SCREENING OF NOVEL GENES INVOLVED IN BIO-FILM MEDIATED RESISTANCE TO ANTIBIOTICS IN MDR *Pseudomonas aeruginosa*

M.Sc. Thesis

2078(2022)

Submitted To:

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Submitted By:

Padma Ratna Manandhar

M.Sc. Biotechnology

T.U. Regd. No.: 5-3-28-230-2016

Supervised By:

Suresh Subedi, PhD

Assistant Professor

RECOMMENDATION

This is to certify that the research work entitled **"SCREENING OF NOVEL GENES INVOLVED IN BIO-FILM MEDIATED RESISTANCE TO ANTIBIOTICS IN MDR *Pseudomonas aeruginosa*"** has been carried out by **Mr. Padma Ratna Manandhar** under my supervision.

This thesis work was performed for the partial fulfilment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. I hereby, recommend this thesis for final evaluation.

.....

Asst. Prof. Suresh Subedi, PhD

(Supervisor)

Central Department of Biotechnology

Tribhuvan University

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Tel. No.: +977 1 4336221

Date……

CERTIFICATE OF EVALUATION

This is to certify that this thesis entitled "**SCREENING OF NOVEL GENES INVOLVED IN BIO-FILM MEDIATED RESISTANCE TO ANTIBIOTICS IN MDR *Pseudomonas aeruginosa***" presented to evaluation committee by **Mr. Padma Ratna Manandhar** is found satisfactory for the partial fulfilment of Master of Science in Biotechnology.

……..

Asst. Prof. Suresh Subedi, Ph.D

(Supervisor)

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

……..

Prof. Krishna Das Manandhar, Ph.D

Head of Department

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

……

Assoc. Prof. Megha Raj Banjara, Ph.D

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

……

Prof. Rajani Malla, Ph.D

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

ACKNOWLEDGEMENTS

This project would not have been possible without the guidance and help of several individuals who in one way or the other contributed and extended their valuable assistance in the preparation and completion of this study.

It is my immense pleasure to express my sincere thanks and deep sense of gratitude to my honourable teacher and supervisor **Dr. Suresh Subedi,** Asst. Prof. of Central Department of Biotechnology for his regular supervision and guidance. He was always there to enhance my understanding of how to carrying out the scientific work and guiding me to perform experiments that could answers the thesis hypothesis. My deepest gratitude to him for his splendid supervision, invaluable suggestion and kind cooperation because it was a painstaking journey and he went through the whole project without losing patience. Without his effort this work would not take this shape in which it has been presented. Through his invaluable discussions and endless endeavours, I have gained a lot.

And no words can express my appreciation to Dr. Pramod Aryal sir, for his guidance at the time of need. He was there to lift my spirits to carry on with the research work.

I owe a deep sense of gratitude to Prof. Dr. Krishna Das Manandhar, Ph.D., Head of Central Department of Biotechnology, Tribhuvan University, Kirtipur for his support to complete my thesis in the department. I would like to give my special thanks to my Senior Ms. Samikshya Kaphle for her excellent support by providing the required bacterial isolates and expert advice during the project. My gratitude to Apsara Parajuli, Sita Ghimire for their valuable suggestions. The support from the laboratory staff and administrative staff was always there.

I take this opportunity to extend my appreciation to Ms. Himani Upreti for providing me with bacterial isolates and other friends of 8th Batch of CDBT for support and help during laboratory work.

I am extremely grateful to Nepal Youth Council, Nepal for providing the “Master thesis Support Grant” as financial support.

Finally, I want to thank my parents and my brothers Punya Ratna Manandhar and Siddhi Ratna Manandhar and other family members for their constant support and encouragement to accomplish this work on time.

Date: …/…/……

……..

Padma R. Manandhar

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY ACRONYMS

ABS: Activation Binding Site

AST: Antibiotic Susceptibility Test

BLAST: Basic local alignment search tool

C-di-GMP: bis-(3', 5')-cyclic dimeric guanosine mono-phosphate (c-di-GMP)

CDO: Catechol 2, 3-Deoxygenase

CF: Cystic Fibrosis

CLSI: Clinical and Laboratory Standard Institute

CRPA: Carbapenem Resistant *Pseudomonas aeruginosa*

CTAB: Cetyltrimethyl Ammonium Bromide

Cup: Chaperone usher pathway

DGC: Diguanyl cyclase

DHP-I: Dehydropeptidase I

DNA: Deoxyribonucleic Acid

EDTA: Ethylene Diamine Tetra Acetic acid

ELISA: Enzyme Linked Immuno Sorbent Assay

EMSA: Electro Mobility Shift Assay

EPS: Exopolysaccharide

ESBL: Extended Spectrum Beta Lactamase

EtBr: Ethidium Bromide

IPM: Imipenem

IPTG: Isopropyl β-D-1-thiogalactopyranoside

LB: Luria-Bertani

LPS: Lipopolysaccharide

LTTRs: Lys-Type Transcriptional Regulators

MIC: Minimum Inhibitory Concentration

MRP: Meropenem

NCBI: National Centre for Biotechnology Information

OD: Optical Density

ODc: Optical Density of Control

PCR: Polymerase Chain Reaction

PDE: Phosphodiesterase

PGA: Poly-beta-1, 6-N-acetyl-D-glucosamine

PTZ: Piperacillin/Tazobactam

PVC: Poly vinyl chloride

QS: Quorum Sensing

RBS: Ribosome Binding Site

RNA: Ribonucleic Acid

Roc: Regulation of cup genes

RT-PCR: Reverse Transcriptase PCR

SOC: Super Optimal broth with Catabolite repression

TCP: Tissue Culture Plate

TCSs: Two Component Signal Transduction Systems

TF: Transcription Factor

TFBS: Transcription Factor Binding Site

TM: Tube Adherence Method

ABSTRACT

The role of biofilm in the pathogenesis of some chronic human infection is now widely accepted. *Pseudomonas aeruginosa* is a model organism for biofilm study, which seems to have complex regulatory circuit that controls biofilm formation. Biofilm forming physiology of *P. aeruginosa* should have some genetic implication. In this study, the potential of biofilm formation in *P. aeruginosa,* was assessed and susceptibility to selected carbapenem antibiotic was determined. The potential regulatory gene was identified using homology searches and used in the study for determining the role of this gene in biofilm formation using PCR amplification and cloning. Assessment of biofilm formation by 8 confirmed *Pseudomonas* isolates were done using normal polystyrene microtitre plate (non-tissue coated), using crystal violet staining and read at 551nm. The AST was carried following Clinical and Laboratory Standard Institute protocol in Muller Hilton Agar plate using 10 mcg disc of imipenem and meropenem antibiotics. BLASTp program identified PA1961 gene as putative regulatory gene of the family LysR- type transcriptional regulator in *P. aeruginosa* for which primer was designed using PrimerBLAST tool of NCBI. PCR was used to determine whether presence or absence of the gene was related to biofilm formation physiology. Out of 8 isolates, two different isolates, 1 isolate showed consistent biofilm formation using cut-off (Mean OD of negative control + 3X SD of negative control) and 1 isolate showed antibiotic resistance to both IPM and MRP, making 12.5%(12.5% biofilm former and 12.5% antibiotic resistance), which is considerably low as compared to previous studies. Next we aimed to draw if there is any relationship of biofilm formation with antibiotic resistance. We identified PA1961 gene using homology search and expected its presence only in biofilm forming carbapenem resistant isolates. In contrary the gene was found to be present in all isolates, including the positive control (PAO1) and the result was inconclusive in drawing direct relationship of biofilm formation with antibiotic resistance in *Pseudomonas*. We could have done protein analysis for the same gene to be sure of this genes role in biofilm formation. But since it was a surface protein, protein quantification would not provide be done. Further study with the increase in

number of fresh isolates, stringent control (inclusion of negative control strain) and robust bioinformatics analysis will provide conclusive result for the identification of novel genes involved in biofilm mediated antibiotic resistance in MDR pathogens.

Keywords: Biofilm, Antibiotic resistance, Carbapenem, *P. aeruginosa*, LysR-type transcriptional regulator, Antibiotic susceptibility test.

CHAPTER 1

INTRODUCTION

1.1 Background

Microorganisms can contend diverse obstacle and have struggled to exist in world for millions of years. Microbiologist have reveal some aspect of microorganism using different tools like biochemistry, molecular biology, microscopy (scanning electron microscopy, confocal microscopy) besides culturing them in nutrient media. While use of microorganism for their useful properties is recently being studied, deleterious effect of microorganism in health has been largely studied. Amongst other properties of microorganisms, antibiotic resistance is a major threat to public health. Mainly believed to be due to improper and massive use of antibiotics, bacteria naturally also evolve to hostile environment. It may be a natural process for bacteria to develop resistance against mostly used antibiotics. Another ancient survival strategy involves bacteria existing within a self-produced polymeric matrix, which is termed as biofilm. Biofilm besides protecting the bacteria from harsh environment, also provide favourable environment for transfer of antibiotic resistance gene between species. Biofilm is physiological characteristic bacteria seems to possess due to change in the regulatory region and hence, either increased or decreased expression of existing genotype. Many mutational studies involving transposon induced mutation, site directed mutagenesis, Electro mobility shift assay (EMSA), different inducer studies which promote biofilm formation (Mg^{+2}, Zn^{+2}, SO_2^{--}, NO_2^{-}, Nitrogen source, NaCl) have been carried out to gather information on biofilm formation characteristic of microorganism.

1.1.1 Biofilm

Biofilms are adherent groupings of microbial cells wrapped in an extracellular matrix (ECM) that are much more resistant to antimicrobial treatments than non-adherent, planktonic cells (Hall & Mah, 2017). In their natural environment, most types of bacteria and fungi alternate between planktonic (free-living) and sessile states in

response to environmental stimuli such as the availability of essential nutrients, shearing stress of flowing water etc. (Lynch & Robertson, 2008). The abundance of such (biofilm) form of bacteria in nature was theorized by Costerton. In 1978, Geesey et al adapted a whole series of quantitative recovery methods to allow us to enumerate biofilm bacteria and found biofilm mode of life dominated in hundreds of aquatic system examined (Costerton et al., 1995). While Hoiby, Bjarnsholt and their successors showed the biofilm mode of growth prevalent in *Pseudomonas aeruginosa* laden in mucus of cystic fibrosis patients and in dental infection (Bjarnsholt, 2013).

Biofilm show profound physio-chemical heterogeneity believed to be due to:

1. Degree of limitation imposed by the matrix e.g. diffusion limits and physical constrains lead to the structural and physiological heterogeneity.

2. The presence of diverse species in the biofilm found in nature.

This may cause variation in microenvironments of biofilm found in different times. The channels and pillars architecture seen in electron microscopy is attributed as the remnants of the organization of founding bacteria because of their rigidity (Ghigo, 2003).

The actual progression of biofilm formation seems to follow the same pattern regardless of the organism. The general pattern includes:
(i)Reversible adhesion, (ii) Irreversible adhesion, (iii) Formation of micro-colonies and maturation, (iv) Mature biofilm & (v) Detachment

The fact that there is an increase in biofilm related complication in the patient with use of indwelling medical devices has been recognised by governmental agencies including Food and Drug Administration (FDA) and the Center for Disease Control and Prevention. According to the web site of the U.S. Food and Drug Administration more than 60% of human infections are biofilm related (Pace et al, 2006).

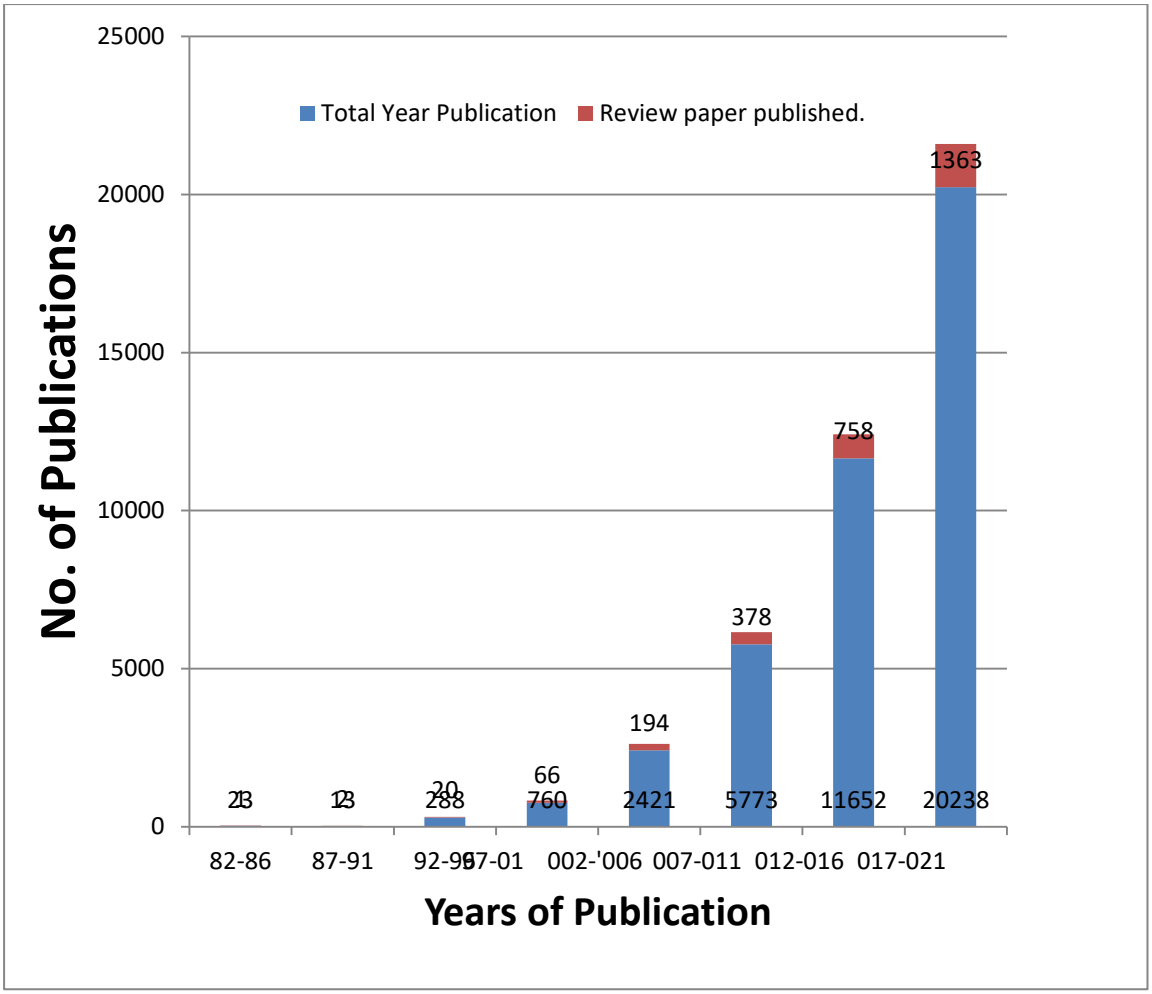


Figure1. 1: Accumulated publications on biofilms per year, derived from the search engine PubMed (http://www.ncbi.nlm.nih.gov/pubmed/).

The bar chart denotes over 2,000 publications each year since 2007 and doubling every succeeding year that have reported different aspects of biofilm's unknown characteristics and the new information that are emerging annually. Moreover, about 8% of published articles are review article. Scientific community already have wealth of knowledge about biofilm but the key role-playing genes and a physiological pathway for biofilm formation is missing.

Overall studies on bio-film forming characteristic of different microbes have concluded different point, which can guide us to do further research. The composition of ECM varies between species but essentially it consist of extracellular polymeric substance mainly polysaccharides, lipids, proteins & nucleic acid. Two types of bacterial biofilms have been reported, one which requires surface

for infection: such as on implant, contact lens, catheters, artificial heart valves and teeth. Non-surface related biofilm are CF, otitis media, chronic osteomyelitis (Bjarnsholt, 2013). Almost all bacterial species(Gram-positive and Gram-negative) and some fungi are found to form biofilm after sensing the environmental cues like shear stress of flowing water, limited nutrition etc (Benítez-Cabello et al., 2015). Benitez studies shows that simple washing procedure is not enough for removing biofilm formed by micro-organism. Biofilm forming bacteria can be retrieved by using sonicator machine for about 5 to 30 min. Other process like enzymatic treatment for over 1hour is both time consuming and expensive for routine examination of bacteria population in biofilm. Successful biofilm quantitation method like Microtiter plate assay capable to differentiate strong biofilm former, mild-biofilm former and weak biofilm former in the laboratory have been developed (O'Toole, 2010). Successful biofilm susceptibility testing to different antibiotics, biocides, and disinfectant method like Minimal Biofilm Eradication Concentration (MBEC) have been developed and applied in laboratories to account for the decreased susceptibility of biofilm. The increase doses or new choice of antibiotic as derived from MBEC test have shown positive co-relation between antibiotic used and clinical outcome in different organism and disease models in vivo (Innovotech, 2012).

Despite so much knowledge and development in the field of biofilm still many pharmaceutical industries use planktonic bacteria for their Minimal Inhibitory Concentration (MIC) estimation for their antibiotic. Though initially the data from MIC between biofilm former and biofilm non-former were in close agreement for harsh antibiotic like Carbapenem (3^{rd} generation of antibiotic) some bacteria have started to show different result in biofilm formers than non -formers in different generation of antibiotic like ampicillin (3^{rd} generation), ceftiofur (3^{rd} generation) , cloxacillin (4^{th} generation), oxytetracyline , penicillin G, streptomycin, tetracyclin, enorfloxacin (3^{rd} generation), erythromycin, gentamicin, tilmicosin and trimetho prim-sulfadoxine for Gram -positive and -negative bacteria as shown by MIC value and Minimal Biofilm Eradication Concentration (MBEC) determined by Olson, et al (Olson et al., 2002). As bacteria involved in chronic infection, burn wound, cystic

fibrosis, osteomyelitis, use of indwelling medical devices and many other condition involves the biofilm mode of life the use of MBEC for antibiotic dose and susceptibility testing provides better chance of success in the treatment.

1.1.2 Carbapenem as last line of antibiotics

Carbapenem is the class of antibiotic which show effect to both Gram positive and Gram negative as well as aerobic and an anaerobic bacteria. Carbapenem were chosen as the study anti-biotic because Carbapenem act as 'the last line agent' or 'Antibiotic of last resort' when every antibiotic treatment fail or start to develop antibiotic resistance (Papp-Wallace et al., 2011).

Structurally 'carbapenem' are 4:5 fused ring lactam of penicillin's with a double bond between C-2 and C-3 but with the substitution of carbon for sulphur at C-1(Papp-Wallace et al., 2011).While imipenem: $C_{12}H_{17}N_3O_4S$ and meropenem: $C_{17}H_{25}N_3O_5S$ both have hydroxyethylene side chain which is a drastic diffentiation from conventional penicillin and cephalosporin, as they have acylamino substituent on the beta-lactam ring. However the imipenem is susceptible to deactivation by dehydropeptidase I (DHP-I), found in human renal brush border while meropenem show some stability against its hydrolysis (Papp-Wallace et al., 2011).

The mechanism of beta lactams antibiotics are inhibition of cell wall synthesis .These synthetic or semi synthetic compound have potential to inhibit several enzymes associated with final step of peptidoglycan synthesis β-lactam basically target the penicillin blinding protein and doesn't allow it to produce transpeptidase or carboxypeptidase enzyme . These enzymes are essential for the formation of peptide bonds which crosslink two peptidoglycans to form bacterial cell wall. Reason for biofilm mediated resistance to carbapenem antibiotic is attributed to the extracellular matrix not allowing entry to antibiotics, the slow growth of microbial cells in biofilm and formation of so called 'persister' cell inside biofilm.

1.1.3 Antibiotic Resistant and biofilm

The 'resistant' tagged microorganism implies that the concentration of antibiotic which the host can tolerate in vivo condition cannot effectively eradicate the micro-organism from host body. Such organism show zone diameter which fall in the range where antibiotic resistant mechanism (e.g. β -lactamase) are likely. This does not mean that the bacteria can grow rampant in the environment ,but yes the clinical trial passed antibiotic regime fall short once the micro-organism invade the host system . Such resistance micro-organisms have been the major cause for the death of patient due to sepsis and systemic infection. The antibiotic that shows efficacy in vitro susceptibility test might fail in showing out come in actual clinical cases. This imprecision might be attributed to presence or absent of biofilm, drug thermodynamics and other factors.

Most literature cite the exopolymeric substance, serving as physical barrier, as a cause of antibiotic resistance and this is seen in some species; *P. aeruginosa* EPS contains negatively-charged alginate that easily slows the diffusion of positively-charged aminoglycosides (Mendoza et al., 2019).

However, this type of resistance is dependent on type of antibiotic and bacteria on which the test is done. e.g., there is unrestricted diffusion of ciprofloxacin and ampicillin through *K. pneumoniae,* ciprofloxacin through *P. aeruginosa* and tetracycline through *E. coli*. While the role of biofilm as barrier is clear but if sufficient antibiotic accumulation occurs, the bacteria will be killed. Hence, biofilm act as temporary barrier which provide time for the bacteria to launch other defensive strategy (Mendoza et al., 2019).

1.1.4 LysR-Type Transcriptional Regulators

The highly conserved LysR-type transcriptional regulators (LTTR) family is one of the well-characterized groups of transcriptional regulators. Found abundantly in prokaryotes such as bacteria, the functional orthologues of LysR-type transcriptional regulators have also been identified in archaea and eukaryotes (Maddocks & Oyston, 2008). In prokaryotes, LTTR regulates the expression of genes whose products are

involved in diverse functions. The proteins under the regulation of LTTR take part in cell division, amino acid biosynthesis, metabolic signaling, motility, and attachment as well as in β-lactamase transportation, detoxification, oxidative stress response, quorum sensing, and virulence (Issa et al., 2018). Different techniques like DNase I protection assay and DNA fingerprinting techniques are used to explain different aspects of LTTR protein regulatory roles. The TF coding gene of the LysR family is situated at <100 nucleotides upstream of TG and occasionally up to 500 bp upstream from the initiation codon. The site at which LTTR proteins (or TF) binds is termed Transcriptional Factor Binding Site (TFBS). The intergenic region consists of two to three different TFBSs with the inter-motif length between the first two being generally seven nucleotides. This, however, isn't true for LyrR where the inter-motif length is six. The third motif when present mostly overlaps with the second at a region called activation binding site (ABS). The LysR is an exception to this as it contains 19 bp inter-motif between second and third TFBSs. The two activated TF dimers, under the influence of one or several inducers, bind in a cooperative manner but with different affinities for transcriptional activation. Cooperative and allostery are two different modes of action of activators, where cooperative binding refers to the simultaneous interaction of activators with DNA and polymerase to recruit enzymes to promoters (Watson, 1987). The TF will either be an activator or a repressor on the basis of its affinity with TFBS.

1.2 Current studies

In cystic fibrosis and other biofilm-based infections, antibiotic prescription is mainly based on standard minimum inhibitory concentration (MIC) methods, but these are based on planktonic cells (Bjarnsholt, 2013). There are many researches suggesting biofilm-based diagnostic for determining suitable antibiotic for treatment of biofilm related infections. There is significant increase in the concentration of antibiotic needed in which biofilm is considered for determination of minimum eradication or inhibition concentration.

The Calgary method has been used to demonstrate that the clinical *P. aeruginosa* strains which were resistant to ciprofloxacin (MIC 2 μg/ml) and sensitive to

meropenem (MIC≤ 0.25 µg/ml) by standard antibiotic susceptibility testing using the broth dilution method had an increase in antibiotic concentration of 2-4 fold and 64-128 fold for ciprofloxacin and meropenem respectively (Hassan et al., 2020) needed to bring the about the same effect.

The latest research on biofilm studies are directed to characterising the three dimensional structure of biofilm providing more factual idea about the interaction of antimicrobial drugs and biofilm structures. To better understand biofilm one paper studied the formation of in vivo-like biofilm structure by *P. aeruginosa* in porcine model of cystic fibrosis lung infection (Harrington et al., 2020).

Biofilm formation in bacteria is facilitated by quorum sensing and in recent trend a group of researchers are interested in quorum quencher as a way for eradiation of biofilm. The area such as inhibition of signal generation, interference with signal dissemination, blocking signal receptors and inhibition of signalling response system are still under research. These elements called quorum quenchers are being used as an adjunct to antibiotic to increase antibiotic effectiveness and to control antibiotic resistance. Several candidate drugs have been identified and are at various stages of drug development. Most of these fascinating findings are still under research.

1.3 Research Hypothesis

1.3.1 Null hypothesis:

Presence or absence of single key regulatory gene can affect the physiology of biofilm formation in *P. aeruginosa.*

1.3.2 Alternative hypothesis:

Presence or absence of a single key regulatory gene cannot affect the physiology of biofilm formation in *P. aeruginosa*.

1.4 Objectives:

1.4.1 General Objective

The general objective of this research is to make a thorough study of biofilm regulatory genes in Gram –ve bacteria and come up with a novel gene which have role in regulation of biofilm in certain pathway.

1.4.2 Specific Objectives

I. Screen *P. aeruginosa* isolates and characterise them for the further study.

II. Search for the specific genes which have role in biofilm formation and antibiotic resistance.

III. Isolate and identify *P. aeruginosa* for carbapenem resistance and screen the isolates as biofilm formers.

IV. PCR detection of desired gene in the carbapenem resistant isolates and characterize whether it has a role in biofilm formation or not.

V. Study the cloning and colony PCR technique to preserve the PA1961 gene in compitenent *E.coli* cells.

1.5 Rationale of the study

We try to discover a novel gene which has role in biofilm formation and antibiotic resistance in biofilm forming Gram negative bacteria. Bacteria must have undergone evolution of new genes in the process of turning themselves sessile from the planktonic life-style or modified the expression level of certain gene. The search for novel genes usually focuses on the formation of new coding sequences. Non the less equally important in the evolution of novel functional genes are the formation of regulatory region which control the role of gene (Ponce et al., 2012). Here we try to focus on the search for a regulatory gene having role in biofilm mediated antibiotic resistance in multi-drug resistant bacteria.

Antibiotic resistance is a global problem and Nepal is not exception to it and biofilm aided antibiotic resistance has dreadful consequences. Many hospitals face biofilm-related problems while dealing with chronic wound in admitted patients. The data from two research articles showed among Gram negative and Gram positive clinical isolates from wound, the frequency of biofilm formation was as follow (Neopane et al., 2018) &(Baniya et al., 2017):

Table 1.1: Prevalence of biofilm in Nepalese hospitals

Biofilm formation method	**Prevalence of Biofilm former**	**Organism**	**Total**	**Research place and time**
TCP	30 (69.8%)	Gram positive *S. aureus*	43	Chitwan Medical College and Teaching Hospital, 2018.
TM	28 (65.1%)			
TM	23 (27.05%)	Gram negative *P. aeruginosa*	85	Bir Hospital from July 2013 to May 2014
Congo red agar	13 (15.29%)			
TM	7 (14%)	Gram negative *Acinetobacter spp.*	50	
Congo red agar	5 (10%)			

Note:

TCP= Tissue Culture Plate

TM= tube adherence Method

These data suggest that, in spite of the various antimicrobial therapies available, the management of bacterial wound infections remains problematic (Schierle et al., 2009) because of underlying problem of biofilm.

Biofilm is responsible for chronic bacterial infection, contamination of medical devices, deterioration of water quality and the contamination of food. While research data shows prevalence of biofilm in hospital infection, Nepal lags behind in considering the role of biofilm in non-healing infections.

1.6 Scope of the study

The number of bacterial infections that involve biofilms varies depending on the reporting agency, with estimation being around 65% of all infections according to the Center for Disease Control (CDC), and 80% according to the National Institutes of Health (NIH).The role of biofilm in the progression of the wound that do not heal is well established especially in the burnt wound patient. The break thorough in the field of biofilm is yet to be found. Many researches in this field are going on worldwide to supplement the antibiotic chemotherapy for the eradication of biofilm based infection. Area of research vary from the development of methods for quick identification of biofilm forming microorganism like Calgary biofilm devices, developing three dimensional in vivo model for correct assessment of susceptibility to antibiotics, microscopic diagnostic approaches and molecular gene level understanding of biofilm formation mechanism. All of these study focuses on efficient eradication of biofilm from infection and to better understand the biofilm forming process.

As the resistance to antibiotic is a global problem, biofilm based antibiotic resistance is also becoming a burning topic. Future breakthrough in biofilm holds one of the key to get ahead in antibiotic resistance.

CHAPTER 2

LITERATURE REVIEW

2.1 *P. aeruginosa*

P. aeruginosa is an organism which possesses threat to human health only in immuno-compromised humans. Largely associated with infection in cystic fibrosis , they are considered opportunistic pathogen with innate resistance to many antibiotic & disinfectant . *P. aeruginosa* is a good model organism for biofilm study since they occasionally produce cell-to- abiotic surface attachment organ like pili and settle as sessile form during their cycle of life.

Tam and colleagues in their study of 2008 showed mortality of *P. aeruginosa* bacteremia due to isolates that had reduced susceptibility to piperacillin/tazobactam (PTZ) (MIC, 32 to 64 µg/mL) but reported as susceptible based on the "previous" CLSI breakpoints ; seven patients received empiric therapy with this drug. Thirty-day mortality was 85.5% in the group treated with PTZ and 22.2% in the control groups who had received other drug therapy (p=0.004).

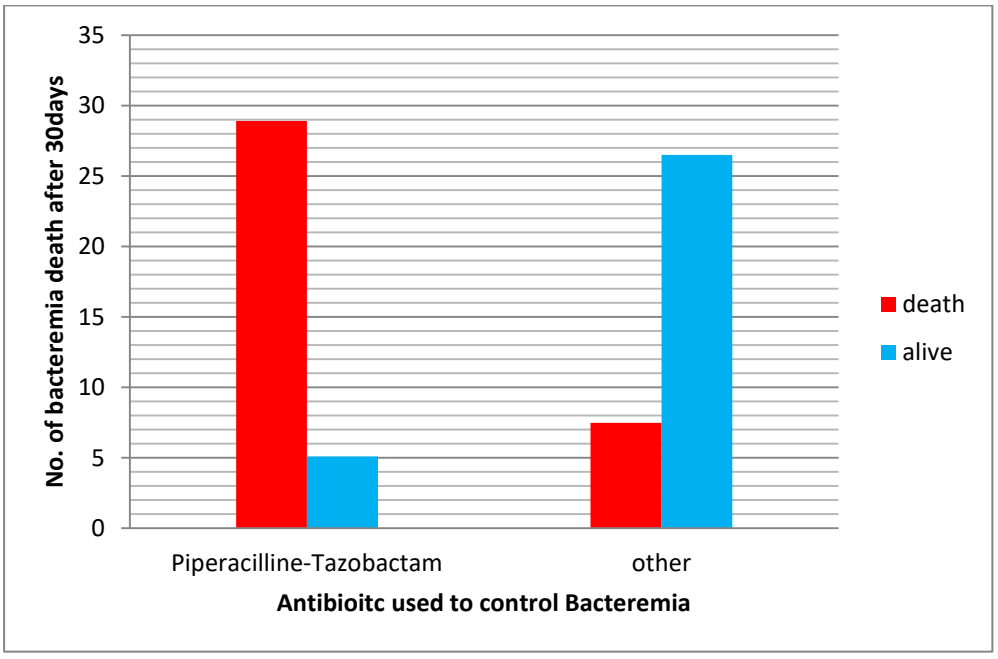


Figure2.1: Death of patient in retrospective study in patient using piperacillin-tazobactam due to reduced susceptibility of *P. aeruginosa*

2.1.1 The increasing resistance of *P. aeruginosa* to different classes of antibiotics:

P. aeruginosa has been a benchmark to check the activity of antibiotic classes. It is taken as if the antibiotic show activity against *P. aeruginosa* then it will most probably affect other Gram- negative bacteria. In the class of antibiotic Penicillin is the first class. Piperacillin antibiotic under sub-class Ureidopenicillin show activity against *P. aeruginosa*. Another class β-lactam/ β-lactamase inhibitor show promise against *P. aeruginosa*. Piperacillin-tazobactam has effective activity against *P. aeruginosa.* It has developed resistance against other antibioitcs under Penicillin and β-lactams with β-lactamase inhibitor. In other word sub-classes penicillin, aminopenicillin, carboxypenicillin, penicillinase-stable penicillins under penicillin class have no effect in *P. aeruginosa*. Popular antibioitics like penicillin, penicillin G, penicillin V, procrain penicillin, benzathine penicillin falls under penicillin major subclass. Amoxicillin, ampicillin falls under aminopenicillin. Azlocillin, mezlocillin under ureidopenicillin. And cloxacillin, dicloxacillin, methicillin, nafcillin, oxicillin under penicillinase stable penicillin. Amoxicillin-clavulanic acid, ampicillin-sulbactam, ceftaroline-avibactam, ceftazidime-avibactam, ticarcilin-clavulanic are other names under β-lactam/β-lactamase inhibitor that has lost their effect over *P. aeruginosa*.

Some *P. aeruginosa* strain has developed to be known as the pan-resistant group of microorganism. Public Health England (PHE) defines a pan-resistant infection as a bacterial resistant to every antibiotic it tested as part of its standard antibiotic panel. Practically it implies the infection is resistant to all commonly used antibiotic. The reason for the resistance to multiple classes of antibiotics is due to several mutations that upregulate AmpC β-lactamase and efflux, decreased cell wall permeability through alterations or loss of porins, and alters in the gyrase enzyme target to convey fluoroquinolone resistance. In addition, *P. aeruginosa* clones have also disseminated to many parts of the world that have acquired plasmid-mediated

resistance to several drug classes carried on integrin gene cassette that encode metallo-β-lactamases and multiple aminoglycoside modifying enzymes (e.g., MBL producing *P. aeruginosa*).

2.2. Genetic basis of resistance to major antibiotic classes

The study of bacterial physiology, pharmacology of antimicrobial drugs, and molecular biology of infectious agent reveals the mechanism of bacterial antibiotic resistance (Procop, et al., 2006). Bacteria show incredible resilience and rapid adaptation to develop resistance under selection pressure from antibiotic use. Antibiotic resistant gene may be resided on the chromosome or the plasmid. If it is located in plasmid the transfer of antibiotic resistant gene becomes much easier as compared to when it is located in the chromosome.

Although bacteria can show antibiotic resistance by mutation in chromosomal gene or inductive expression of a latent chromosomal gene, the transfer of one or more antibiotic resistance gene and exchange of genetic information is a more common phenomenon. Among different methods of gene transfer (transformation, transduction and conjugation), the most common method by which resistance genes are transferred is conjugation. Most commonly, antibiotic resistance genes are carried on plasmids as part of a transposable genetic element called a transposon or "jumping gene" (Procop et al., 2006). The antibiotic resistance genes located in the plasmid are disseminated to intra- or inter species more commonly among *Enterobacteriaceae, Pseudomonas* and anaerobic species (Procop et al., 2006).There is a study that shows transfer of antibiotic resistance determinants across a major barrier between Gram positive and Gram-negative bacteria but the reverse has not been found commonly (Courvalin, 1994).

Antibiotic genes show two types of expression pattern, constitutive and inducible. Some antibiotic genes are expressed constitutively regardless of exposure to antibiotic while others are expressed only when induced by the presence of sub-inhibitory concentration of antibiotic. The location of many β-lactamases of Gram-negative bacteria is the chromosome and show constitutive expression, but they may be induced to express even greater level of enzymes when exposed to β-lactam

agents. These bacterial enzymes inactivate this major class of antibiotics by cleaving the amide bond in the four-atom β- lactam ring structure (Procop et al., 2006) .

Finally, some resistance mechanisms are expressed homogenously, while others are expressed heterogeneously. While taking sampling of the bacteria showing heterogeneous antibiotic resistant pattern error on reporting can occur, while if it is expressed homogenously such error would be minimised during sampling.

2.3 Role of alginate in biofilm formation

Alginate (hetero-polymer of beta -1,4-linked D-manuronic and D-guluronic acid) constitute the major component of *P. aeruginosa* biofilm matrix (Davies et al., 1993; Govan & Deretic, 1996). While some non -mucoid strain's biofilm composition do not contain polysaccharide but form biofilm by exo-polysaccharide(Deretic et al., 1990).

Study shows mutation in muc loci affect transcription of algD and algR gene and produce alginate in mucoid strains of *P. aeruginosa* which is dependent on environmental stimuli (Deretic et al., 1990). Specifically, mutation in muc loci (muc-2, muc-22 and muc-23) affect transcription of algD and algR in environmental dependent manner. Alginate producing strains are characterized by their mucoid appearance. Most CF patient have mucoid colony but scientist have found mucoid colony to be present in laboratory isolates as well which were indistinguishable from CF isolates(Fyfe & Govan, 1980). Actually, it is the environmental condition that affect algR and algD transcription which transform the bacteria into mucoid type.

2.3.1 Different environmental stimuli affecting algR gene regulation in *P. aeruginosa*

A. Nitrogen concentration / Nitrogen source:

It discusses the responsiveness of the algD and algR promoters to nitrogen limitation. Strain PAO568 and PAO578 harbouring plasmid pPAMO3 (an algD-xylE transcription fusion) or pPr 1 (algR-xylE transcription fusion) were grown on minimal medium

with the different nitrogen source (ammonia, potassium nitrate, or potassium nitrate + glutamine). Cells were harvested and CDO activity was determined. Here more CDO means algD gene expression while less CDO means algR gene expression . PA0568 had mutation in muc-2 while PA0578 had mutation in muc-22. Different site mutation resulted in same environmental cue (different nitrogen source to have different effect in mucoidy. PA0578 cells were mucoid in nitrate containing media i.e. both potassium nitrate or potassium nitrate +glutamine while PA0568 were mucoid in media with only nitrate i.e. potassium nitrate and in all other i.e. ammonia and potassium nitrate +glutamine stayed non-mucoid.

B. Salt concentration

Different response of muc strains to increase in salt concentration was determined. Strain PAO568, PAO578 and PAO579 were used. Added NaCl resulted in the increase of NaCl concentration in LB as indicated i.e. 0mM, 90mM, 300mM. PAO568 was mucoid on +300mM but non-mucoid on other media. PAO579 was non-mucoid on +300 NaCl. PAO578 was non-mucoid in all media.

Note: PAO568, PAO578 and PAO579 had mutation in muc-2, muc-22 and muc-23 respectively.

Different mutations in the muc loci determine the type of response of algD and algR promoters to environmental stimuli.

2.3.2. Early assumptions that alginate production is related to regulation by

Alginate production was successfully related to algR gene. Now, considering the homology between PhoM and algR which are both response regulator. A new set of experiment was designed. PhoM region gene include NtrC, OmpR, etc. as being regulated by corresponding sensor/second component (e.g. NtrB, EnvZ etc.). In search of sensor for algR gene it was found algZ was corresponding for algR as sensor. Now, algZR two component system is one of the two component system found active in *P. aeruginosa* and said to be absolutely required for two phenotype :twitching motility and alginate production (Deretic et al., 1990).

2.4 Surface attachment interactions signals biofilm formation cascade to initiate

The study by Palmer and White, 1997 shows the processes involved in the early stages of biofilm formation. It suggested two interactions cell to cell and cell to surface interaction were involved in the initiation of biofilm formation. When the study was carried the genes involved in the process were not identified. They observed the physical and chemical determinants that still hold true .They suggested that biofilm formation occurs through set of common steps applicable to all. According to them the types of organic molecules on the substratum plays role in initial attachment. First one layer of cells attaches to a surface and grows, from which additional cell start to form resulting in bulk. Hence, biofilm formation is a four dimensional process which is similar to organ development(Palmer & White, 1997). Two structural entities related to surface attachment has been found important during biofilm formation in *P. aeruginosa* viz. flagella and type IV pili shown by different mutant study. Studies have developed mutant defective in surface attachment on abiotic surface called sad mutant (surface attachment defective). They show crc (catabolite repression control) gene is missing in sad mutant.While *P. aeruginosa* uses succinic acid preferentially over glucose as carbon source, this genes silencing cause it to use organic acid and glucose simultaneously as carbon source. Crc genes are also shown to have effect in twitching motility and in particular the regulation of genes required for type IV pili synthesis (O'Toole et al., 2000). These study conclude crc to be involved in signal transduction which according to carbon availability switch from initial attachment activation resulting in biofilm formation or otherwise .High carbon availability induces bacterial cell to form biofilm and remain attached to the site.

Physical contact to surface can also induce gene expression in some cases of *P. aeruginosa.* Particularly the expression of gene algC for exopolysaccharide (EPS) production is found to be up-regulated three to five fold in recently attached cells vs their planktonic counterpart(Davies et al., 1993; Davies & Geesey, 1995).

2.5 Role of intracellular c-di-GMP concentration in transformation of *P. aeruginosa* from sessile to biofilm nature

One of the regulator controlling cell adhesiveness and biofilm formation is (c-di-GMP). It is the regulator of flagella and pili formation process which are required for cell surface appendage mediated bacterial aggregation (Valentini & Filloux, 2016).

The high concentration of c-di -GMP in the cell extract and incidence of biofilm forming characteristic and low concentration of c-di -GMP associated with motility has been demonstrated in many bacterial species, e.g.-*P aeruginosa* (Roy & Sauer, 2015).

The history of c-di-GMP shows that it was discovered as an allosteric effector involved in cellulose synthesis in *Gluconacetobacter xylinus* (Ross et al., 1987), where as it role in biofilm formation have been revealed later on .

2.5.1 The c-di-GMP metabolism

The enzyme diguanylate cyclase (DGCs) is involved in the synthesis of c-di-GMP. In Wsp signal transduction system of *P. aeruginosa* the surface bound WspA sense surface attachment and transduce signal by phosphorylating WspR which then catalyse the c-di-GMP synthesis (Valentini & Filloux, 2016). The two molecules of GTP aggregate to form c-di-GMP.

Whereas the enzyme involved in breakdown is phosphodiesterases (PDEs) (Valentini & Filloux, 2016). Bioinformatics tools, biochemical and structural approaches reveal the catalytic domains of DGCs and PDEs.

The DGC has GGDEF domain which are abundantly found across all branches of the phylogenetic tree of bacteria. These are regulatory domain linked to many roles like phosphorylation receiver or oxygen sensing domain. They play critical role in exopolysaccharide synthesis, biofilm formation, motility and cell differentiation.

2.5.1.1 DGCs: GGDEF domain

DGCs, GGDEF domain works as homo-dimer. The catalytic site lies in junction between two domains which have GGDEF which contain site for GTP attachment and have role in conversion to c-di-GMP with use of Mg^{+2} as co-factor. The first characterised DGC of *P. aeruginosa* is WspR which is shown to have REC-GGDEF domain (Valentini & Filloux, 2016).

2.5.1.1.1 DGC of *P. aeruginosa* named as Wsp for the phenotypic characteristics wrinkly spreader phenotype it renders to the organism

The naming of diguanylate cyclase in *P. aeruginosa* is according to physical characteristic or appearance it renders to the strain. The DGC is named Wsp for wrinkly spreader phenotype. The wrinkly spreader phenotype is found to be regulated by WspR and WspF. The WspR increase production of exopolysaccharide while WspF conrols WspR activity to reduce exopolysaccharide production (Hickman et al., 2005).

WspR is a protein, which act as response regulator is known to be necessary for wrinkly spreader phenotype appearance. It is a part of chemosensory operon and found to be activated upon phosphorylation triggered by sensing favourable surface for growth (Güvener & Harwood, 2007; Hickman et al., 2005). Not only phosphorylation, second step involves subcellular WspR oligomerisation resulting in cluster formation. Oligomer of WspR further increase DGC activity (Valentini & Filloux, 2016). The phosphorylation mediated activation occurs at Asp residue while feedback inhibition occurs when excess c-di-GMP binds to the I-site of WspR protein (De et al., 2008). A globular dimer (active form), a tetramer (more active) and an elongated dimer (less active due to c-di-GMP binding) (Valentini & Filloux, 2016).

2.5.1.2 PDE: EAL or HD-GYP Domain Protein

Another study deals with the features of PDE (Phosphodiesterase) having EAL domain which act on the response regulator of two component system to regulate virulence (antibiotic resistance and curli formation). In EAL all three amino acid are not critically important for function. While glutamine residue is essential, the conversion of alanine into tyrosine or valine (ETL and EVL) is allowed for smooth

functioning of PDEs. RocR is the PDE, which act as response regulator in RocSAR signaling system (Kulasekara et al., 2005). It consists of sensor kinase RocS1 and two response regulators RocA1 and RocR. The phosphorylation site of RocR is the cheY domain. The PDE protein struggles for phosphorylation with RocA1, which ever gets phosphorylated results in subsequent outcome (i.e. cup fimbrae or type III secretion system activation) (Kulasekara et al., 2005; Rao et al., 2008).

2.5.2 Molecular mechanisms involved in c-di-GMP mediated regulation in bacteria

The functions of bacterial cell are affected using different mechanism because of c-di-GMP. It may be any of these mechanism (i) allosteric regulation, (ii) changes in related transcriptional factor or (iii)direct interaction with non-coding RNA affecting gene expression (Hickman, Jason; Harwood, 2009).

2.5.3 The c-di- GMP receptors and effectors

The c-di-GMP receptors are those molecules which detect c-di-GMP levels in cells and translates the information into activation of specific cellular response or signaling. While c-di-GMP effectors senses level of c-di-GMP by allosterically being bound by them and causing response.

2.5.4 PelD as c-di-GMP receptor containing GGDEF domain similar to DGCs

PelD is an example of c-di-GMP receptor having GGDEF domain bound by c-di-GMP at the motif for polysaccharide production (Lee et al., 2007). PelD has GAF domain, a degenerated GGDEF domain but conserved I-site (Valentini & Filloux, 2016). Further studies required for its role in Pel polysaccharide production mechanism to be revealed.

2.6 FleQ protein whose function as activator of flagellar gene expression and repressor of Pel and Psl genes is further regulated in the presence of c-di-GMP

Studies have shown FleQ to be enhancer binding protein which has role in flagella production and motility. FleQ-like enhancer binding protein is rarely found in bacteria that directly repress transcription. It is evident that fleQ is involved in motility. Also they show wrinkly phenotype evident of biofillm formation. It is found that fleQ is master activator of flagella gene expression and found to be affected in case of high c-di-GMP in cell.

The high concentration of c-di-GMP in cell is found to bind the FleQ and convert its repressing property for pel, psl and cdr gene to neutral or these gene are freely expressed. These genes are involved in production of exopolysaccharide and adhesin hence, the wrinkly phenotype emerge in fleQ mutants. Studies have shown that fleN and ATP might be required for repression of pel by FleQ. Whereas when c-di-GMP get involved the attachment of FleQ as repressor in pel promoter is disrupted and pel can freely express it (Fig appendix 2). Hence role of fleQ as activator or flagella along with repression of pel gene is evident.

2.7 Role of regulator (which contain GGDEF or EAL domain) in transition of lifestyle from planktonic to sessile and vice-versa beyond c-di-GMP particularly regulating cup genes

The overall life style change related to the transition to a sessile lifestyle is contributed by different physiological changes. Extracellular appendages (e.g., flagella and pili) constitute one of the major physiological change that is involved in initial surface attachment (O'Toole & Kolter, 1998). Similarly production of polysaccharides such as Pel and Psl (Friedman & Kolter, 2004), help to form envelop and protect the bacterial colony (Branda et al., 2005). The genes involved in these physiological changes are probably not constitutively expressed due to which the microbes can switch from sessile to free-living. Like other genes they might be controlled by regulators or have regulatory system that control their expression in response to environmental cues. In *P. aeruginosa* many of these regulators are two-

component systems. The genome of *P. aeruginosa* encodes 127 members of TCSs (Rodrigue et al., 2000).

2.8 Two-component system

Bacteria are always sensing the surrounding environment to cope with their environment or host environment. One of the mechanism with which bacteria sense the outer environment is by the use of two component regulatory system (TCS). This system consists of a sensor (histidine kinase) capable of auto-phosphorylation on a conserved histidine residue, and a response regulator on which the phosphate is transferred.

2.8.1 The Roc1 system was the first TCS found to control cup genes in *P. aeruginosa*

The TCS Roc1 (regulation of chaperon-usher pathway genes also called cup genes) encodes components of a machinery allowing assembly of fimbrae. The Roc1 locus (PA3946-PA3948) was identified in transposon screening of mutant which was active for cupB or cupC gene before transposon insertion was done. Later, these locus was found to contain genes encoding one sensor kinase and two response regulators (Kulasekara et al., 2005) viz. RocS1 , RocA and RocR. Among them RocS1 is membrane bound unorthodox senior kinase. RocA1 is conventional response regulator and RocR an output domain with EAL motif. It is found that RocR code for phosphodiesterase whose activity degrades the second messenger c-di-GMP (Rao et al., 2008).

2.8.2 Roc S1A1R system in *P. aeruginosa* was described to control cupC gene expression

The study by Kulasekara and colleagues were able to show that RocS1 sensor signals through both RocA1 and RocR, which have opposite effect in cupC gene expression. The link of RocS1 to RocA1 stimulates cupC gene expression and promotes fimbriae synthesis and biofilm formation while the link with RocR reduces cupC gene expression. The activity of RocS1, whether it will activate RocA1 or RocR is still under

research. While the signalling molecule for Roc TCS is unknown, the downstream signal transduction to RocA1 or RocR could be controlled by their relative abundance or efficiency of anyone being more than other (Mikkelsen et al., 2011).

2.8.3 A paralogue of Roc1 system was found in P. aeruginosa as Roc2 system

Though it was thought, the sensor and response regulators were working as couple and cross-regulation was unlikely to avoid unwanted cross-talk. Further exploration of Roc2, a homologue to Roc1 showed potential cross-regulation between Roc2 component. Their study demonstrated RocS1 and RocS2 could downstream their sensory signals to either RocA1 or RocA2 response regulators. And the response arising from the activation of either the response regulator was completely different. RocA1 induce cupC gene expression while RocA2 inhibit mexAB-oprM which is responsible for efflux of antibiotic. They concluded that biofilm formation and antibiotic resistance can be downstreamed in opposite way (i.e. RocS1 activate curli formation and promote biofilm formation while decreasing mexAB-oprM activity makes them susceptible to antibiotic or decrease their resistance to antibiotic (Sivaneson et al., 2011b).

2.8.4 Cross-regulation between the components of TCS has been reported

Sivaneson et al, study showed that cross regulation occurs between Roc1 and Roc2 signaling pathways. RocS2 and RocS1 control cupC gene expression in a RocA1-dependent and RocA2-independent manner.

2.8.5 RocS1/A1 and RocS2/A2 form a complex and interactive network:

As the cross regulation between the component of TCS have been reported, a detail study of Roc1 and Roc2 system was devised using the *E. coli* bacterial two-hybrid method as previously described (Karimova et al., 1998). RocS1 and RocS2 being unorthodox sensor their conserved domain Hpt (Histidine Phosphotransfer) was extracted and cloned into vector pKT25 (Sivaneson et al., 2011a). Another conserved domain D2 receiver of RocA1 and RocA2 response regulator were cloned on pUT18c.

The interaction between Hpt and D2 was documented. It was found that RocS1 and RocS2 could interact with both response regulators.

To further support the result, RocS1 and RocS2 interaction was only limited to RocA1 and RocA2 was proved, when sensors interactions was studied using a control that was unrelated to Roc system i.e. D2 domain of unrelated response regulator TrpO/ PA0034. Similarly, *P. aeruginosa* sensor kinase RocS1 and RocS2 were also substituted with GacS and data collected. It was shown that GacS firmly interact with GacA but showed no interaction with RocA1 or RocA2. Hence, cupC gene expression was initiated by both RocS1 and RocS2 activating the RocA1. RocS1 and RocS2 activating RocA2 is another part of this TCS.

While RocR is a negative regulator of Roc System and further studies revealed that it also negatively regulate Roc2 response. Similar cloning mediated technique has been devised to verify these phenomena.

2.9 Role of response regulator in effflux pump suppression was identified: RocA2 controls the expression of the mexAB-oprM genes:

The continued explorations of Roc TCS lead to the discovery of RocAZ role in expression of the efflux pump mexAB-oprM gene. It is seen that rocAZ has no role in regulation of cupC gene. Instead RocS1 and RocS2 relay signal to RocA2 response regulator to repress the mexAB-oprM gene. These results were reported first using microarray analysis and then validated again using RT-PCR.

2.9.1 Roc2 is a signalling pathway involved in antibiotic resistance:

The mexAB-oprM is a multidrug efflux system known to contribute to the natural resistance to several antibiotic in *P. aeruginosa.* Sivaneson et al further analysed whether the repression of RocA2 will contribute to antibiotic susceptibility in vitro. RocA2 mutant PAK and PAK strain were subjected to antibiotic treatment by antibiotic disc diffusion and minimal inhibitory concentration value was also determined. both of these strain had overexpressing RocS2. In both antibiotic disc

diffusion assay and MIC RocA2 mutant showed higher resistance to antibiotic than PAK parental strain. Further they tested mutant RocA2 or PAK having plasmid that does not carry carbenicillin resistant gene but gentamycin resistance cassette (pMMB67-rocS1). The result showed rocA2 mutant had decreased susceptibility towards β-lactam carbenicillin, when compared with parental PAK strain.

This showed mutant RocA2 was unable to efficiently produce efflux pump mexAB-oprM resulting in increased susceptibility to antibiotics including β-lactams such as aztreonam and carbenicillin, which are specific substrates of the mexAB-oprM efflux pump.

2.10 Biofilm formation associated with decrease in antibiotic resistance shown in Roc system

The counterintuitive observation that cupC gene expression and hence biofilm formation via RocA1 is linked with the repression of antibiotic resistance mechanism via RocA2 is worth a discussion.

As characteristic of biofilm nature unfolds to scientific community there seems to be challenges for accepted norms that biofilm formation supports or induce antimicrobial resistance. Cepas in his 2018 study have shown that multi-drug resistant isolates do not show a trend towards biofilm production. But biofilm formation and antibiotic resistant properties are random events. The evident cascade of events in TCS involving Roc1 and Roc2 system shows biofilm formation circuitry is linked with repression of efflux pump. Although biofilm mediated resistant to antibiotic is not attributed only to efflux pump but greater impact is due to difficulty for antibiotic molecules to penetrate the matrix and enter bacterial cell (De Kievit et al., 2001). It is shown that gfp fused mexAB-oprM were down regulated in positive biofilm forming bacteria. Another study by Vettoretti and collaborators suggested that greater portion of biofilm forming *P. aeruginosa* isolates (28%) from cystic fibrosis patient showed higher sensitivity to β-lactam antibiotic ticarcillin (Vettoretti et al., 2009). In this study it was shown that the lack of resistance is essentially associated with a non-functional MexAB- OprM pump. In other words, in CF patients *P. aeruginosa* strains grow predominantly as biofilms in the respiratory

tract (Bjarnsholt, 2013) and at the same time large numbers of these strains appear to become more susceptible to antibiotics. There is not known benefits that bacteria can get by suppressing the already functional efflux system. While the speculation that bacteria might have lost their properties of efflux pump while they shift from host environment to laboratory condition was not eliminated because down regulation of mexAB-oprM suppression was conformed using gfp mexAB-oprM fusion and found to be down regulated only in vitro strain after two-three subculture in laboratory environment. This might cause bacteria to switch off efflux pump because they are now in lavish environment which have rich supply of nutrients and good environment to florish amid the antibiotic testing results in death.

Further, other pumps may still provide reasonable level of antibiotic resistant to cope with selection pressure imposed by the antimicrobial treatments given to CF patients in vivo. Finally other mechanisms have been shown to contribute biofilm resistance to antibiotic, such as the NdvB-dependent production of cyclic glucans (Mah et al., 2003; Sadovskaya et al., 2010).

2.11 LTTR protein family

Study by Stragier et al, 1983 identifies an LTTR protein that regulates the gene encoding the enzyme necessary for the synthesis of amino acid lysine. This is the same experiment which identified the first LTTR in bacteria responsible for the regulation of last enzyme for the synthesis of Lysine amino acid, and hence from the same regulatory protein the name of the family Lys type transcriptional regulator was kept.

2.11.1 DNA Bending Mechanism of LTTR regulation:

DNase I protection assay has shown that the LTTRs protect 50 to 60 bp of DNA establishing themselves as functionally active (Muraoka et al., 2003). The extent of DNA binding to the Transcription Factor Binding Site (TFBS) is dependent on the binding of the co-inducer. Only after the Apo form binds to the ribosome binding site, the Activation binding site (ABS)is occupied (Tropel & van der Meer, 2004).

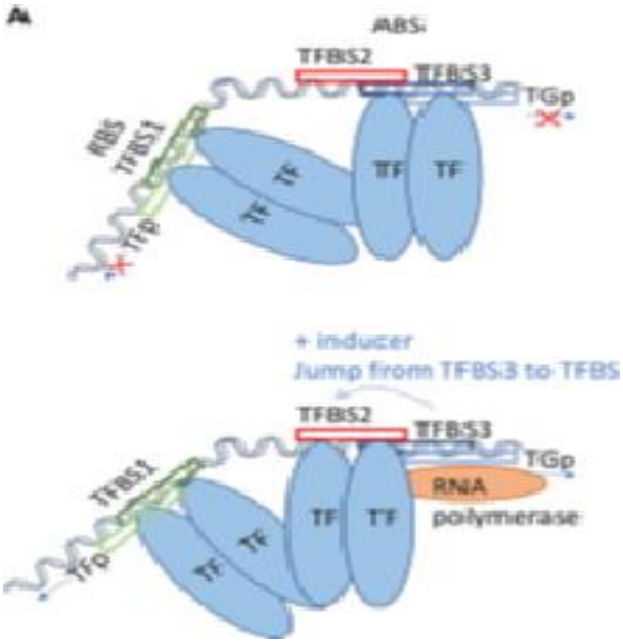


Figure2.2 Diagram representing the mechanism of LysR regulation. Two dimers of transcription factors bind at RBS and ABS for the regulation. The promoter sequence of LysR and transcription binding site (TFBS1) are present in RBS while the ABS contains TFBS2 and TFBS3 overlapped with the target gene promoter (TGp). The second dimer binds with TFBS3 during repression and dimers of dimers of TF cause the bending of DNA fragments. However, the dimers of dimer slide from TFBS3 to TFBS2 due to the conformational change in the presence of the inducer. This way the DNA bending is released and TGp can access RNAP and initiate TG transcription.

The bending of DNA allows ABS and RBS to come in contact and form an active tetrameric structure. A higher-order complex containing RNA polymerase is formed which initiates the transcription. The position of the LTTR binding region and the scale of DNA bending has no significance in determining the nature of LTTR, whether transcriptional activator or repressor.

2.12 Roles of LTTRs in therapeutics, diagnostics and vaccine development

2.12.1 Vaccines:

Various research has highlighted the importance of global transcriptional regulators in the development of attenuated and protective vaccines (Maddocks & Oyston, 2008). This is applied to the BGC vaccine development (Keller et al., 2008). Daughter strains obtained from the numerous subcultures of *Mycobacterium bovis* at varying laboratory conditions displayed different efficacy.

The bacterial strains with attenuation in particular genetic backgrounds can be identified with LTTRs transcriptional studies. Those strains can be used as a vaccine candidate. The attenuated and protective LTTR in one bacterium can have applications in other bacteria with orthologous LTTRS as LTTRs are found in diverse bacterial species (Maddocks & Oyston, 2008)

2.12.2 Therapeutics and diagnostics:

The study of the MarR family of global transcriptional regulators shows the application of global regulators in therapeutics. The MarR transcriptional regulators contain winged Helix-Turn-Helix (wHTH) and are distributed in prokaryotes and archaea. They are responsible for the regulation of multi-drug resistance genes in bacteria such as *E. coli*, *P. aeruginosa*, and *B. subtilis*. The multi-drug resistant strain within a species can be screened by studying MarR-like transcriptional regulator. This is applicable to the rapid screening of clinical isolates (Wilkinson & Grove, 2006).

CHAPTER 3

MATERIALS AND METHODS

3.1 Selection and identification of strain

In order to identify the isolates, they were subjected to standard bacteriological technique. The bacteria were inoculated in Luria-Bertani (LB) broth. After overnight incubation in static condition at 37ºC, turbid bacterial growth was inoculated on nutrient agar plates. After incubations the bacterial colonies appeared on the plates were used for Gram's staining.

3.2 Gram's staining

Gram's staining was done from the isolated colony from NA plates. Those bacteria colonies which were smooth, large and translucent were chosen. Greenish blue pigments secreting colonies were given special consideration. A thin smear of sample was made on clean glass slide. Then it was kept for drying and then heat fixed using slight flame of burner. Then, sequentially stained with CV, Gram's iodine for 1 min (in between washed by tap water). After that it was decolorized by adding Gram's decolouriser drop by drop until the purple colour stopped flowing. It was then washed immediately with water. Then the slide was drained and finally stained with safranin for 1 min. washed with water, blotted to make it dry and finally observed under microscope using oil immersion.

3.3 Biochemical test for identification of *P. aeruginosa*

Different biochemical tests were performed in order to identify the *P. aeruginosa* isolates.

3.3.1 Citrate utilization test

Inoculum was streaked over the slant of Simmon's citrate agar in a test tube. The cultured test tube was then incubated at 37ºC for 24hrs. The positive result was confirmed by the growth on the slant and change in color of medium to blue.

3.3.2 Indole test

Bacteria were transferred into the tube containing SIM medium by the inoculating needle straight down into the tube going about two-third and then pulling the needle straight out. It was incubated at 35-37 ºC for 24-48 hrs.

3.3.3 Methyl Red test (MR test)

The MR/VP broth was inoculated with the pure culture of the organism and incubated at 35°C for 48 to 72 hrs. 5 drops of MR reagent was added to the broth.

3.3.4 Voges-Proskauer test (VP test)

Pure culture of the test organism was inoculated in MR/VP broth and incubated for 24 hrs at 37ºC. Reagents A (Barritt's reagent) was added followed by Reagent B (α-napthol) shaking the tube gently after each addition to bring organism in contact with atmospheric oxygen and the tube was allowed to remain undisturbed for 10-15 min.

In case of positive reaction the media colour changes to pinkish red on the surface of the medium, while negative result shows yellow colour on the surface.

3.3.5 Triple Sugar Iron test

Isolated colony was inoculated with a sterile straight wire by first stabbing the centre of the medium to the bottom of the tube and then streaking the surface of the slant and incubated at 37°C for 18-24 hrs.

3.3.6 Oxidase test

Oxidase paper from Hi-media Company (containing tetramethyl-p-phenylene-diamine dihydrochloride) was used to perform oxidase test. A colony was then picked with a sterile tooth pick and smeared on the oxidase paper. A positive result shows intense deep-purple colour, appearing within few seconds. But coloration after 60 second or more was considered negative result.

3.3.7 Catalase test

Freshly prepared 3% hydrogen peroxide was kept in a slide. The pure isolated colony was then spread over the hydrogen peroxide. The positive test results in bubble formation.

3.4 Confirmatory test for *P. aeruginosa*

3.4.1 O/F Test

This method utilized a semi-solid tubed medium containing the carbohydrate (usually glucose) together with a pH indicator. If acid was produced only at the surface of the medium in open tube without paraffin oil the attack on the sugar was oxidative. If acid was produced in both the open tube without paraffin oil and closed tube with paraffin oil the attack on the sugar was fermentative. The acid production was indicated by the change in media colour from green to yellow.

Caution: The media should have pH of 6.8±0.2 while keeping for autoclave.

3.4.2 Nitrate Broth test

The nitrate present in the medium is first converted to nitrite in the form of NO2- which combine with reagents A (sulphanillic acid) and reagent B (alpha-napthylamine) to produce pink color. If there is no reduction, the color of broth remains unchanged after addition of reagent. While in the case of *P. aeruginosa,* there is complete reduction of nitrate to nitrogen gas or ammonia. Hence, there is no presence of NO_2^- and the test is then further conformed for complete oxidation of Nitrate by addition of zinc powder. In the presence of zinc powder NO_3 gets reduced to NO_2^- and gives pink color. If no pink color is produced after addition of zinc powder then it shows there is complete reduction of Nitrate to Nitrogen gas or ammonia.

Caution: The test should be carried out in the absence of any metal during incubation.

3.4.3 Growth in Cetrimide agar

Cetrimide is a selective agar used for the Gram-negative bacteria like P. aeruginosa which contain the detergent called cetrimide acting as the selective component against alternate microbial flora. *P. aeruginosa* can grow on cetrimide while other Gram-negative organism cannot. Another component glycerol and $MgCl_2$ including cetrimide also enhances the production of *Pseudomonas* pigments such as pyocyanin & fluorescin which show characteristic blue-green & yellow green colour respectively.

3.5 Antibiotic susceptibility test

Imipenem-10 mcg (IPM) and Meropenem-10 mcg (MRP) were used to perform antibiotic susceptibility testing according to the CLSI guidelines by Kirby-Bauer disc diffusion method using Mueller Hinton agar (MHA). The diameter of each zone of inhibition (in mm) was measured and value compared to zone size interpretation chart.

3.6 Biofilm formation assay

The protocol for Microtitre disc biofilm formation assay was published earlier (O'Toole, 2010) and cut off value calculation was based on Stepanovic et al, 2007. In brief, overnight cultures of bacteria were grown at 37ºC under static condition. The OD of overnight grown culture was measured at 600nm and diluted to an OD_{600} of 0.08-0.1 in LB. Prior to seeding on 96 well microtitre plate, the culture was re-suspended at a 1:100 dilution in fresh LB medium for biofilm assays. The 100µl of diluted culture were inoculated in the wells of flat bottom, 96 well, polystyrene, non-coated microtitre plate. This was incubated at 37°C under static condition for 18-24 hrs, covered with lid or aluminium foil. Planktonic bacteria were discarded and the plate was washed using tap water in water-tube 2-3 times. The plates were left in inverted position to remove excess water. Then when dry, 0.1% CV was added to each well and then they were left at RT for 10-15 min. Then again the plates were washed 3-4 times with water by submerging in a tub of water, shook well and then blotted extensively on a stack of paper towel to get rid of all excess dye. Then the plate was turned upside down and allowed to dry for few hours. For qualitative

assays, the wells can be photographed when dry. For quantitative assay 125µl of 30% acetic acid in water was added to each well and incubated for 10-15 min. Then 125µl of the solubilised CV was transferred to a new flat bottom microtitre disc. The absorbance was then read in an ELISA plate reader at 551 nm using 30% acetic acid in water as blank. The absorbance associated with glacial acetic acid, i.e. blank, was subtracted from each sample absorbance value to correct for background effects. *P. aeruginosa* strains were considered biofilm producers when their corrected absorbance values were greater than three times standard deviations from the mean corrected absorbances of the uncultured media OD value.

3.7 Cut off value calculation

We need to define the cut-off value to interpret the obtained value each time we perform the biofilm assay which separates biofilm producing from non- biofilm producing strain. Cut-off value is defined as 3 times standard deviation of negative control (uncultured LB) above the mean OD of negative control. i.e.:

ODc= average OD of negative control+ (3X SD of negative control)

For easier interpretation of the results, strains may be divided into the following categories code (0) for no biofilm producer, (+ or 1) for weak biofilm producer,(++ or 2) for moderate biofilm producer and (+++ or 3) for strong biofilm producer. This was based upon the previously calculated OD values : OD ≤ODc=no biofilm producer; ODc ≤ OD ≤2XODc = weak biofilm producer; 2XODc ≤ OD≤_4XODc = moderate biofilm producer; 4XODc ≤ OD = strong biofilm producer(Stepanović et al, 2003).

3.8 Sequence Similarity Search using BLAST

The whole genome of *P. aeruginosa (*PAO1) was searched for genes from other species *like Esherichia coli, Salmonella* etc to have homology with some genes of *P. aeruginosa,* using both protein and nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

We wanted to find the region which comes out to have similarity from *P. aeruginosa* genome to lie in some hypothetical proteins whose role is unknown in *P. aeruginosa*.

There are hundreds if not thousands of hypothetical proteins in *P. aeruginosa* genome.

The candidate genes from *Escherichia coli, Salmonella* etc for BLAST were identified by reading different research articles. The research articles were searched in NCBI PubMed section using key words biofilm, *Pseudomonas aeruginosa* and antibiotic resistance.

3.9 Primer designing using NCBI Primer Tools

Sequences of primer for PA1961 gene of *P. aeruginosa* was designed using Integrated DNA technology's Oligoanalyzer tool. Forward and reverse primers were designed with desiarable qualitites to have higher GC/AT ratio and minimal probability of primer dimerization. The NCBI database (http://www.ncbi.nlm.nih.gov/) was used to gather information of the gene and to predict the PCR product sizes. Primers were ordered from Macrogen Inc., South Korea.

3.10 Primer designing and setting parameter

Primer with restriction site BamHI (forward primer) and NdeI (reverse primer) was designed for PA1961 gene.

While calculating the melting temperature the restriction sites were excluded (using oligocalc from www.northwest.edu, Kibbe WA. 'OligoCalc: an online oligonucleotide properties calculator'. (2007)

Primer sequence

Forward primer: 5'- AACCCAAACCAT ATGCCCCACGACCTCAACGACC -3'

Reverse primer: 5'- TTAAGGATCCTT ACTCGCGGGCGCGGC -3'

Table 3.1: Forward and reverse primer for PA1961 gene amplification

Primer Type	Length	Start	Stop	Tm	GC%	Self-complementarity	Self- 3' complementarity
Forward Primer	22	2145745	2145724	67	55.88	2.0	0.0
Reverse Primer	15	2144812	2144826	62	86	6.00	4.00

* The underlined sequences are restriction enzyme sites for subsequent cloning.

Product size: 960 bp

3.11 Preparation of genomic DNA

Bacterial genomic DNA extraction protocol was carried out as previously described (William et al., 2004).

For the extraction of genomic DNA, bacteria were grown in 5ml of Luria- Bertani (*LB*) broth media and incubated at 37ºC until the culture was saturated. From the primary culture, 1.5 ml of culture was centrifuge at 12000 rpm for 2 min. Then the supernatant was discarded and the pellet was re-suspended well in 567µl (TE) Tris-EDTA buffer by repeated pipetting. Then 30 µl of 10% Sodium dodecyl sulfate and 3 µl of 20 mg/ml proteinase K was added, mixed and incubated for 1-2 hour at 56 ºC. After incubation, 100 µl of 5M NaCl was added and mixed thoroughly. Afterwards 100 µl of CTAB/NaCl solution (heated to 65ºC) was added, incubated at 65ºC for 10 min. After incubation, equal volume of chloroform: isoamyl alcohol (24:1) was added,

mixed well and centrifuged at 12000 rpm for 10 min. Aqueous supernatant was transferred to a fresh tube, leaving the interface and organic solution behind. Then, 0.5µl of phenol:chloroform:isoamylalcohol (25:24:1) was added, mixed well and centrifuged at 12000 rpm for 10 min. Aqueous supernatant was then transferred to clean micro-centrifuge tube. Finally 0.5µl of chloroform:isoamylalcohol(24:1) was added, mixed well and centrifuged at 12000 rpm for 10 min. Then the supernatant was transferred to a clean centrifuge tube. To the supernatant, 0.6 times volume of isopropanol was added and mixed gently until the DNA precipitate (e.g., if 400µl of aqueous phase is transferred, add 240µl of isopropanol). Then it was incubated at -20ºC for 2hrs to overnight. Then the tube was centrifuged at 13500 rpm for 15 min to remove isopropanol. 1ml of 70% ethanol was added and centrifuged at 13500 rpm to wash the salt away from the DNA. The ethanol was pipette out without disturbing the DNA pellet and the excess of ethanol were allowed to dry at room temperature. This may take some time (20 min. to several hours, depending on humidity). Finally, the pellet was re-suspended in 25µl of TE buffer and stored at 4ºC.

3.12 Agarose gel electrophoresis

0.8% of the agarose gel was prepared by dissolving 0.8 gram of agarose in 100 ml of 1X TAE buffer and boiled to dissolve completely. After cooling, 5µl EtBr (ethidium bromide) was added from the stock of 10mg/ ml. The gel was poured onto gel casting tray and allowed to set. The extracted DNA was mixed with loading dye and was loaded in the well of gel. Electrophoresis was carried out at constant volt (80V) for 1 hr. After completion, the gel was observed under UV transilluminator. Then pictures were taken using gel documentation system.

3.13 Polymerase chain reaction

After DNA extraction polymerase chain reaction was carried out. The thermal cycler was programmed using annealing temperature from primer manufacturer. 5X master-mix was used from Solisbiodyne. This master-mix contains thermostable Taq polymerase FIREPol®, $MgCl_2$, dNTPs and buffer with detergent. We just added template, primers and water. Overall 5X master-mix was diluted using 2.5µl in 25µl total volume making final concentration 1X.

The mixture contained:

I. FIREPol DNA Polymerase
II. 5X reaction buffer B: 0.4M Tis HCl, 0.1M $(NH_4)_2SO_4$, 0.1% w/v Tween-20.
III. 7.5mM dNTPs of each 1X PCR solution-200µM dATP, 200µM dCTP, 200µM dGTP and 200µM dTTP.

Primers were ordered from Macrogen Inc., South Korea having final concentration 100pM/µl each, after addition of suggested volume of nuclease free water (see annex 6). Template concentration was determined to be at least 40ng/µl using nano drop machine.

3.14 Optimisation of PCR for amplification of PA1961 gene:

The thermal cycler was programmed according to the annealing temperature of the primer, the length of the gene to be amplified and the concentration of the primer was fixed so that no primer dimer was formed.

Table 3.2: Optimisation of PCR for PA1961 primer:

S.No.	Process/Reagent	Condition/ Concentration
1	Annealing Temperature	57-55-60-67 ºC
2	Primer Concentration	10X-8X-5X-3X-1X (1X=1pM/µl)

The PCR amplification process was optimized at 67ºC annealing temperature and 10X dilution of both reverse and forward primer.

Table 3.3: Reaction components of PCR, 5U master mix used.

S.N.	Reagent	Final Concentration	Volume (µl)
1	Template	40ng/µl	2.5
2	Forward Primer	10pM/µl	2.5
3	Reverse Primer	10pM/µl	2.5
4	Master Mix	1U	5
5	Nuclease Free Water		12.5
	Total		25

3.15 PCR amplification of PA 1961 gene with restriction site

The primer for the PA1961 gene was designed using the gene sequence of PA1961 gene from the NCBI whole genome of PAO1. The addition of BamHI site in forward and in NdeI reverse primer was done so that the gene can be retrieved by using these restriction enzymes in future.

Table 3.4: PCR condition for PA1961 gene

Stage	Name	Cycle	Temperature(ºC)	Time (min)
1	Initial Denaturation	1	95	5
2	Denaturation	30	95	0.5
3	Annealing		67	1
4	Extension		72	1
5	Final Extension	1	72	10
6	Hold	1	4	Hold

3.16 Cloning

Cloning was done to preserve the gene PA1961. PCR product of PA1961 was cloned in competent cells DH5α using cloning vector plasmid pCR® 2.1 from Invitrogen company.

Taq polymerase adds deoxyadenine residue (A) to the 3´ ends of PCR products, independent of template. The linearized vector supplied with this kit has single 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

3.17 Steps in cloning strategy

3.17.1 PCR amplification of target gene

PCR was carried out and the PCR product was analysed in agarose gel electrophoresis. Then it was stored at -20 ºC until further use.

3.17.2 Cloning into pCR® 2.1 plasmid vector

Table 3.5: Preparation of ligation mixture

Components	**Volume**
Sterile water	3 µl
5X ExpressLink™ T4 DNA Ligase Buffer	2 µl
pCR2.1vector (25 ng/µL)	2 µl
Fresh PCR product (~10 ng)	2 µl
ExpressLink™ T4 DNA Ligase (5 units)	1 µl
Total volume	**10 µl**

1. The ligation mixture was incubated at room temperature for 15 minutes.
2. Then the ligation mixture was centrifuged briefly and then placed on ice.
3. Then we proceed to one shot chemical transformation.

3.18 One shot chemical transformation

1. The provided competent cells with the kit (Top 10) and lab made competent cells DH5α cells were used for transformation.

   Note: SOC media and positive control was provided in the kit along with one shot ® chemically competent cells.

2. 50µl vials of competent cells (One shot ® or DH5α) was thawed on ice for each transformation.

3. 2 µl of ligation mixture was pipetted into the competent cell and mixed by stirring gently with pipette tip.

4. The vial was then incubated on ice for 30 minutes. The remaining ligation mixture was stored at -20 ºC.

5. Then the cells were heat shocked for 30 seconds at 42 ºC without shaking. Then immediately it was transferred into ice.

6. Then 250µl of super optimal broth with catabolite repression (SOC media) was added to each vial.

7. Then the vials were shaken at 37 ºC for 1hr at 225 rpm in a shaking incubator.

8. Then 50 µl of each transformation vial was spread in LB agar containing X-gal and 100µg/ml ampicillin. IPTG was also used if DH5α was used as competent cell. The volume of cells spread on plate was changed according to the cell's concentration. Two plates were used with different volumes so that at least one plate had well-spaced colony.

9. The plates were then incubated at 37 ºC overnight. Then the next day it was kept at 4 ºC for few hours to allow proper colour development.

3.19 Schematics of cloning strategy

The cloning of PA1961 gene was done using the pCR 2.1 vector.

Step 1. Ligation of PCR product into pCR 2.1 vector

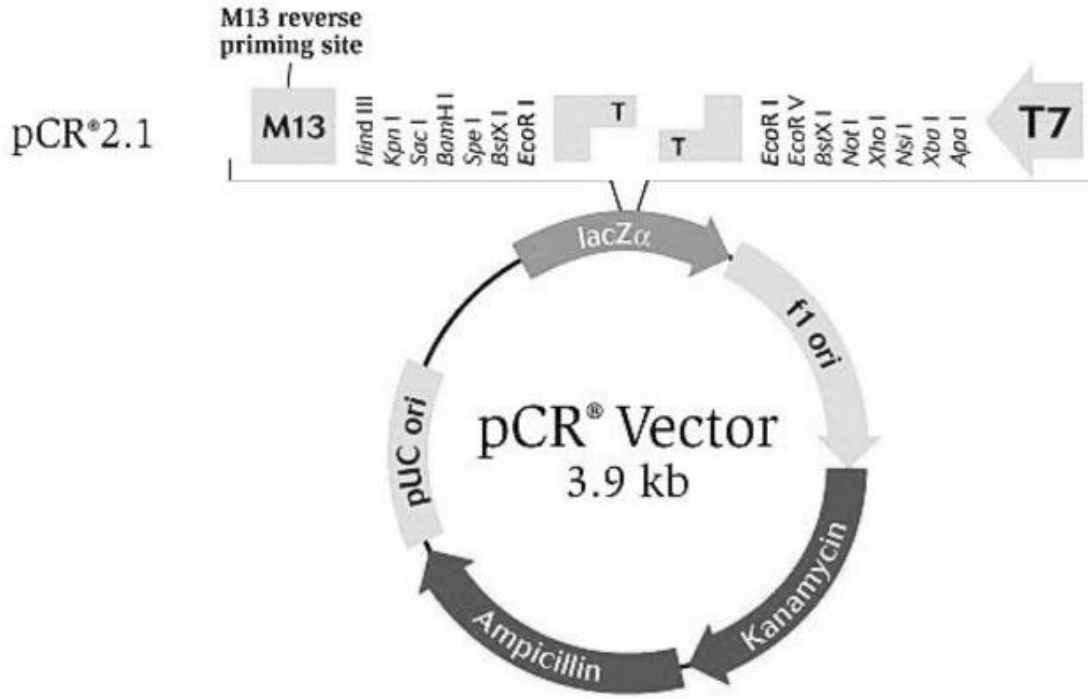
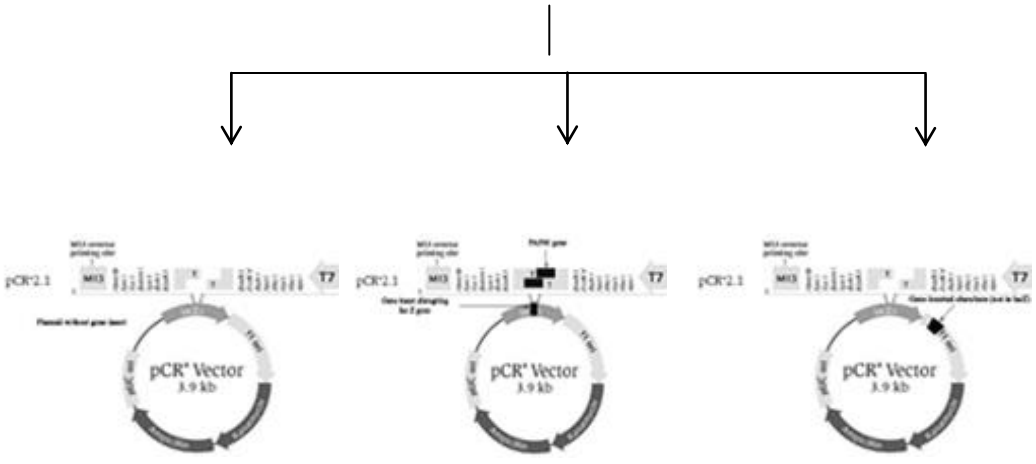


Figure 3.1: pCR vector with lacZ gene insertion site and kanamycin and ampicillin resistant gene.

The PCR amplified gene was ligated in to the vector using T4 DNA ligase in 15 min incubation of PCR product in ligation mixture.



Result 1 Result 2 Result 3

Possible result in ligation mixture

Step 2. Transformed into competent cells:

Then transformation was carried out in the tube containing 50µl of competent cells by using heat shock method.

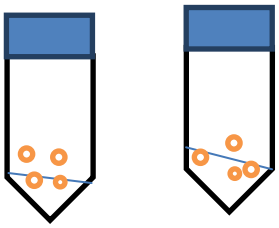
Requirements:

Ice cubes

Water bath at 42°C

Shaking incubator at 37°C with 225 rpm

50µl of Top 10 competent cells provided in the kit.



50µl of competent DH5 α prepared in the laboratory (CDBT)

Step 3. Blue White method for selection of cells carrying recombinant plasmid:

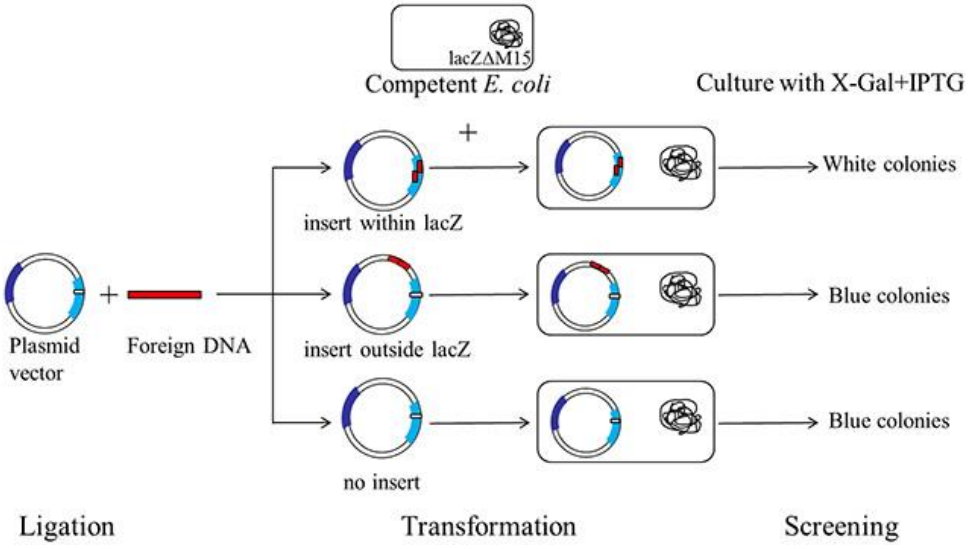


Figure 3. 2: Schematic of cloning procedure.

3.20 Colony counting

The colonies were counted to be between 150-200 colonies. But colour differentiation on X-gal plate was not observed.

3.21 Colony PCR

Colony PCR was performed to conform the transformed cells have the insert:

3.21.1 Steps in colony PCR

1. 30-40 µl of nuclease free autoclaved water was kept in eppendorf tubes.
2. With the help of autoclaved tooth pick, several bacterial colonies are transferred into the eppendorf tube. It was then mixed well by stirring the tooth pick in the nuclease free water.
3. For convenience and accuracy colony can be picked from replica plated disc but for our experiment here we took the colony from transformed colony plates.
4. Then 15µl of the diluted colony in nuclease free water was used in place of template and nuclease free water in the PCR reaction mixture as shown in Table 3.3 above.
5. The PCR reaction cycle was set with an initial 15 min of heating as the first step followed by the steps shown in Table 3.4.
6. After the PCR reaction, the result was analysed by running the DNA in agarose gel electrophoresis using a 100 bp ladder.
7. The presence of DNA band near the location of the size of the target gene indicates the presence of the target gene in the plasmid.

3.22 Preparation of Glycerol stock of the *P.aeruginosa* isolates and transformed *E.coli* DH5α:

1. Pure isolates of *P. aeruginosa* were picked from an overnight culture in a nutrient agar and diluted in fresh LB.
2. In case of transformed E. coli DH5α, the colony corresponding to the presence of insert in Colony PCR was then diluted in fresh 5 ml LB for glycerol stock preparation.
3. Then it was incubated at 37ºC overnight or until turbid growth was evident.

4. Then 1.5ml culture was transferred in eppendorf tube and centrifuged at 12,000 rpm for 5 min. The supernatant was discarded, the process was repeated once again with 1.5ml culture.
5. Then 300µl of fresh LB was added to the cell palettes and incubated at 37^{o}C for 1 hr.
6. After incubation 700µl of autoclaved 50% glycerol was added to the eppendorf making total volume 1000ml.
7. Then the culture was incubated overnight at 37^{o}C.
8. Next day it was taken out from incubator and kept in 4ºC freezer for few hours and then stored at -20ºC with proper labelling.
9. For long term storage they can be transferred to -40ºC freezer for lesser glycerol content (15 to 25%).

3.23 Data management and analysis

All the data were processed using Microsoft excel. The bar-chart was built using excel features. The transeq online platform was used for the retrieval of NhaR DNA sequence from NhaR protein. Subsequently, BLAST program from NCBI was used for the alignment of NhaR DNA sequence to the whole genome of PAO1 and discovery of PA1961 transcriptional regulator region.

CHAPTER 4

RESULTS

4.1 Microbiological analysis

The clinical isolates of *P. aeruginosa* were used from the glycerol stock maintained by Samikshya Kafle, Central Department of Biotechnology. The isolates were revived and biochemically tested for the presence of only *P. aeruginosa.* In this research PAO1, P3, P4, P6, P10, P20, P30 (A), P30 (B), P31 were used as sample *P. aeruginosa* strains.

4.1.1 Colony characteristics

Table 4.1: Colony characteristic of *P. aeruginosa*

Shape	Colour	Odour	Elevation	Consistency	Opacity
Round	Greenish	Grape-like	Convex	Consistent	Opaque

Figure 4.1: *P. aeruginosa* isolate P6 colony grown on cetrimide agar

4.1.2 Gram's staining

Isolates were subjected to Gram's staining in order to confirm they were Gram negative bacteria.

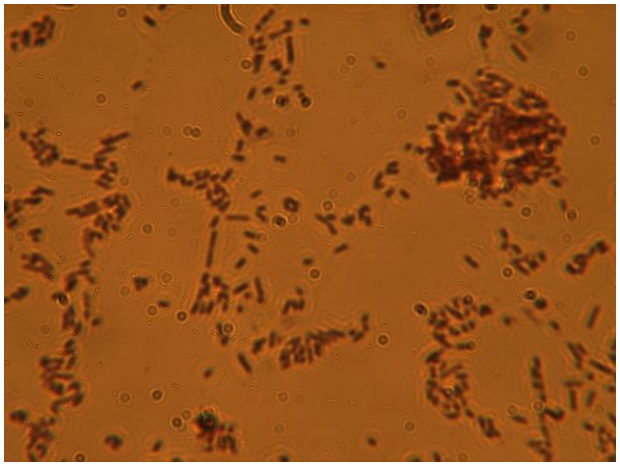


Figure 4.2: *P. aeruginosa* isolate P6 colony grown on cetrimide agar

4.1.3 Biochemical test for the identification of *P. aeruginosa*

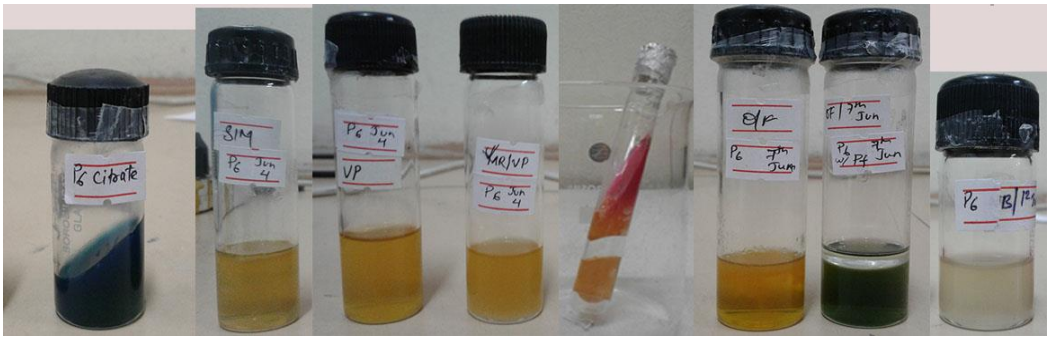


Figure 4.3: Biochemical test of *P. aeruginosa* (P6 isolate)

Table 4.2: Results for biochemical test of *P. aeruginosa*

S.N.	Biochemical test	Results
1.	Citrate Test	Positive
2.	Oxidase Test	Positive
3.	Indole Test	Negative
4.	Methyl red (MR) Test	Negative
5.	Voges- Proskauer (VP) Test	Negative
6.	Triple Sugar Iron (TSI) Test	R/R, alk/alk, H2S Negative
7.	Catalase Test	Positive
8.	O/F Test	Oxidative
9.	Nitrate Test	Positive (shown by no color change even after addition of Zn powder)

4.2 Antibiotic Susceptibility pattern

P. aeruginosa of two types were found: both Imipenem and Meropenem sensitive: PAO1, P3, P4, P6, P10, P30(A), P30(B) and P31 or both Imipenem and Meropenem resistant: P20 but none was found to be Imipenem resistant and Meropenem sensitive or otherwise (i.e. Meropenem resistant and Imipenem sensitive).

Table 4.3: Antibiotic resistance and sensitive percentage

Antibiotics	**$IMP^{S}MRP^{S}$**	**$IMP^{S}MRP^{R}$**	**$IMP^{R}MRP^{S}$**	**$IMP^{R}MRP^{R}$**
Imipenem and Meropenem	87.5%	0%	0%	12.5%

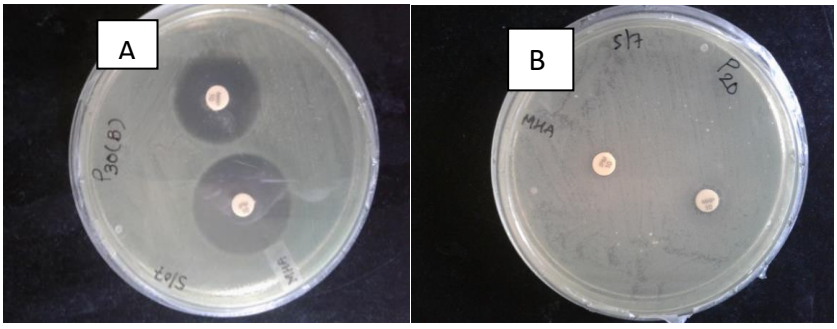


Figure 4.4: Both IMP & MRP sensitive (A) and Both Ipm & Mrp resistant (B)

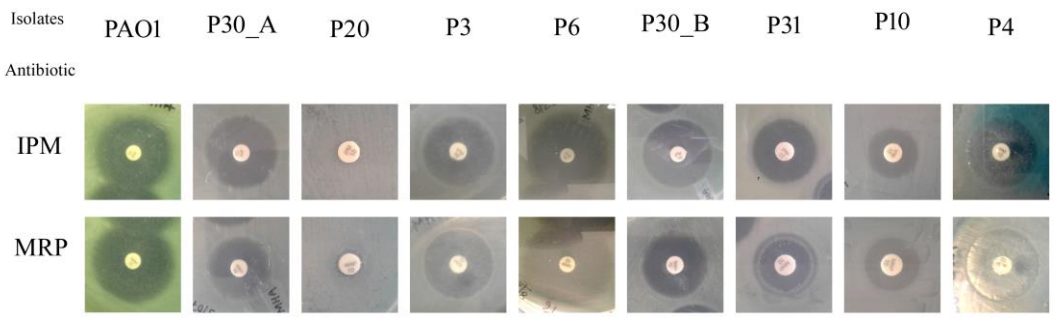


Figure 4.5: Antibiotic disc diffusion assay following guideline of CLSI

Table 4.4: Zone of inhibition shown by different isolates of *P. aeruginosa*

Isolates	Diameter of Zone of Inhibition (in mm)	
	IPM(10mcg)	**MRP(10mcg)**
PAO1	34	33
P30_A	28	23
P20	0	0
P3	27	26
P6	20	22
P30_B	29	24
P31	22	16
P10	16	16
P4	26	25

Note: Data are the average of 3 independent zones of inhibition data.

4.3. Biofilm assay of isolates

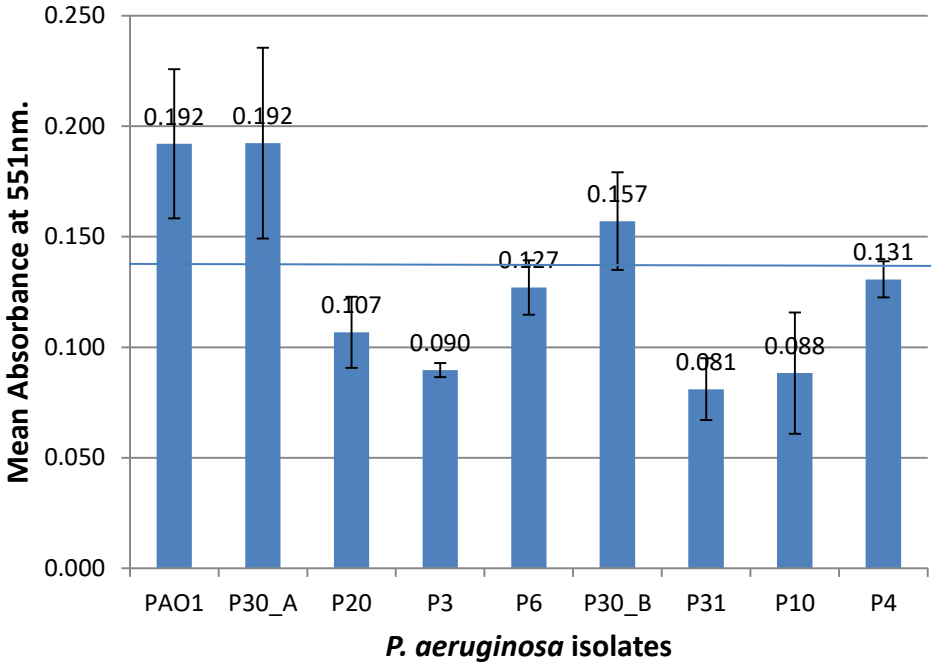


Figure 4.6: Biofilm forming capacity of 8 *P. aeruginosa* isolates with PAO1 as positive reference strain

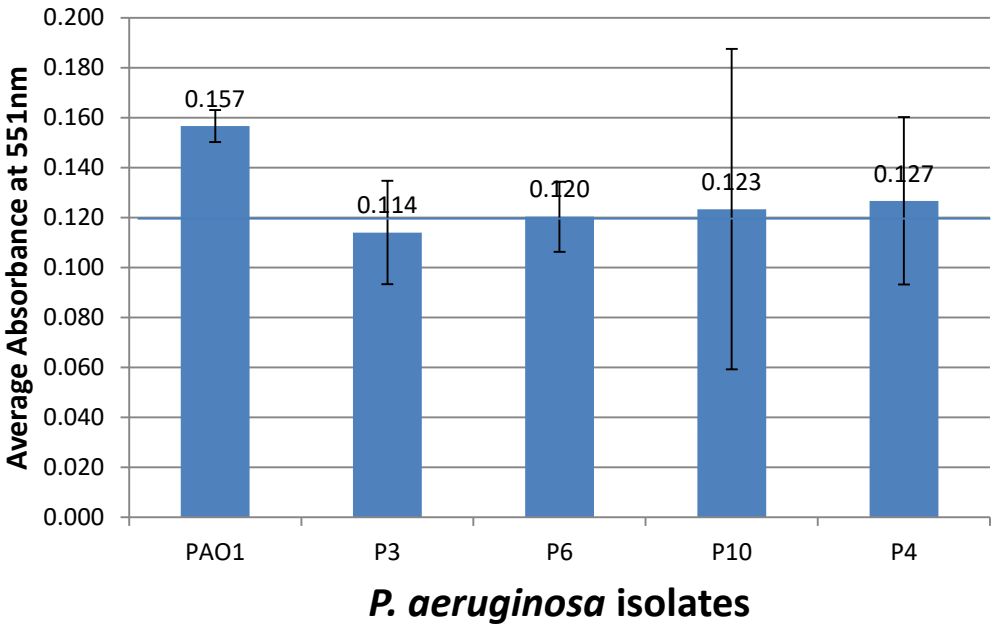


Figure 4.7 Biofilm forming capacity of 5 *P. aeruginosa* isolates with PAO1 as positive reference strain

Out of the eight isolates, one was found to be biofilm producer and seven were biofilm non-producer. While the antibiotic resistant strain P20 was found biofilm non-producer. And the biofilm producer was not antibiotic resistant.

Table 4.5: Summary of biofilm formation assay done at different time

Isolates	**PAO1**	**P30_A**	**P20**	**P3**	**P6**	**P30_B**	**P31**	**P10**	**P4**	**Cut- off value**
Average D	0.192	0.192	0.107	0.089	0.127	0.157	0.081	0.088	0.131	0.168
Biofilm Characteristic	+	+	0	0	0	0	0	0	0	
Average OD	0.157			0.114	0.120			0.123	0.127	0.1417
Biofilm Characteristic	+			0	0			0	0	

4.3.1 Cut- off value calculation

Table 4.6: Cut- off value for *P. aeruginosa* isolates

Triplicate negative control (LB without bacteria) values	0.073, 0.141, 0.133
Mean OD of negative control	0.116
Standard deviation of negative control	0.030
3*standard deviation of negative control	0.090
Cut-off value	0.206
Cut-off – average of absorbance of acetic acid	0.168

4.4 Search for the gene having role in biofilm formation and antibiotics resistance

Related articles in NCBI were searched using the key words biofilm, antibiotic reistance and *P. aeruginosa* for understanding different aspects of biofilm formation and their details were studied. Other online sites like KEGG Pathway, Biocyc, Regulon DB were searched for regulatory genes for biofilm synthesis or regulation.

The protein sequence of NhaR, a LysR transcriptional regulator which responds to elevated [Na+] and alkaline pH and activate the transcription of the pga ABCD operon (Pannuri et al., 2012) was found homologous to the protein translated from PA 1961 gene in *P. aeruginosa.*

NhaR was found to activate pga ABCD operon and promote poly-n-acetyl glucosamine (PGA a polysaccharide adhesin) synthesis.

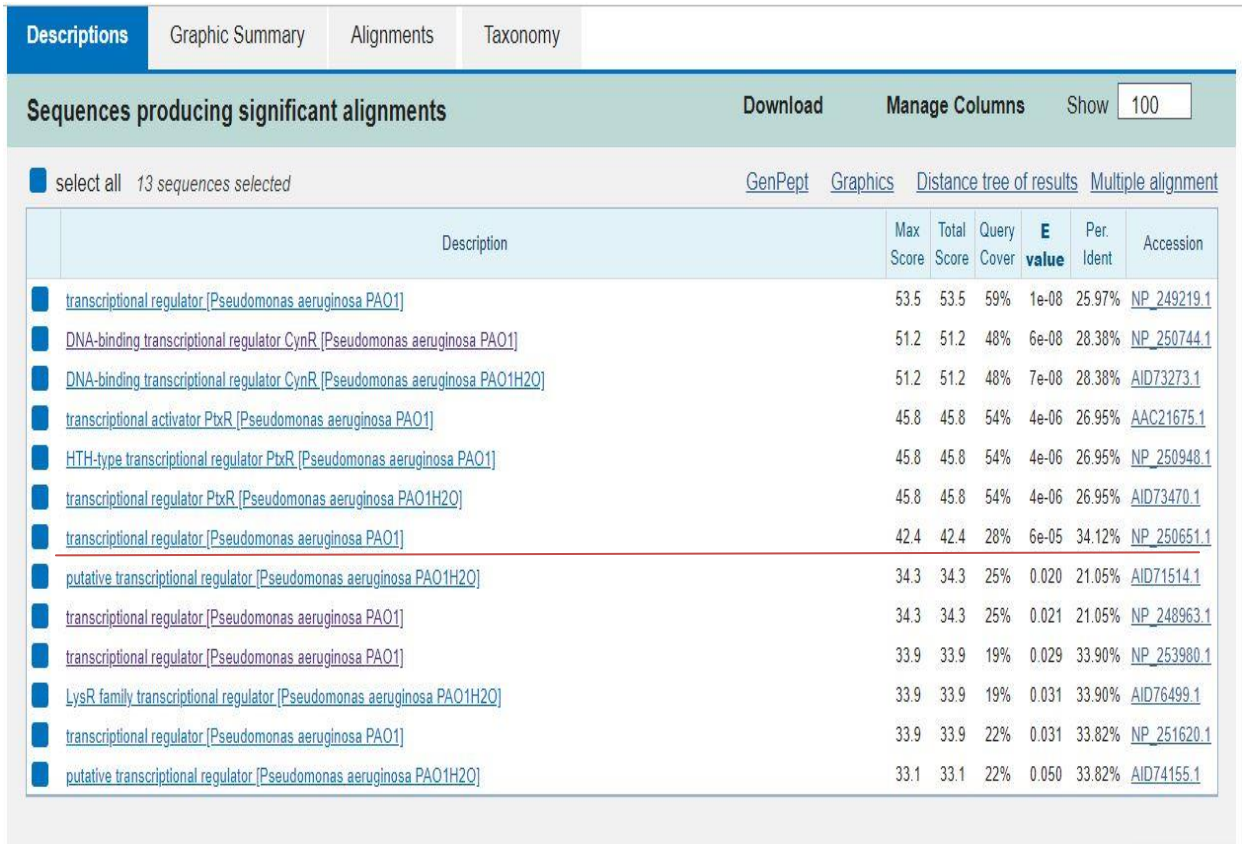
The study showed CsrA represses biofillm formation by repressing the NhaR and hence establish CsrA as a global regulator of stress response system (Pannuri et al., 2012).

Similarly, a study that identifies the LysR-type transcriptional regulator in Enterrohemorrhagic *E. coli* having role in mobility and Type III secretion system.

Hence, we try to find the role of PA1961 in biofilm formation or antibiotic resistance in *P. aeruginosa.*

4.5 Homology search using BLASTp tool in NCBI

1. NhaR gene sequence was retrieved from NCBI gene database.
2. The gene sequence was then converted to protein sequence using transeq tool of ensemble (the first frame was used which gave minimum stop codon)(https://www.ebi.ac.uk/Tools/st/emboss_transeq/).
3. Then the translated protein sequence was used to BLASTp with the whole genome of *P. aeruginosa* (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).
4. There was a hit having maximum score 42.4, total score 42.4, query cover 28%, E-value 6X10^-5, and percentage identity 34.12% having accession NP 250651.1.
5. The associated gene detailed comes out to be PA1961 gene in *P. aeruginosa* PAO1.



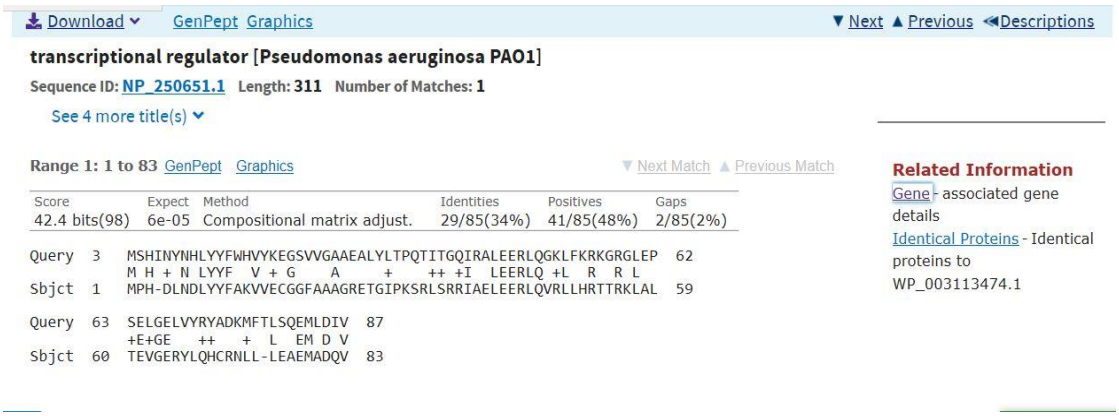
Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Manage Columns Show 100

select all *13 sequences selected* GenPept Graphics Distance tree of results Multiple alignment

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
transcriptional regulator [Pseudomonas aeruginosa PAO1]	53.5	53.5	59%	1e-08	25.97%	NP_249219.1
DNA-binding transcriptional regulator CynR [Pseudomonas aeruginosa PAO1]	51.2	51.2	48%	6e-08	28.38%	NP_250744.1
DNA-binding transcriptional regulator CynR [Pseudomonas aeruginosa PAO1H2O]	51.2	51.2	48%	7e-08	28.38%	AID73273.1
transcriptional activator PtxR [Pseudomonas aeruginosa PAO1]	45.8	45.8	54%	4e-06	26.95%	AAC21675.1
HTH-type transcriptional regulator PtxR [Pseudomonas aeruginosa PAO1]	45.8	45.8	54%	4e-06	26.95%	NP_250948.1
transcriptional regulator PtxR [Pseudomonas aeruginosa PAO1H2O]	45.8	45.8	54%	4e-06	26.95%	AID73470.1
transcriptional regulator [Pseudomonas aeruginosa PAO1]	42.4	42.4	28%	6e-05	34.12%	NP_250651.1
putative transcriptional regulator [Pseudomonas aeruginosa PAO1H2O]	34.3	34.3	25%	0.020	21.05%	AID71514.1
transcriptional regulator [Pseudomonas aeruginosa PAO1]	34.3	34.3	25%	0.021	21.05%	NP_248963.1
transcriptional regulator [Pseudomonas aeruginosa PAO1]	33.9	33.9	19%	0.029	33.90%	NP_253980.1
LysR family transcriptional regulator [Pseudomonas aeruginosa PAO1H2O]	33.9	33.9	19%	0.031	33.90%	AID76499.1
transcriptional regulator [Pseudomonas aeruginosa PAO1]	33.9	33.9	22%	0.031	33.82%	NP_251620.1
putative transcriptional regulator [Pseudomonas aeruginosa PAO1H2O]	33.1	33.1	22%	0.050	33.82%	AID74155.1

Figure 4.8: Protein homology search using NhaR protein as template.



Download GenPept Graphics ▼ Next ▲ Previous ◀Descriptions

transcriptional regulator [Pseudomonas aeruginosa PAO1]

Sequence ID: NP_250651.1 Length: 311 Number of Matches: 1

See 4 more title(s)

Range 1: 1 to 83 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
42.4 bits(98)	6e-05	Compositional matrix adjust.	29/85(34%)	41/85(48%)	2/85(2%)

```
Query  3   MSHINYNHLYYFWHVYKEGSVVGAAEALYLTPQTITGQIRALEERLQGKLFKRKGRGLEP  62
           M H + N LYYF  V + G    A     +    ++ +I  LEERLQ +L  R  R L
Sbjct  1   MPH-DLNDLYYFAKVVECGGFAAAGRETGIPKSRLSRRIAELEERLQVRLLHRTTRKLAL  59

Query  63  SELGELVYRYADKMFTLSQEMLDIV  87
           +E+GE   ++   +  L  EM D V
Sbjct  60  TEVGERYLQHCRNLL-LEAEMADQV  83
```

Related Information

Gene - associated gene details

Identical Proteins - Identical proteins to WP_003113474.1

Figure 4.9: BLASTp result in detail shows 34% identity to PA1961 gene product

4.6 Preparation of genomic DNA

Genomic DNA of 9 *P. aeruginosa* were extracted using standard protocol bacterial genomic DNA isolation using CTAB (William et al., 2004).

4.7 Agarose gel electrophoresis

After the extraction, the genomic DNAs were run in 0.8% agarose gel electrophoresis at 80V for 45 min and the bands were visualized under UV transilluminator. L2, L3, L4, L5, L6, L7, L8, & L9 showing positive band for genomic DNA. Note: L1 was left empty.

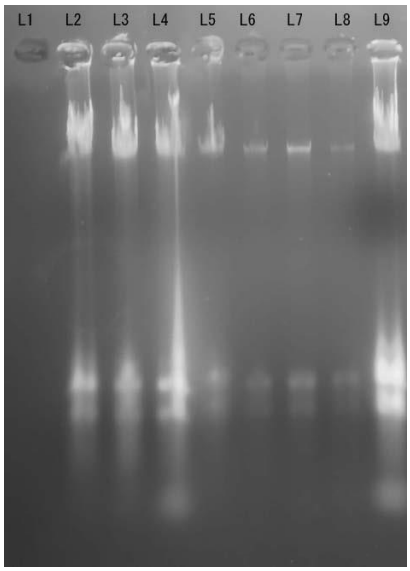


Figure 4.10: Genomic DNA extraction of isolates of *P. aeruginosa.*

4.8 PCR amplification of PA1961 genes in different isolates of *P. aeruginosa*

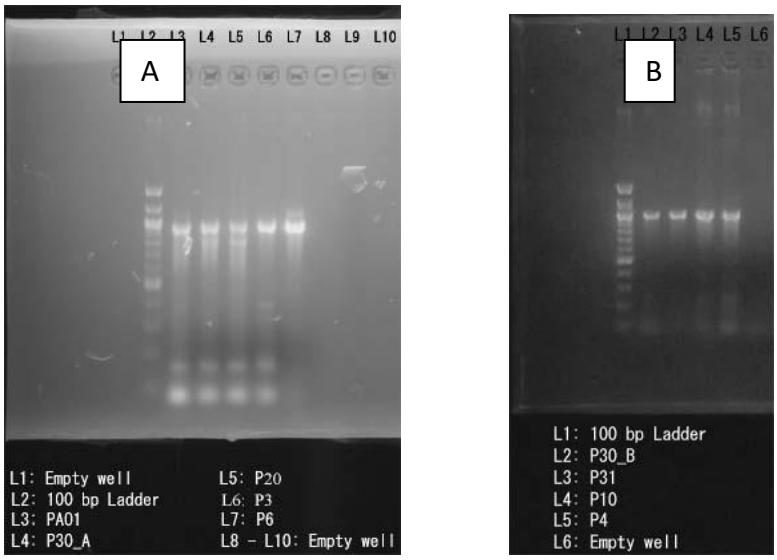


Figure 4.11: PCR amplification of PA1961 using genomes of different isolates as template (A)- 5 isolates & (B)- 4 isolates

Amplification of PA1961 gene was seen in all the isolates, verified by the bright band in 1,000bp using 1kb ladder.

4.9 Cloning

Transform colony on LB agar containing 100µg/ml ampicillin flooded with X-gal and IPTG.

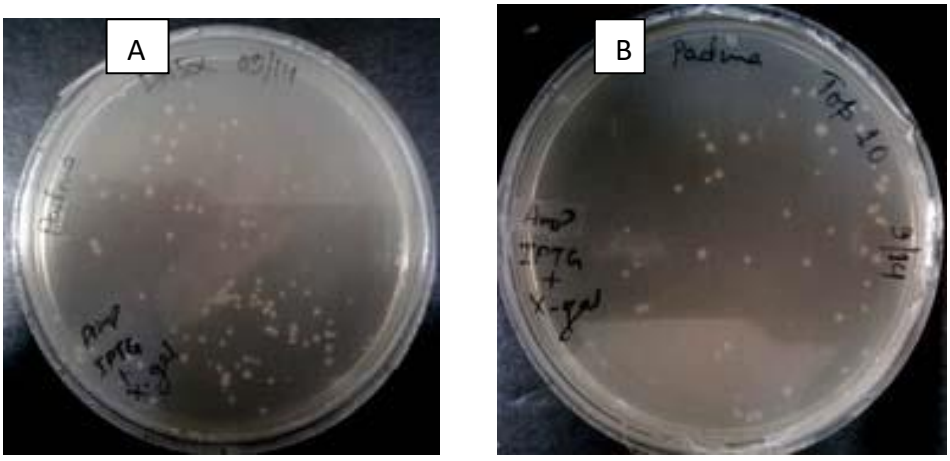


Figure 4.12: Transformation of pCR® vector with PA1961 gene in (A) DH5α and (B) Top10 competent cells

4.10 Colony PCR of transformed cells

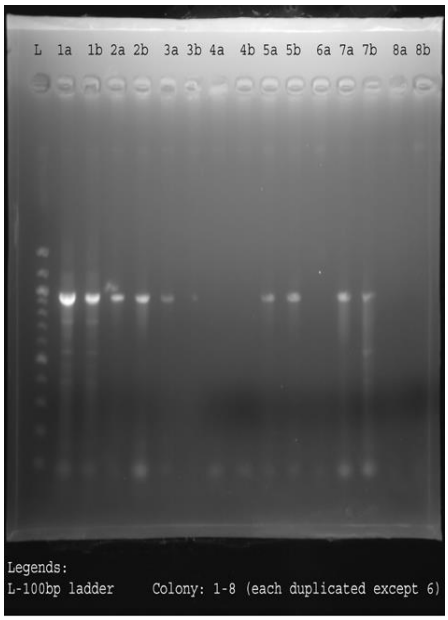


Figure 4.13: Colony PCR of 8 isolated colonies with amplification in 5 colonies

E.coli cells transformed with PA1961 genes was successful shown by large percentage of white colony showing the amplification of PA1961 gene in Electrophoresis after PCR amplification around 1Kb region of reference 100bp ladder.

CHAPTER 5

DISCUSSION

The cases of biofilm related infection in hospital environment, mainly in immune-compromised patient and patient who use indwelling medical devices like artificial heart valves, ventilators, catheters etc are on the rise. *P. aeruginosa* (potential biofilm former) is common bacteria causing such biofilm related infection. In 2017 list of bacteria, published by WHO for which better treatment option are urgently needed, *P. aeruginosa* is of critical priority. In a study carried out in isolates from ventilator associated pneumonia (VAP) patient from intensive care unit, for their potential for biofilm formation and antibiotic resistance, the prevalence of *P. aeruginosa* exceeds other organism. Of the total bacteria isolated from bronchoalveolar lavage (BAL) and deep tracheal aspirate, *P. aeruginosa* (31.0%) was predominant followed by *Acinetobacter calcoaceticus baumanii* complex (16.9%), *Klebsiella pneumoniae* (16.9%), *Citrobacter freundii* (15.5 %) and others (Baidya et al., 2021).

This study examines the antibiotic susceptibility and biofilm formation characteristic of *P. aeruginosa* isolates. As it is found, the frequency of biofilm formation is very much less as compared to previous studies. In our study, Carbapenem antibiotic is found to be effective for inhibition of growth of *P. aeruginosa* bacteria in-vitro and hence the relationship between biofilm formation and antibiotic resistance is not significant. Due to inaccessibility to hospital isolates, we had to use isolates available in laboratory (for biofilm study) which yielded only 8 isolates showing *P. aeruginosa* like biochemical character. So, there was insufficient data for any conclusive relation between antibiotic resistance and biofilm formation.

We want to relate the physiological change (biofilm formation) to some genotypic expression; suppression or enhancement. The aim was to check the role of a potential regulatory gene in biofilm forming characteristic of *P. aeruginosa*. One putative gene of *P. aeruginosa* was studied for their role in biofilm formation. The

potential regulatory gene was chosen based on their homology to a characterized gene from other species, shown to have role in biofilm formation from literature. The NhaR protein of *E.coli* produced significant percent identity (34%) with translated protein of PA1961 gene, so further laboratory experiments were carried out to show role of this gene in biofilm formation.

Cloning was done on pCR2.1 vector and the gene was preserved inside the *E.coli* cells for future use. The success of cloning experiment will help in directing the further investigation about the role of PA1961 gene.

5.1 Relation between biofilm formation and antibiotic susceptibility

Isolates were tested for their susceptibility to antibiotic that belongs to Carbapenem class. Clinically, routinely used antibiotic are beta-lactam antibiotic which include penicillin and its derivatives, such as methicillin and amoxicillin others in use are cephalosporin, carbapenems and monobactam. Among beta lactams currently available, carbapenems are unique because they are resistant to commonly available beta-lactamases secreted by bacteria. Carbapenem are beta-lactam with slight change in their side chain. They possess hydroxyethylene side chain in place of acyl amino substitute. Disc of sub groups of carbapenem: meropenem and imipenem, of 10mcg used show susceptibility to 7 isolates and one isolate (P20) showed resistance. As these drugs are recommended for multidrug resistance bacteria, resistance against these drug calls for concern. Bacteria can become antibiotic resistance either by acquired resistance involving acquisition of different mutation and transferable resistance mechanism or by intrinsic resistance, which occurs through phenotypic changes manipulating existing genetic material according to bacterial need. Bacteria going from the planktonic to the biofilm state exemplify an intrinsic shift that may result in antibiotic resistance (Schachter, 2003). Biofilm have been found to be associated with antibiotic resistance in different studies. But, the biofilm forming isolates in our study was sensitive to antibiotic in AST using MHA plate. It might be because biofilm may not have been formed in MHA plate due to the lack of favourable environment. The MH broth is shown to promote less biofilm

formation characteristic in a study involving *Campylobacter jejuni,* in vitro as compared to another media(Melo et al., 2017). Also, culture in broth and determination of MIC for susceptibility was used to get this result.

Similar results have been observed in *P. aeruginosa* isolated from VAP (ventilator associated Pneumonia) in a recent study performed in Tribhuvan University Teaching Hospital ICU. Antibiotic susceptibility assay done in 22 isolates of *P. aeruginosa*, using Imipenem and Meropenem antibiotic the number of susceptible isolates was greater than resistant in biofilm former i.e. 8 and 6 respectively. While in biofilm non-former there were more resistant isolate as compared to sensitive i.e. 6 resistant and 2 sensitive (Baidya et al., 2021).

5.2 Biofilm formation phenotype

Out of 8 isolates only 1 showed biofilm formation capacity, using the cut-off calculated according to Stepanović. The prevalence of biofilm forming isolates of *P. aeruginosa* shown by other studies is much higher for e.g. 76.5% (decreasing order: sample isolated from respiratory, urine and blood) (Cepas et al., 2019). Biofilm formation prevalence in other species is also higher. One study done in *Acinetobacter baumanii* by microtiter plate assay at 650nm showed 41%, 10% and 18% increasing biofilm former from weak, medium to strong; making a total of 69% biofilm former. The study was done on 75 *A. baumanii* strains isolated from burn wounds and urine catheters(Abdi-Ali et al., 2014). A recent study done using *P. aeruginosa* isolated from lower respiratory tract of VAP patient, the biofilm formation was reported in 63.3%. All these study suggest that our biofilm formation ratio is quite at lower end and needs to be revised.

One reason for isolates showing lesser biofilm formation in our study is the use of laboratory adapted microorganism instead of fresh hospital or on site isolates. The biofilm formation property of microorganism is their intrinsic properties, which can be supressed in laboratory condition. Bacteria are more robust in the host body because they have to face different hostile environment, accordingly they adapt to the environment; producing biofilm. As the traditional bacterial culture

system utilized highly enriched solid or liquid media the natural characteristic (or robust nature of bacteria like biofilm formation) remains hidden (Palkova, 2004).

One study has shown that the Lactic acid bacteria in the outer epidermis of green Gordal olives profoundly forms biofilm by scanning electron microscopy and the bacteria recovered by physical methods like sonication was cultured and found to be *Lactobacillus pentosus.* Normally, lactic acid bacteria like *Lactobacillus pentosus* when grown in laboratory environment gradually lose their capacity to form biofilm or their biofilm formation capacity remains unexplored (Benítez-Cabello et al., 2015).

Another study demonstrates that, the previous physiological state of bacteria affected their ability to form biofilm. The originally planktonic population showed significantly lower production of biofilm compared with that of the sessile population, irrespective of the inoculum size (Rollet et al., 2009).

PAO1 and P30_A were consistent in forming biofilm suggesting in natural or in-vivo condition they existed in sessile form. The consistency in biofilm formation in-vitro showed that there is some transition at genetic level of these *Pseudomonas* isolates. Whereas other 7 isolates must have lost their biofilm formation capacity.

The decreased biofilm forming percentage is not due to cut-off variation. Cut-off calculated remain at the range of 0.1417-0.168, each time we used new plate. However, we can try using the stain in same genus which does not produce biofilm as the negative control to check if percentage change in biofilm former occurs (Fox et al., 2005).

The error bar indicates SD of three triplicate values. The error bar in P_30A and P10 in Fig 4.6 is comparably higher as compared to other isolates. These error bars indicate variability in biofilm quantification.

5.3 Search for homologous protein in putative region of *Pseudomonas*

As protein search is much more sensitive than DNA: DNA searches, the NhaR genes was transcribed in-silico to produce protein sequence which was later used to

perform BLASTp. We found 34% identity, query cover 28%, E-value 6X10^-5, and bit score 42. According to an article published in National Institute of Health, beyond percentage identity, more reliable indicators to infer homology between sequences are E-value and bits. The accepted criteria in general for homology is more than 30% identical over their entire length, the article suggest, this may miss out homology, and it is wise to consider other indicators to infer homology when identity percentage is below 30%. The E-value < 10^{-10} and bits score above 50 are always worth considering. Though the E-value score and bit score is not as indicated, we found the two domains common in our protein and query protein i.e., The HTH helix and LysR_substrate.

5.4 Primer designing

Primer designing was done manually using the sequence derived from NCBI. OligoCalc (http://biotools.nubic.northwestern.edu) online software was used to calculate GC content and Tm value prediction. The primer was over-hanged with Restriction Enzyme site for future cloning application. The target site of 934bp was increased to 960 because of the addition of restriction-site. The restriction sites were not used while calculating annealing temperature because we don't want the restriction site to anneal during initial annealing. The software suggested annealing temperature for forward primer was 69 ^{o}C and for reverse primer was 67.9 ^{o}C (salt adjusted). Annealing temperature was optimized for our laboratory at 67 ^{o}C. The primer thus designed was checked for specificity using Primer BLAST tool of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Though the software showed off-target hit of 1455bp, 2712bp, 1196bp, 3887bp, 1337bp and 1934bp, we found exactly or below 1,000bp proves that intended target has been amplified.

5.5 PCR amplification of PA1961 gene

The PCR amplification was done to verify the presence of the PA1961 gene in our laboratory isolates. PCR amplification was seen at 1000bp or below, signifying amplification of intended target. Low repeatability of PCR amplification of same isolates in different PCR reactions is attributed to degradation of extracted DNA, efficiency of PCR machine and use of low quality nuclease free water in our

laboratory. This study show amplification at 1000 bp or lower region in 5 isolates namely PAO1, P_30A, P3, P10 and P20. Other isolates showed in consistent amplification due to old genomic DNA. The stability of bacterial genome is dependent on the storage condition and the time of extraction.

Many rounds of PCR amplification confirmed the presence of PA1961 gene in biofilm former PAO1 and P_30A and biofilm non-former P3, P4, P6, P10, P20, P30_B and P31.

The amplification of PA1961 gene in both biofilm former (PAO1, P_30A) and biofilm non-former (P3, P10 and P20) indicated the role of the gene is dependent on level of expression of the gene. The mere presence or absence of the gene cannot predict biofilm formation status. In other words physiology of biofilm formation (phenotype) cannot be correlated with presence or absence of gene (genotype). Further exploration of the level of gene expression may better predict the role of the gene in biofilm formation phenotype.

5.6 PA1961 gene

The purpose of this study was to find out a putative gene and show its role in the process of biofilm mediated antibiotic resistance in Multi-drug resistant bacteria (*P. aeruginosa*).Here, we PCR amplified PA1961 a Lys-type transcriptional regulator which was found to be homologous to NhaR in their protein sequence. NhaR protein was found to be activator of pgaABCD operon required for biofilm formation. In general LTTRs have wide range of functions including β-lactamase production which help in antimicrobial resistance

5.7 Cloning of PA1961 gene for future study

Cloning of the gene was done in pCR 2.0 vector by following the manufacturer protocol (Invitrogen). Cloning result was verified by transforming the cloned vector into competent *E.coli* bacteria. The selection of cloned vector inserted was done by blue-white screening using X-gal and IPTG. Since, very few only 1 blue in 200 white colonies was observed, the white colonies was searched for the insert (PA1961 gene) using colony PCR. Amplification was not observed in the untransformed *E.coli* (DH5-α) genome using primer of PA1961 initially (Data not shown). But when same

experiment was carried out using transformed competent cell of DH5-α, amplification was observed in less than 100bp region. Hence, cloning was successfully inserted in competent cells.

5.8 Glycerol stock to continue the experiment for further evidence collection

Finally, the biofilm forming isolates, antibiotic resistant isolates and vector inserted E. coli cells were preserved in glycerol stock for further analysis.

CHAPTER 6

SUMMARY

Hospital acquired multidrug resistant strains of *P. aeruginosa* were revived from the glycerol stock. After confirmatory test for *P. aeruginosa*, we could only revive 8 confirmed isolates of *P. aeruginosa.* Antibiotic susceptibility testing was done again to confirm whether the isolates retain the antibiotic sensitivity pattern as reported earlier or not. And found high variability in antibiotic susceptibility profile in preserved colonies in glycerol stock as only one bacterium isolate showed antibiotic resistant to tested carbapenem (IPM & MRP).

After that biofilm forming *P. aeruginosa* were characterised in microtiter plate. NCBI data for whole genome of *P. aeruginosa* was searched for unreported gene (putative genes). Earlier literature were searched for the genes involved in biofilm mediated antibiotic resistance in other species and the gene was blast for presence of homology with *Pseudomonas* to found a region identified as PA1961 which was annotated as LysR transcriptional regulator. The presence of the putative regulatory gene -PA1961 in the isolates was confirmed.

Finally, the amplified gene was then cloned into a vector pcR2.1 for future studies.

CHAPTER 7

CONCLUSION AND RECOMMENDATIONS

7.1 Conclusion

Out of 95 MDR suspected isolates stored in glycerol stock, approximately 30 bacteria colonies of *P. aeruginosa* were revived. Biochemical characterisation showed that 8 isolates were of *P. aeruginosa.* Out of 8 isolates, 2 isolate showed distinguishing character. Among the two, one isolate showed biofilm forming properties in microtiter plate assay. Antibiotic susceptibility testing was done again to confirm whether the isolates retain the antibiotic sensitivity pattern as reported earlier or not. And found high variability in antibiotic susceptibility profile in preserved colonies in glycerol stock. Only one bacterial isolate showed antibiotic resistance against carbapenem tested (IPM & MRP) and the resistant bacterium was non-biofilm producer.

The targeted region of amplified gene that was suspected to be involved in biofilm production was amplified in all of the isolates. Since, we have already observed in the wet laboratory that only one of the isolates was able to produce biofilm and the isolate which showed antimicrobial resistance did not produce any biofilm. Thus, it can be concluded that the suspected targeted gene may not have any relationship with either biofilm production or antibiotic resistance. It may also be possible that these targeted genes may express proteins which indirectly controls the genes required for biofilm formation. This requires further investigation.

7.2 Future research avenues

The biofilm mediated antibiotic resistance is an emerging topic in the world, which is facing antibiotic resistance problem in medical settings. World Health Organisation recommends health authorities of all country to track Carbapenem-resistant bacteria all around the world.

P. aeruginosa are categorized as second most critical group of pathogen by WHO. The role of *P. aeruginosa* biofilm in CF lungs, indwelling medical devices infection in

surgeries is current avenues in which related mechanism of biofilm formation regulatory genes can be researched. New ex-vivo pig lung (EVPL) models of *P. aeruginosa* CF lung infection is developed to increase understanding of chronic CF biofilm infection. The research is going on in different parts of the world to discover novel infection prevention methods and treatment and enhance exploration of biofilm architecture.

Our research was able to isolate biofilm forming strains amongst the vast unconfirmed strains and the isolates are preserved hence, can be made available for future researcher. The target gene of the LTTR PA1961 can further be explored using other techniques such as EMSA, mutant used to compare the transcriptomic profile with wild type, site-directed mutagenesis and lacZ gene fusion technique to see enhancement of pgaABCD type operon if present in *P. aeruginosa*.

If PA1961 inserted vector is able to induce biofilm forming characteristic in non-biofilm forming Pseudomonas by transformation technique. We could use this technique to turn non-biofilm former to biofilm forming *Pseudomonas* and use them in bioremediation process of pollution eradication from waste water.

7.3 Recommendations

Based on this work, the following things should be addressed for betterment of results in future works with related protocols.

We recommend that mutant strain of *P. aeruginos*a be used to check the difference in transcriptomic profile of wild type *P. aeruginosa* and the mutant *P. aeruginosa* ΔPA1961 to find out the target gene of transcriptional regulator (PA1961).

The isolate number could be increased by visiting hospitals for example Kirtipur Hospital, Nepal Cleft and Burn Center and isolating the bacteria from the burned skin wound. There is high chance of isolation of *P. aeruginosa* from the pus sample of burn patient's wound.

While making inoculum for biofilm assay, it is advantageous to grow the isolates in solid media and then diluted in autoclaved water or saline water to maintain O.D. as,

it has been reported in *Staphylococci* that when it was grown on solid medium, it expressed cell associated molecules more which help in future adhesion with negatively charged substrata.

It is recommended to use broth culture based Minimum inhibitory concentration in place of AST-for better assessment of role of biofilm in antibiotic resistance. Further, Microbial Biofilm Eradication Concentration (MBEC) creates environment for biofilm formation in-vitro and help clinician and researcher understand how biofilm hinders in antimicrobial working mechanism. Its use in clinical practice could help clinician to get in-vivo result corresponding to their in-vitro analysis. Till 2009, the MBEC Assay has been introduced in more than 100 peer-reviewed publications. Microbial Eradication concentration (MBEC) test for determining the concentration and dosage of antibiotic, biocide, disinfectant and heavy metal for eradication of biofilm from site of infection is commercialized by Innovotec Inc., a Canada based company.

REFERENCES

©Innovotech. (2012). MBEC™ Assay For High-Throughput Antimicrobial Susceptibility Testing of Biofilms PROCEDURAL MANUAL. *©Innovotech, Inc.*, *1.0*, 1–15.

A. Mendoza, R., Hsieh, J.-C., & D. Galiano, R. (2019). The Impact of Biofilm Formation on Wound Healing. In *Wound Healing - Current Perspectives* (pp. 235–250). https://doi.org/10.5772/intechopen.85020

Abdi-Ali, A., Hendiani, S., Mohammadi, P., & Gharavi, S. (2014). Assessment of biofilm formation and resistance to imipenem and ciprofloxacin among clinical isolates of Acinetobacter baumannii in Tehran. *Jundishapur Journal of Microbiology*, *7*(1), 1–5. https://doi.org/10.5812/jjm.8606

Baidya, S., Sharma, S., Mishra, S. K., Kattel, H. P., Parajuli, K., & Sherchand, J. B. (2021). Biofilm Formation by Pathogens Causing Ventilator-Associated Pneumonia at Intensive Care Units in a Tertiary Care Hospital: An Armor for Refuge. *BioMed Research International*, *2021*. https://doi.org/10.1155/2021/8817700

Baniya, B., Pant, N. D., Neupane, S., Khatiwada, S., Yadav, U. N., Bhandari, N., … Chaudhary, R. (2017). Biofilm and metallo beta-lactamase production among the strains of Pseudomonas aeruginosa and Acinetobacter spp. at a tertiary care hospital in Kathmandu, Nepal. *Annals of Clinical Microbiology and Antimicrobials*, *16*(1), 3–7. https://doi.org/10.1186/s12941-017-0245-6

Benítez-Cabello, A., Romero-Gil, V., Rodríguez-Gómez, F., Garrido-Fernández, A., Jiménez-Díaz, R., & Arroyo-López, F. N. (2015). Evaluation and identification of poly-microbial biofilms on natural green Gordal table olives. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *108*(3), 597–610. https://doi.org/10.1007/s10482-015-0515-2

Bjarnsholt, T. (2013). The role of bacterial biofilms in chronic infections. *APMIS. Supplementum*, (136), 1–51. https://doi.org/10.1111/apm.12099

Branda, S. S., Vik, Å., Friedman, L., & Kolter, R. (2005). Biofilms: the matrix revisited. *Trends in Microbiology*, *13*(1), 20–26. https://doi.org/10.1016/j.tim.2004.11.006

Cepas, V., López, Y., Muñoz, E., Rolo, D., Ardanuy, C., Martí, S., … Soto, S. M. (2019). Relationship between Biofilm Formation and Antimicrobial Resistance in Gram-Negative Bacteria. *Microbial Drug Resistance*, *25*(1), 72–79. https://doi.org/10.1089/mdr.2018.0027

Chua, S. L., Liu, Y., Yam, J. K. H., Chen, Y., Vejborg, R. M., Tan, B. G. C., … Yang, L. (2014). Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles. *Nature Communications*, *5*. https://doi.org/10.1038/ncomms5462

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., & Lappin-Scott, H. M. (1995). Microbial Biofilms. *Annual Review of Microbiology*, *49*(1), 711–745. https://doi.org/10.1146/annurev.mi.49.100195.003431

Courvalin, P. (1994). *MINIREVIEW Transfer of Antibiotic Resistance Genes between Gram-Positive and Gram-Negative Bacteriat*. *38*(7), 1447–1451.

Davies, D. G., Chakrabarty, A. M., & Geesey, G. G. (1993). Exopolysaccharide production in biofilms: Substratum activation of alginate gene expression by Pseudomonas aeruginosa. *Applied and Environmental Microbiology*, *59*(4), 1181–1186. https://doi.org/10.1128/aem.59.4.1181-1186.1993

Davies, D. G., & Geesey, G. G. (1995). Regulation of the alginate biosynthesis gene algC in Pseudomonas aeruginosa during biofilm development in continuous culture. *Applied and Environmental Microbiology*, *61*(3), 860–867. https://doi.org/10.1128/aem.61.3.860-867.1995

De Kievit, T. R., Gillis, R., Marx, S., Brown, C., & Iglewski, B. H. (2001). Quorum-sensing genes in Pseudomonas aeruginosa biofilms: their role and expression patterns. *Applied and Environmental Microbiology*, *67*(4), 1865–1873. https://doi.org/10.1128/AEM.67.4.1865-1873.2001

De, N., Pirruccello, M., Krasteva, P. V., Bae, N., Raghavan, R. V., & Sondermann, H. (2008). Phosphorylation-independent regulation of the diguanylate cyclase WspR. *PLoS Biology*, *6*(3), 0601–0617. https://doi.org/10.1371/journal.pbio.0060067

Deretic, V., Govan, J. R. W., Konyecsni, W. M., & Martin, D. W. (1990). Mucoid Pseudomonas aeruginosa in cystic fibrosis: mutations in the muc loci affect transcription of the algR and algD genes in response to environmental stimuli. *Molecular Microbiology*, *4*(2), 189–196. https://doi.org/10.1111/j.1365-2958.1990.tb00586.x

Fox, L. K., Zadoks, R. N., & Gaskins, C. T. (2005). Biofilm production by Staphylococcus aureus associated with intramammary infection. *Veterinary Microbiology*, *107*(3–4), 295–299. https://doi.org/10.1016/j.vetmic.2005.02.005

Friedman, L., & Kolter, R. (2004). Genes Involved in Matrix Formation in Pseudomonas aeruginosa PA14 Biofilms. *Molecular Microbiology*, *51*, 675–690. https://doi.org/10.1046/j.1365-2958.2003.03877.x

Fyfe, J. A. M., & Govan, J. R. W. (1980). Alginate synthesis in mucoid Pseudomonas aeruginosa: a chromosomal locus involved in control. *Journal of General Microbiology*, *119*(2), 443–450. https://doi.org/10.1099/00221287-119-2-443

Ghigo, J. M. (2003). Are there biofilm-specific physiological pathways beyond a reasonable doubt? *Research in Microbiology*, *154*(1), 1–8. https://doi.org/10.1016/S0923-2508(02)00012-8

Govan, J. R. W., & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: Mucoid Pseudomonas aeruginosa and Burkholderia cepacia. *Microbiological Reviews*, *60*(3), 539–574. https://doi.org/10.1128/mmbr.60.3.539-574.1996

Güvener, Z. T., & Harwood, C. S. (2007). Subcellular location characteristics of the Pseudomonas aeruginosa GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces. *Molecular Microbiology*, *23*(1), 071119190133004-??? https://doi.org/10.1111/j.1365-2958.2007.06008.x

Hall, C. W., & Mah, T. F. (2017). Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiology Reviews*, *41*(3), 276–301. https://doi.org/10.1093/femsre/fux010

Harrington, N. E., Sweeney, E., & Harrison, F. (2020). Building a better biofilm - Formation of in vivo-like biofilm structures by Pseudomonas aeruginosa in a porcine model of cystic fibrosis lung infection. *Biofilm*, *2*(November 2019), 100024. https://doi.org/10.1016/j.bioflm.2020.100024

Hassan, M. M., Harrington, N. E., Sweeney, E., & Harrison, F. (2020). *Predicting Antibiotic-Associated Virulence of Pseudomonas aeruginosa Using an ex vivo Lung Biofilm Model*. *11*(September), 1–9. https://doi.org/10.3389/fmicb.2020.568510

Hickman, Jason; Harwood, C. (2009). Identification of FleQ from Pseudomonas aeruginosa as c-di-GMP-responsive transcription factor. *Mol Microbiol*, *69*(2), 206–221. https://doi.org/10.1111/j.1365-2958.2008.06281.x.Identification

Hickman, J. W., Tifrea, D. F., & Harwood, C. S. (2005). A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(40), 14422–14427. https://doi.org/10.1073/pnas.0507170102

Issa, K. H. B., Phan, G., & Broutin, I. (2018). Functional mechanism of the efflux pumps transcription regulators from Pseudomonas aeruginosa based on 3D structures. *Frontiers in Molecular Biosciences*, *5*(JUN), 1–20. https://doi.org/10.3389/fmolb.2018.00057

Karimova, G., Pidoux, J., Ullmann, A., & Ladant, D. (1998). A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proceedings of the National Academy of Sciences*, *95*(10), 5752 LP – 5756. https://doi.org/10.1073/pnas.95.10.5752

Keller, P. M., Böttger, E. C., & Sander, P. (2008). Tuberculosis vaccine strain Mycobacterium bovis BCG Russia is a natural recA mutant. *BMC Microbiology*,

8(1), 120. https://doi.org/10.1186/1471-2180-8-120

Kulasekara, H. D., Ventre, I., Kulasekara, B. R., Lazdunski, A., Filloux, A., & Lory, S. (2005). A novel two-component system controls the expression of Pseudomonas aeruginosa fimbrial cup genes. *Molecular Microbiology*, *55*(2), 368–380. https://doi.org/10.1111/j.1365-2958.2004.04402.x

Lee, V. T., Matewish, J. M., Kessler, J. L., Hyodo, M., Hayakawa, Y., & Lory, S. (2007). A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. *Molecular Microbiology*, *65*(6), 1474–1484. https://doi.org/10.1111/j.1365-2958.2007.05879.x

Lynch, A. S., & Robertson, G. T. (2008). Bacterial and fungal biofilm infections. *Annual Review of Medicine*, *59*, 415–428. https://doi.org/10.1146/annurev.med.59.110106.132000

Maddocks, S. E., & Oyston, P. C. F. (2008). Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. *Microbiology*, *154*(12), 3609–3623. https://doi.org/10.1099/mic.0.2008/022772-0

Mah, T.-F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S., & O'Toole, G. A. (2003). A genetic basis for Pseudomonas aeruginosa biofilm antibiotic resistance. *Nature*, *426*(6964), 306–310. https://doi.org/10.1038/nature02122

Melo, R. T., Mendonça, E. P., Monteiro, G. P., Siqueira, M. C., Pereira, C. B., Peres, P. A. B. M., … Rossi, D. A. (2017). Intrinsic and extrinsic aspects on Campylobacter jejuni Biofilms. *Frontiers in Microbiology*, *8*(JUL). https://doi.org/10.3389/fmicb.2017.01332

Mikkelsen, H., Sivaneson, M., & Filloux, A. (2011). Key two-component regulatory systems that control biofilm formation in Pseudomonas aeruginosa. *Environmental Microbiology*, *13*(7), 1666–1681. https://doi.org/10.1111/j.1462-2920.2011.02495.x

Muraoka, S., Okumura, R., Ogawa, N., Nonaka, T., Miyashita, K., & Senda, T. (2003).

Crystal Structure of a Full-length LysR-type Transcriptional Regulator, CbnR: Unusual Combination of Two Subunit Forms and Molecular Bases for Causing and Changing DNA Bend. *Journal of Molecular Biology*, *328*, 555–566. https://doi.org/10.1016/S0022-2836(03)00312-7

Neopane, P., Nepal, H. P., Shrestha, R., Uehara, O., & Abiko, Y. (2018). In vitro biofilm formation by Staphylococcus aureus isolated from wounds of hospital-admitted patients and their association with antimicrobial resistance. *International Journal of General Medicine*, *11*, 25–32. https://doi.org/10.2147/IJGM.S153268

O'Toole, G. A. (2010). Microtiter dish Biofilm formation assay. *Journal of Visualized Experiments*, (47), 10–11. https://doi.org/10.3791/2437

O'Toole, G. A., Gibbs, K. A., Hager, P. W., Phibbs, P. V., & Kolter, R. (2000). The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by Pseudomonas aeruginosa. *Journal of Bacteriology*, *182*(2), 425–431. https://doi.org/10.1128/JB.182.2.425-431.2000

O'Toole, G. A., & Kolter, R. (1998). Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. *Molecular Microbiology*, *30*(2), 295–304. https://doi.org/10.1046/j.1365-2958.1998.01062.x

Olson, M. E., Ceri, H., Morck, D. W., Buret, A. G., & Read, R. R. (2002). Biofilm bacteria: Formation and comparative susceptibility to antibiotics. *Canadian Journal of Veterinary Research*, *66*(2), 86–92.

Pace, John L., Rupp, Mark E., Finch, R. G. (2006). *Biofilms, infection and Antimicrobial Therapy* (R. G. Pace, John L., Rupp, Mark E., Finch, ed.). Retrieved from http://www.ghbook.ir/index.php?name=فرهنگ و رسانه های نوین&option=com_dbook&task=readonline&book_id=13650&page=73&chkhashk=ED9C9491B4&Itemid=218&lang=fa&tmpl=component

Palkova, Z. (2004). Multicellular microorganisms: Laboratory versus nature. *EMBO Reports*, *5*(5), 470–476. https://doi.org/10.1038/sj.embor.7400145

Palmer, R. J., & White, D. C. (1997). Developmental biology of biofilms: implications for treatment and control. *Trends in Microbiology*, *5*(11), 435–440. https://doi.org/10.1016/S0966-842X(97)01142-6

Pannuri, A., Yakhnin, H., Vakulskas, C. A., Edwards, A. N., Babitzke, P., & Romeo, T. (2012). Translational repression of NhaR, a novel pathway for multi-tier regulation of biofilm circuitry by CsrA. *Journal of Bacteriology*, *194*(1), 79–89. https://doi.org/10.1128/JB.06209-11

Papp-Wallace, K. M., Endimiani, A., Taracila, M. A., & Bonomo, R. A. (2011). Carbapenems: Past, present, and future. *Antimicrobial Agents and Chemotherapy*, *55*(11), 4943–4960. https://doi.org/10.1128/AAC.00296-11

Ponce, R., Martinsen, L., Vicente, L. M., & Hartl, D. L. (2012). Novel Genes from Formation to Function. *International Journal of Evolutionary Biology*, *2012*, 1–9. https://doi.org/10.1155/2012/821645

Procop, G.W.; Church, D.L.; Hall, G.S.;Janda, W. M. (2006). *Koneman's Color Atlas and Textbook of Diagnostic Microbiology* (7 th; E. W. Koneman, ed.). Lippincott Williams & Wilkins.

Rao, F., Yang, Y., Qi, Y., & Liang, Z. X. (2008). Catalytic mechanism of cyclic di-GMP-specific phosphodiesterase: A study of the EAL domain-containing RocR from Pseudomonas aeruginosa. *Journal of Bacteriology*, *190*(10), 3622–3631. https://doi.org/10.1128/JB.00165-08

Rodrigue, A., Quentin, Y., Lazdunski, A., Méjean, V., & Foglino, M. (2000). Two-component systems in Pseudomo- nas aeruginosa: why so many? *Trends Microbiol.*, *8*(11), 498–540. https://doi.org/10.1016/s0966-842x(00)01833-3

Rollet, C., Gal, L., & Guzzo, J. (2009). Biofilm-detached cells, a transition from a sessile to a planktonic phenotype: A comparative study of adhesion and physiological characteristics in pseudomonas aeruginosa. *FEMS Microbiology Letters*, *290*(2), 135–142. https://doi.org/10.1111/j.1574-6968.2008.01415.x

Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., … Benziman, M. (1987). Regulation of cellulose synthesis in Acetobacter xylinum by cyclic diguanylic acid. *Nature*, *325*(6101), 279–281. https://doi.org/10.1038/325279a0

Roy, A. B., & Sauer, K. (2015). Diguanylate cyclase NicD based signaling mechanism of nutrient-induced dispersion by Pseudomonas aeruginosa Ankita. *Mol Microbiol*, *94*(4), 771–793. https://doi.org/10.1111/mmi.12802.Diguanylate

Sadovskaya, I., Vinogradov, E., Li, J., Hachani, A., Kowalska, K., & Filloux, A. (2010). High-level antibiotic resistance in Pseudomonas aeruginosa biofilm: the ndvB gene is involved in the production of highly glycerol-phosphorylated β-(1→3)-glucans, which bind aminoglycosides. *Glycobiology*, *20*(7), 895–904. https://doi.org/10.1093/glycob/cwq047

Schachter, B. (2003). Slimy business - The biotechnology of biofilms. *Nature Biotechnology*, *21*(4), 361–365. https://doi.org/10.1038/nbt0403-361

Schierle, C. F., De La Garza, M., Mustoe, T. A., & Galiano, R. D. (2009). Staphylococcal biofilms impair wound healing by delaying reepithelialization in a murine cutaneous wound model. *Wound Repair and Regeneration*, *17*(3), 354–359. https://doi.org/10.1111/j.1524-475X.2009.00489.x

Sivaneson, M., Mikkelsen, H., Ventre, I., Bordi, C., & Filloux, A. (2011a). Two-component regulatory systems in Pseudomonas aeruginosa: an intricate network mediating fimbrial and efflux pump gene expression. *Molecular Microbiology*, *79*(5), 1353–1366. https://doi.org/10.1111/j.1365-2958.2010.07527.x

Sivaneson, M., Mikkelsen, H., Ventre, I., Bordi, C., & Filloux, A. (2011b). Two-component regulatory systems in Pseudomonas aeruginosa: An intricate network mediating fimbrial and efflux pump gene expression. *Molecular Microbiology*, *79*(5), 1353–1366. https://doi.org/10.1111/j.1365-2958.2010.07527.x

Stepanović, S;Djukić, N; Opavski, N; Djukić, S. (2003). No Title. *Significance of Inoculum Size in Biofilm Formation by Staphylococci.*, *26*(1), 129–132.

Toole, G. O., Kaplan, H. B., & Kolter, R. (2000). Biofilm formation as microbial development. *Annual Review of Microbiology*, *54*, 49–79.

Tropel, D., & van der Meer, J. R. (2004). Bacterial Transcriptional Regulators for Degradation Pathways of Aromatic Compounds. *Microbiology and Molecular Biology Reviews*, *68*(3), 474 LP – 500. https://doi.org/10.1128/MMBR.68.3.474-500.2004

Valentini, M., & Filloux, A. (2016). Biofilms and Cyclic di-GMP (c-di-GMP) signaling: Lessons from Pseudomonas aeruginosa and other bacteria. *Journal of Biological Chemistry*, *291*(24), 12547–12555. https://doi.org/10.1074/jbc.R115.711507

Vettoretti, L., Plésiat, P., Muller, C., El Garch, F., Phan, G., Attrée, I., … Llanes, C. (2009). Efflux unbalance in Pseudomonas aeruginosa isolates from cystic fibrosis patients. *Antimicrobial Agents and Chemotherapy*, *53*(5), 1987–1997. https://doi.org/10.1128/AAC.01024-08

Watson, J. D. (1987). *Molecular Biology of the gene* (4th ed.). Menlo Park, California: The Benjamin/Cummings.

Wilkinson, S., & Grove, A. (2006). Ligand-responsive transcriptional regulation by members of the MarR family of winged helix protein. *Current Issues in Molecular Biology*, *8*, 51–62.

William, S., Feil, H., & Copeland, A. (2004). Bacterial DNA Isolation CTAB Protocol Bacterial genomic DNA isolation using CTAB Materials & Reagents. *Doe Joint Genome Institute*, 4. Retrieved from http://my.jgi.doe.gov/general/protocols/JGI-Bacterial-DNA-isolation-CTAB-Protocol-2012.pdf

APPENDICES

APPENDIX 1:

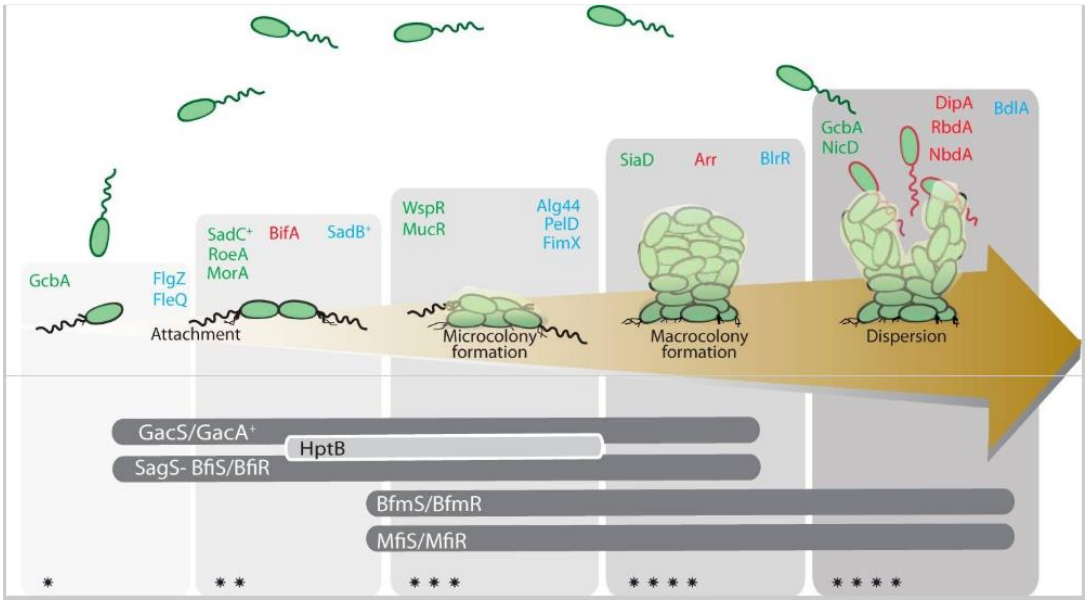


FIGURE 1. Coordinated action of c-di-GMP signaling pathways and two-component system cascades in the control of *P. aeruginosa* biofilm development. In the laboratory, biofilm formation is shown to be a cyclic process that initiates with attachment to the surface of planktonic bacteria (first reversible and then irreversible). A bacteria microcolony is subsequently formed, which evolves into a mature mushroom-shaped macrocolony until the biofilm-associated cells disperse to resume again a planktonic lifestyle. Planktonic, biofilm, and dispersed cells possess distinct physiological stages (green, black, and red outline, respectively) (Chua et al., 2014; Toole et al., 2000). The upper panel illustrates DGC (green), PDE (red), and c-di-GMP receptors/effectors (blue) and the developmental stage in which they are proposed to act. The lower panel illustrates biofilm stage-specific two-component regulatory systems (Mikkelsen et al., 2011). The gradient of the gray panels in the background of the figure indicates increasing intracellular c-di-GMP levels (also indicated with *, **, ***, and ****).

APPENDIX 2:

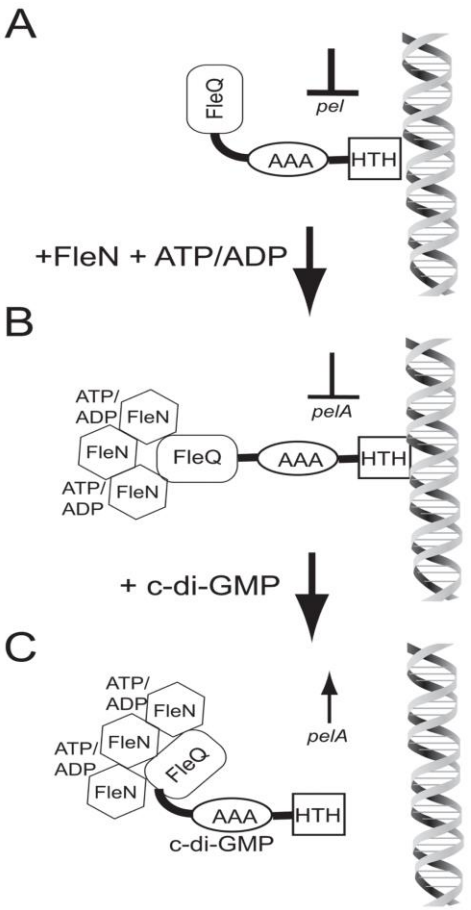


Figure 2. Model for the regulation of gene expression by FleQ, FleN, and c-di-GMP

A) FleQ in the absence of FleN or c-di-GMP maximally represses *pel* transcription. B) The situation in wild-type cells; FleQ binding at the *pelA* promoter is reduced by FleN and ATP/ADP, resulting in less *pel* repression than the situation in panel A. C) C-di-GMP binds to FleQ to cause it to dissociate from DNA thereby causing derepression of transcription from the *pel* promoter.

APPENDIX 3

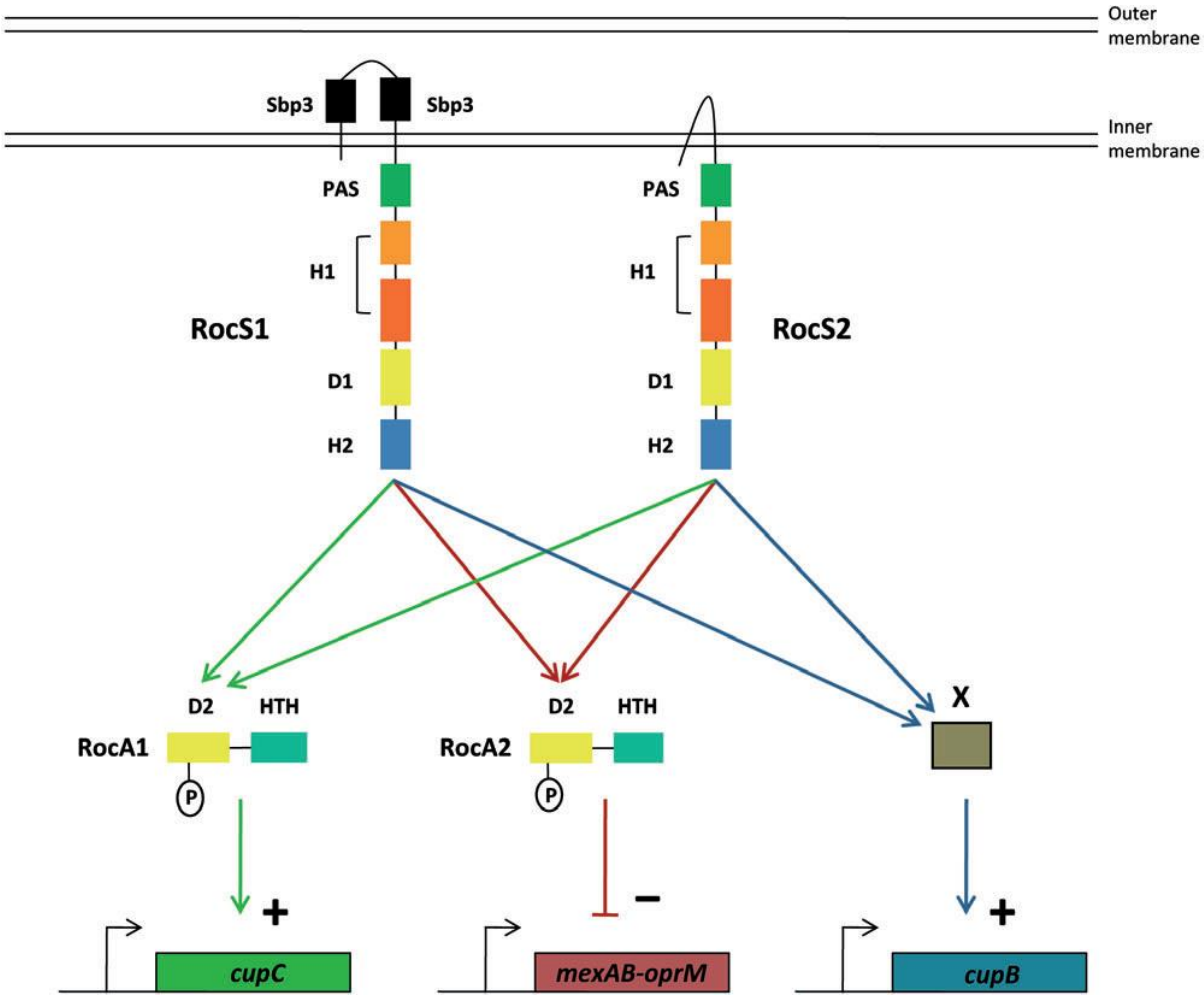


Figure 3: Schematic model showing the cross-regulation between Roc1 and Roc2 signalling pathways. The Roc1 and Roc2 components are represented as in Fig. S1. The RocS1 and RocS2 sensors are shown as integral inner membrane proteins. The positive regulation on the *cupC* gene expression is shown with green arrows. The negative regulation on the *mexAB-oprM* genes is shown with brown arrows. The positive regulation on *cupB* genes is shown with blue arrows and involves a yet unknown regulator indicated as X(Sivaneson et al., 2011b).

APPENDIX 4

Preparation of Reagents for DNA extraction:

Table 1:CTAB Extraction Buffer

Reagent	**Amount to be added (for 10ml)**	**Final concentration**
Cetyltrimethyl ammonium bromide (CTAB) (10% in H2O)#	3 ml	3 %
5M NaCl	2.8 ml	28 %
0.5M EDTA (pH 8.0)	0.4 ml	4 %
1 M Tris-Cl (pH 8.0)	1.0 ml	10 %
Polyvinylpyrrolidone (PVP) (MW 40kDa)	0.3 g	3 %
β- Mercaptoethanol	0.02 ml	0.2 %
H2O	2.48 ml	24.8 %

Table: Preparation CTAB/NaCl Buffer(cshprotocols.cshlp.org):

*Prepare CTAB extraction buffer immediately before use; buffer is only good when freshly prepared.

#Store 10% CTAB stock solution at 37ºC to avoid precipitation. It may be stored at 37ºC for several years.

Table 2: 50X Tris Acetate-EDTA(TAE) Buffer:

Buffer	**Working Solution**	**Stock solution / Ltr.**
TAE	**1X** 40 mM Tris-acetate 1 mM EDTA	**50X** 242g of Tris base 57.1 ml of glacial acetic acid 100ml of 0.5M EDTA (pH 8.0)

Final volume 1000ml to be made with water.

Table 3: Tris-EDTA

Buffer	**Working Solution**	**Stock solution / Ltr.**
TE	**1X** 10 mM Tris(pH 8.0) 1 mM EDTA	**10X** 15.759 g of Tris base 2.92 g EDTA (pH 8.0)

Final volume 1000ml to be made with water.

APPENDIX 5

Lay out of Microtiter plate for biofilm Assay in Fig4.3:

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D	PAO1	P6	P20	P30_A	P31	P30_B	P3	P10	P4	C1	C2	Acetic acid
E												
F	1	1	1	1	1	1	1	1	1	1	1	1
G	2	2	2	2	2	2	2	2	2	2	2	2
H	3	3	3	3	3	3	3	3	3	3	3	3

1,2,3: Triplicate of biofilm reading of isolates.

PAO1: Positive control for biofilm formation

C1: Same LB without isolates, after 24hrs incubation as Negative control.

C2: Next lot LB without 24hrs incubation.

APPENDIX 6

Oligo data from the Macrogen Inc., South Korea (the manufacturer).

Oligo	**PA1961_F**					
SEQ	5'- TTAAGGATCCTT ACTCGCGGGCGCGGC -3'(34mer)					
GC%	**MW**		**Yield**		**scale (umoles)**	**Tm(c)**
	Calculated	**Measured**	**OD**	**nmol**		
55.88	10223.8	10311.3	9.9	27.0	0.05	77.7
vol for 100pmol/ul		**Purification**		**Modification**		
270.0		MOPC				

Oligo	**PA1961_R**					
SEQ	5'- TTAAGGATCCTT ACTCGCGGGCGCGGC -3'(27mer)					
GC%	**MW**		**Yield**		**scale (umoles)**	**Tm(c)**
	Calculated	**Measured**	**OD**	**nmol**		
62.96	8292.4	8335.0	8.2	29.0	0.05	74.4
vol for 100pmol/ul		**Purification**		**Modification**		
290.0		MOPC				