server used for primer design

Following server were used to design the primer for *cob*A and *lac*Z gene

https://www.ncbi.nlm.nih.gov/

http://biotools.nubic.northwestern.edu/OligoCalc.html

http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form

https://www.idtdna.com/calc/analyzer

https://www.snapgene.com/snapgene-viewer/

**For *cob*A promoter cloning (Suprim Tha and Sita Ghimire)**

*Pseudomonas aeruginosa* PA96 3642816..3643553

gene - *cobA* Vector - puc19

3639601 cagcgcatcg aacagccgca attgaagaag gagttcgaac ggattccgct caagcaactg
3639661 gaaaacgtct gaatcccttg cccgggccgt cccacggcgg ccctcccccc accggagtga
3639721 accgatgaac tggctggata tctgctccct cgatgaaatc aacccgctgg gctcgcgcgt
3639781 ggtcgccggc ccgaaaggcg acatcgccat cttccgcgcc gccgacgacc aggtcttcgc
3639841 cctcgatgac cgctgcccgc acaagggcgg cccgctgtcc cagggcctga tctacggcaa
3639901 gcgagtggcc tgtccgttgc acaactggca gatcgaactg gagagcggcg aagccgtggc
3639961 cccggaccag ggctgcgccc atcgccaccc ggtgcgggtg gagaacggac gggtgctgct
3640021 cggcctggac agcgtggcgc tgtgcgcctg atctctcgcc gataacagga gccgagcatg
3640081 tcgacagatt ccccgctgcg caccaccgct tcgacctgct gctactgcgg cgtgggttgt
3640141 ggcgtcctga tcgaacacga cggcgaacgc atcctcggcg tgcaaggcga cccgcggcac
3640201 ccggccaact tcggcagact gtgcagcaaa ggcgccagcc tgcacctgac cggcgacctc
3640261 caggcccgcg cgctgtaccc ccaactgcgc ctcggcaagc aactggcgcg ggcccgcagc
3640321 gactgggaga gtgccctgga acatgccgcc gggcgtttcg ccgagaccat tcgcgaacac
3640381 gggccggaca gcgtggcctt ctatatatcc ggccagttgc tgaccgagga ctactacgcc
3640441 ttcaacaagc tggcgcgggc cctggtcggc accaacaaca tcgacagcaa ttcgcgcctg
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3640741 ctgcttcccg gtaccgacgt cgcactgttc cacggcatcc tgcatatcct gctctgggag
3640801 gactggatcg accgttcgtt catcgccgag cataccgagg gtttcgccga cctgaaggaa
3640861 ctggtgcgcg actacacccc ggccgccgtc gccgacatct gcggcatcga ccgcgccgac
3640921 ctgcagcgct gcgccgaatg gatcggccgt tcgccgcgct tcctttcgct ctggtgcatg
3640981 gggctcaacc agtccagcgc cggcagcgcc aagaacagcg cactgatcaa cctgcacctg
3641041 gccaccggca agatcggccg cgccggatgc gggccgtttt ccctcaccgg tcagccgaac
3641101 gccatgggcg gtcgcgagac cggcagcctg gccaacctgc tgcccggcca ccgcgaagcc
3641161 gccgatcccg gccatcgcgc cgaggtggcg cactactggg gtgtggaaca gttgcccacg
3641221 tcccccggtc tcagcgccat cgagctgttc gatgcggtcc acgacggtcg gatcaaggcg
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3641521 caggactgga acatcgtctg cgacttcgcg cgccgcctgg aagggcacct gcggcccgct
3641581 aaacccggcc tgttcgcctt tgccgacagc cgcagcctgt tcgacgaata caagctgctc
3641641 accgccgggc gcgacctcga tctttccggc ctcagctacg cactgctcga ccgcctgggg
3641701 ccgcagcaat ggccatttcc cactggcgcg gagcacggca ccgcacggct ctatgcagac
3641761 ggccgcttcc ctaccgcctc gggacgcgcg caactggtcg ccgaacccta ccgcgccgcc

3641821 caggaaaaac gcgacgcgcg ctacccgctg accctcaata ccggacgctt gcgcgaccag
3641881 tggcacggca tgagccggac cggcacctgc gcacgcctgt tcggccatga agaggaggcc
3641941 ctggtgcacc tgcatcccga ggaactgcgc cgccgccaac tgcaggacgg gcagctcgtg
3642001 cgcctgaaga gccggcgtgg cgccctgatc ctgccagtga gtgcagacga ctcggtgcgc
3642061 cccggccagg ccttcctgcc gatgcactgg ggcgatcgct tcctcaaggg cctgggctgc
3642121 aatgtcctga ccctgccggc gttcgacccc ctgtcgaaac aacccgaact caagcacgcc
3642181 ggggttcagg tcgaatccgt cgaactgccc tggcgcctgt tcgcgctggt ggaaaccgat
3642241 atccaggcgc gtttcgaggc cctccgtgcg ctgtgcgaag tcttcgacca cgccagcttc
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3642361 ggcgcagacc tgctggagcg gatagatcgc caactcgaat tgctcgacgg gccgatcctc
3642421 gcctacgacg acccacggcg cgccatcggc aagcgcgtgc gcctcgaaga cgggcgcatc
3642481 gtcgccgtcc ggctcgccgg cgaaaccctc gcccgagact ggttgaaaga gctctggctt
3642541 accggccggg ccgatagcga gctacgccgc tggctgctcg ccccactcgg cgcggcaccg
3642601 ggacgaccca gccagggtgc gggcggcaag accctgtgca gttgccagaa cgtcagccag
3642661 caaacggtgc tcggcggcat cgcccgcgga ttggacctgg acggattgaa gcgcgagttc
3642721 ggctgcggca ccggctgcgg ctcctgcgta ccggaaatca agagactgct ggcggccccc
3642781 cggcccatgg cggcgaatgc ctgaggagaa cgacgatgag cggtaaggtc tggctggtgg
3642841 gtgcgggtcc cggcgatccg gaactgttga ccctgaaggc agttcgcgcc ctgcaggacg
3642901 cggatgtggt gatggtcgac gatctggtca acccgagcat actggagcac tgtccgtccg
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3643441 tcggcgagct actgcgggac gccggacgtt tcgccttgaa aagcccggcg atcctggtga

3643501 tcggcgaagt aacccgcgac atcgtgtcgc aaccgatttc cctgagcgcc tga…3643553

**For lacZ cloning (Suprim Tha and Sita Ghimire)**

E.coli CFT073

lacZ gene 3075 bp

Complement (448924 – 451998)

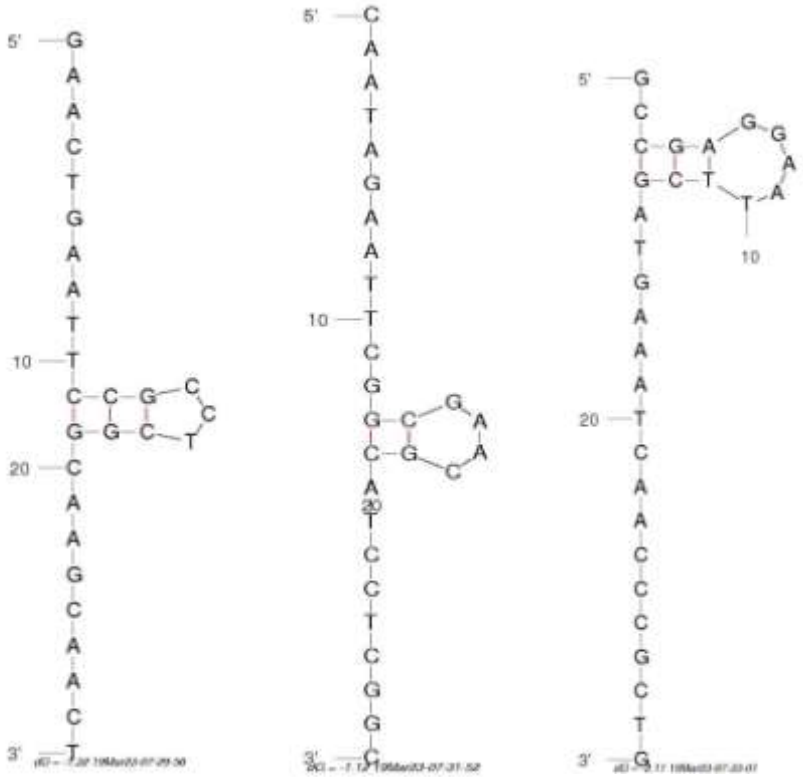
GTC TTT ACA CTC TTA TGT GTC CGG CTC GTA TGT TGT GTG AAA TTG TGA GCG GAT AAC AAT TTC ACA CAG GAT ACA GCT

451998- ATG ACT ATG ATT ACG GAT TCT CTG GCC GTC GTA TTA CAA CGT CGT GAC TGG GAA AAC CCT GGC GTT ACC CAA CTT AAT CGC CTT GCG GCA CAT CCC CCT TTC GCC AGC TGG CGT AAT AGC GAA GAG GCC CGC ACC GAT CGC CCT TCC CAA CAG TTG CGC AGC CTG AAT GGC GAA TGG CGC TTT GCC TGG TTT CCG GCA CCA GAA GCG GTG CCG GAA AGC TGG CTG GAG TGC GAT CTT CCT GAC GCC GAT ACT GTC GTC GTC CCC TCA AAC TGG CAG ATG CAC GGT TAC GAT GCG CCT ATC TAC ACC AAC GTG ACC TAT CCC ATT ACG GTC

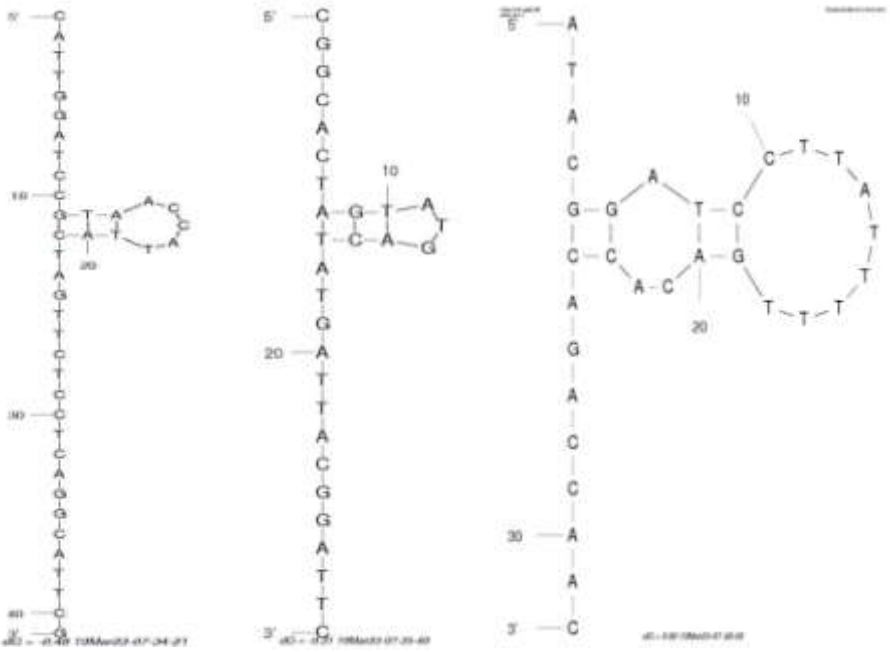
AAT CCG CCG TTT GTT CCC GCG GAG AAT CCG ACA GGT TGT TAC TCG CTC ACA TTT AAT ATT GAT GAA AGC TGG CTA CAG GAA GGC CAG ACG CGA ATT ATT TTT GAT GGC GTT AAC TCG GCG TTT CAT CTG TGG TGC AAC GGG CGC TGG GTC GGT TAC GGC CAG GAC AGC CGK TTG CCG TCT GAA TTT GAC CTG AGC GCA TTT TTA CGC GCC GGA GAA AAC CGC CTC GCG GTG ATG GTG CTG CGC TGG AGT GAC GGC AGT TAT CTG GAA GAT CAG GAT ATG TGG CGG ATG AGC GGC ATT TTC CGT GAC GTC TCG TTG CTG CAT AAA CCG ACC ACG CAA ATC AGC GAT TTC CAA GTT ACC ACT CTC TTT AAT GAT GAT TTC AGC CGC GCG GTA CTG GAG GCA GAA GTT CAG ATG TAC GGC GAG CTG CGC GAT GAA CTG CGG GTG ACG GTT TCT TTG TGG CAG GGT GAA ACG CAG GTC GCC AGC GGC ACC GCG CCT TTC GGC GGT GAA ATT ATC GAT GAG CGT GGC GGT TAT GCC GAT CGC GTC ACA CTA CGC CTG AAC GTT GAA AAT CCG GAA CTG TGG AGC GCC GAA ATC CCG AAT CTC TAT CGT GCA GTG GTT GAA CTG CAC ACC GCC GAC GGC ACG CTG ATT GAA GCA GAA GCC TGC GAC GTC GGT TTC CGC GAG GTG CGG ATT GAA AAT GGT CTG CTG CTG CTG AAC GGC AAG CCG TTG CTG ATT CGC GGC GTT AAC CGT CAC GAG CAT CAT CCT CTG CAT GGT CAG GTC ATG GAT GAG CAG ACG ATG GTG CAG GAT ATC CTG CTG ATG AAG CAG AAC AAC TTT AAC GCC GTG CGC TGT TCG CAT TAT CCG AAC CAT CCG CTG TGG TAC ACG CTG TGC GAC CGC TAC GGC CTG TAT GTG GTG GAT GAA GCC AAT ATT GAA ACC CAC GGC ATG GTG CCA ATG AAT CGT CTG ACC GAT GAT CCG CGC TGG CTA CCC GCG ATG AGC GAA CGC GTA ACG CGG ATG GTG CAG CGC GAT CGT AAT CAC CCG AGT GTG ATC ATC TGG TCG CTG GGG AAT GAA TCA GGC CAC GGC GCT AAT CAC GAC GCG CTG TAT CGC TGG ATC AAA TCT GTC GAT CCT TCC CGC CCG GTA CAG TAT GAA GGC GGC GGA GCC GAC ACC ACG GCC ACC GAT ATT ATT TGC CCG ATG TAC GCG CGC GTG GAT GAA GAC CAG CCC TTC CCG GCG GTG CCG AAA TGG TCC ATC AAA AAA TGG CTT TCG CTG CCT GGA GAA ATG CGC CCG CTG ATC CTT TGC GAA TAT GCC CAC GCG ATG GGT AAC AGT CTT GGC GGC TTC GCT AAA TAC TGG CAG GCG TTT CGT CAG TAC CCC CGT TTA CAG GGC GGC TTC GTC TGG GAC TGG GTG GAT CAG TCG CTG ATT AAA TAT GAT GAA AAC GGC AAC CCG TGG TCG GCT TAC GGC GGT GAT TTT GGC GAT ACG CCG AAC GAT CGC CAG TTC TGT ATG AAC GGT CTG GTC TTT GCC GAC CGC ACG CCG CAT CCG GCG CTG ACG GAA GCA AAA CAC CAA CAG CAG TAT TTC CAG TTC CGT TTA TCC GGG CGA ACC ATC GAA GTG ACC AGC GAA TAC CTG TTC CGT CAT AGC GAT AAC GAG TTC CTG CAC TGG ATG GTG GCA CTG GAT GGC AAG CCG CTG GCA AGC GGT GAA GTG CCT CTG GAT GTT GGC CCG CAA GGT AAG CAG TTG ATT GAA CTG CCT GAA CTG CCG CAG CCG GAG AGC GCC GGA CAA CTC TGG CTA ACG GTA CGC GTA GTG CAA CCA AAC GCG ACC GCA TGG TCA GAA GCC GGA CAC ATC AGC GCC TGG CAG CAA TGG CGT CTG GCG GAA AAC CTC AGC GTG ACA CTC CCC TCC GCG TCC CAC GCC ATC CCT CAA CTG ACC ACC AGC GGA ACG GAT TTT TGC ATC GAG CTG GGT AAT AAG CGT TGG CAA TTT AAC CGC CAG TCA GGC TTT CTT TCA CAG ATG TGG ATT GGC GAT GAA AAA CAA CTG CTG ACC CCG CTG CGC GAT CAG TTC ACC CGT GCG CCG CTG GAT AAC GAC ATT GGC GTA AGT GAA GCG ACC CGC ATT GAC CCT AAC GCC TGG GTC GAA CGC TGG AAG GCG

GCG GGC CAT TAC CAG GCC GAA GCG GCG TTG TTG CAG TGC ACG GCA GAT ACA CTT GCC GAC GCG GTG CTG ATT ACA ACC GCC CAC GCG TGG CAG CAT CAG GGG AAA ACC TTA TTT ATC AGC CGG AAA ACC TAC CGG ATT GAT GGG CAC GGT GAG ATG GTC ATC AAT GTG GAT GTT GCG GTG GCA AGC GAT ACA CCG CAT CCG GCG CGG ATT GGC CTG ACC TGC CAG CTG GCG CAG GTC TCA GAG CGG GTA AAC TGG CTC GGC CTG GGG CCG CAA GAA AAC TAT CCC GAC CGC CTT ACT GCA GCC TGT TTT GAC CGC TGG GAT CTG CCA TTG TCA GAC ATG TAT ACC CCG TAC GTC TTC CCG AGC GAA AAC GGT CTG CGC TGC GGG ACG CGC GAA TTG AAT TAT GGC CCA CAC CAG TGG CGC GGC GAC TTC CAG TTC AAC ATC AGC CGC TAC AGC CAA CAA CAA CTG ATG GAA ACC AGC CAT CGC CAT CTG CTG CAC GCG GAA GAA GGC ACA TGG CTG AAT ATC GAC GGT TTC CAT ATG GGG ATT GGT GGC GAC GAC TCC TGG AGC CCG TCA GTA TCG GCG GAA TTC CAG CTG AGC GCC GGT CGC TAC CAT TAC CAG TTG GTC TGG TGT CAA AAA TAA – 448924

## M-fold Structure of designed Primer of *cob*A and *lac*Z gene



**CobAF100** **CobAF200** **CobAF600**



**CobA R** **LacZ F** **LacZ R**

## Composition of LB per 100 ml

| | |
|---|---|
| Yeast extract | 0.5 gm |
| Enzyme casein hydrolase | 1 gm |
| Sodium chloride | 1 gm |

## Alkaline Lysis Solution I

50 mM Glucose

25 mMTris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

The solution was sterilized by autoclaving and stored at 4$^{o}$C.

## Alkaline Lysis Solution II (Freshly prepared)

0.2 N NaOH

1 % (w/v) SDS

## Alkaline Lysis Solution III (per 100 ml)

| | |
|---|---|
| 5 M Potassium Acetate | 60.0 ml (Final concentration in 100 ml is 3 M) |
| Glacial Acetic Acid | 11.5 ml |
| Autoclaved Distilled Water | 28.5 ml |

### Tris EDTA buffer

| | |
|---|---|
| Tris pH 8 | 1Mm |
| EDTA pH 8 | 10Mm |

### 50X Tris Acetate –EDTA (TAE) buffer

| | |
|---|---|
| Tris base | 24.2 gram |
| Glacial acetic acid | 5.7 ml |
| 0.5 M EDTA (pH 8) | 10 ml |

Final volume 100 ml to be made with distilled water

## Preparation of buffer used during β-galactosidase activity

### Z-buffer composition

| | |
|---|---|
| $NaH_2PO_4.2H_2O$ | 0.31 gm |
| $Na_2HPO_4$ | 0.425 gm |
| KCL | 0.0372 gm |
| $MgSO_4$ | 0.0123 gm |

### Assay Buffer for 10 ml

| | |
|---|---|
| Z-buffer | 9.6 ml |
| 0.1% SDS | 0.4 ml |
| β-mercaptoethanol | 27 µl |

### Phosphate buffer

| | |
|---|---|
| $NaH_2PO_4.2H_2O$ | 0.31 gm |
| $Na_2HPO_4$ | 0.425 gm |

ONPG (4 mg/ml) should be dissolved in phosphate buffer and should be used fresh.

## Reagent used in Lowry method

A. 2% $Na_2CO_3$ in 0.1 N NaOH (Reagent A)
B. 1% NaK in 0.5% $CuSO_4$(Reagent B)
C. Reagent A and Reagent B in the ration 49:1
D. Folin's reagent