Genomic diversity in biofilm forming pathogenic bacteria isolated from clinical samples

PhD Thesis

DEPARTMENT OF MICROBIOLOGY
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SUBMITTED BY
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Genomic diversity in biofilm forming pathogenic bacteria isolated from clinical samples

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF DHAKA IN THE FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Dedicated to…

My Beloved Parents, Who Cherished My Life with Their Blessings

Quotation…

"Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world."

Louis Pasteur

Certification

It is hereby certified that student bearing Reg. No. 164, Session 2017-2018 has carried out the research work entitled "**Genomic diversity in biofilm forming pathogenic bacteria isolated from clinical samples**" for the fulfillment of his PhD Degree from University of Dhaka, Bangladesh, under my academic supervision in the Microbial Genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka.

..……

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Examiner's Copy

Abstract

Biofilms has become an emerging health concern as bacteria in biofilm are more resistant to antimicrobials and disinfectants than their planktonic counterparts. The multifactorial nature of biofilm development imposes great challenges for the use of conventional antimicrobials. It is therefore very important to understand the molecular basis of biofilm formation to find effective therapeutics to block the early steps of biofilm. In this study, a total of 45 MDR clinical isolates from different Bangladeshi hospitals were tested for their biofilm forming abilities. Of the 45 clinical isolates tested, 38 (85%) produced biofilm and 13 (29%) were characterized as strong biofilm formers (SBF). Among the SBFs, *P. aeruginosa* isolates were prevalent. Fluorescent image data analysis and viable cell count after 10 minute exposure with 0.1% SDS and NaOCl on *P. aeruginosa* biofilms did not show significant lethal effect on attached cells viability. On the other hand, 2 to 4 log cycle reduction on viable cell count observed after 70% ethanol and Savlon treatment, but the bacterial load remained as high as 10^3-10^4 cells/ mm^2, indicating the failure of these agents to eradicate the biofilms completely. Despite of having variable biofilm forming ability, common biofilm related genes were detected in all of 10 the *P. aeruginosa* isolates tested by conventional PCR. Whole genome sequencing of a strong (27b), a moderate (20c) and a weak biofilm former (30b) isolate was performed and analysis of biofilm related genes in the sequenced genomes revealed that, 80 of the 88 biofilm related genes possess 98-100% sequence identity to the reference PA01 strain. Complete and partial sequence of LecB of 10 *P. aeruginosa* isolates revealed that, isolates that have PA14 like LecB sequence produced strong biofilms, while PAO1 like LecB containing isolates are either moderate or weak biofilm formers. All of the 7 Pel operon protein coding genes in weak biofilm former isolate 30b showed significant nucleotide and amino acid sequence variation with other tested isolates, and these proteins are 99% identical with the Pel operon proteins of PA7. Bioinformatics analyses identified distinct sequence and structural features that separates PA7 like Pel operon proteins from reference PAO1 like Pel operon. Congo red and Pellicle forming assays revealed that, the sequence and structure variations may have interfered with Pel production pathway and resulted in impaired Pel production in 30b. Expression analysis also showed that, both PelB and LecB proteins were about 5-6 folds upregulated in SBF 27b in comparison with WBF 30b. Our findings indicate significant genomic divergence in biofilm related genes of *P. aeruginosa* strains that affect their biofilm phenotypes.

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ABBREVIATIONS

3D – Three Dimensional

aa – Amino Acid

AI – Autoinducer

Bp – Base pair

BLAST– Basic Local Alignment Search Tool

BLASTN– Nucleotide BLAST

BLASTP– Protein BLAST

c-di-GMP – Cyclic dimeric Guanosine Monophosphate

CDS – Coding DNA Sequence

CF – Cystic Fibrosis

DSB – Dry Surface Biofilm

eDNA – Extracellular DNA

EPS – Extracellular Polymeric Substances

Et- Br – Ethidium Bromide

IUPAC – International Union of Pure and Applied Chemistry

MEGA – Molecular Evolutionary Genetics Analysis

MBF – Moderate Biofilm Former

NBF – Non Biofilm Former

NCBI – National Centre for Biotechnology Information

OD – Optical Density

PBS – Phosphate buffer saline

PCR – Polymerase Chain Reaction

PDB – Protein Data Bank

PI – Propidium Iodide

QS – Quorum Sensing

Rpm – Rotation per Minute

SBF – Strong Biofilm Former

SD – Standard Deviation

SDS – Sodium Dodecyl Sulphate

RQ – Relative Quantification

RT-PCR – Real Time PCR

TA – Tracheal Aspirate

TAE – Tris Acetate EDTA

TSA – Tryptic Soya Agar

TSB – Tryptic Soya Broth

UTI – Urinary Tract Infection

WBF – Weak Biofilm Former

WS – Wound Swab

ABBREVIATED NAMES OF AMINO ACIDS

G - Glycine

V - Valine

L - Leucine

I - Isoleucine

F - Phenylalanine

P - Proline

Y - Tyrosine

W - Tryptophan

S - Serine

T - Threonine

A - Alanine

M - Methionine

N - Asparagine

Q - Glutamine

D - Aspartate

E - Glutamate

K - Lysine

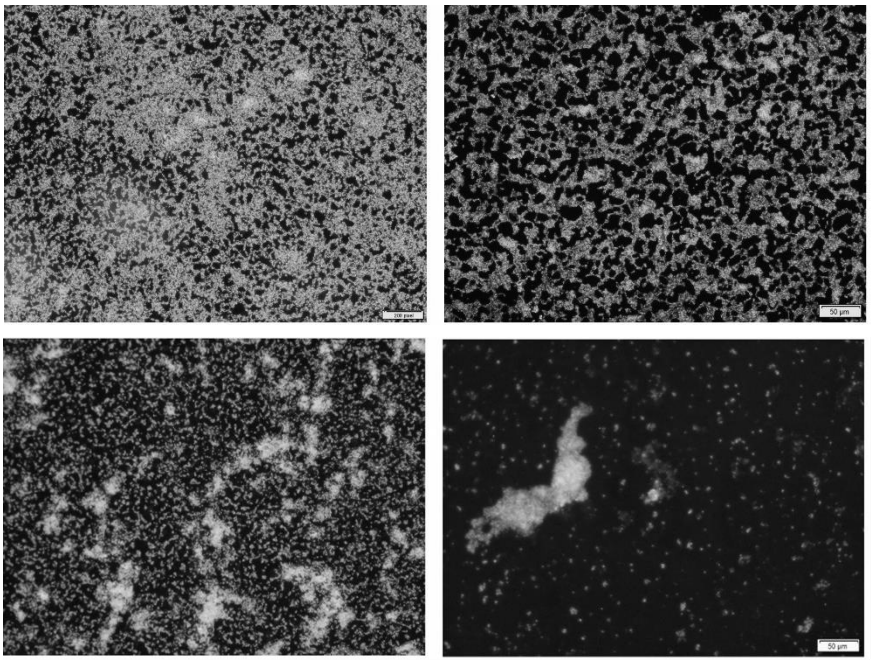
R - Arginine

C - Cysteine

H - Histidine

Chapter 1

INTRODUCTION AND LITERARTURE REVIEW



1.1 General Introduction

Biofilms has become an emerging health problem as they are reported to be more resistant to antimicrobials than their planktonic equivalents (P. S. Stewart & Costerton, 2001). National Institutes of Health (NIH) revealed that 65% microbial and 80%, chronic infections are associated with biofilms (Jamal et al., 2018). Moreover, in hospital environments, biofilms formed by pathogens on openings and medical equipment allows them to persist as reservoirs and thus can freely spread to patients (Kostakioti et al., 2013). Due to the increased resistance and protective mechanisms against disinfectants and antimicrobials, biofilms have become a key target for therapeutics.

In hospital environment, the elimination of biofilms formed by pathogenic bacteria is a big challenge as there is no consistent approach for the control of biofilm (Simões et al., 2009). The risk of this type of biofilm related hospital acquired infection has been estimated as high as two to twenty times in developing countries than that of developed countries (Islam et al., 2016). In densely populated country like Bangladesh, where hygiene is poor, hospital managements are inappropriate and antibiotic resistance is in a high note due to the improper use, the risk is even bigger. In this context, this study primarily emphasized on determination of biofilm forming ability of multidrug resistant pathogenic bacteria isolated from clinical samples of Bangladesh.

In Bangladesh, some common disinfectants are frequently used to clean the hospital surfaces. This study also focused on determining the efficacy of common disinfectants on strong biofilm former pathogenic strains. Fluorescence microscopy is frequently being used as a noninvasive method to evaluate the antimicrobial potential and efficacy in biofilms and also contributes to our understanding of the efficacy of disinfectants on complex biofilm structures (Bridier et al., 2011; Davison et al., 2010; Takenaka et al., 2008). As the use of conventional antimicrobials are failing to eradicate complex biofilms , the need for multi-targeted or combinatorial therapies is becoming essential to control the multifactorial nature of biofilm growth (Koo et al., 2017). Till date several approaches also have been used to block the early step of biofilm formation or to destroy the already formed biofilms (Sigurdsson et al., 2012). To introduce an effective therapeutics against biofilm, biofilm formers need to be extensively studied for genetic determinants that contribute to biofilm formation. As biofilm formation is dependent on various genetic and environmental

factors, control strategies against biofilms formed by pathogenic bacteria should be directed on a case-by-case basis (Magdalena A et al., 2016).

Among the biofilm producing bacteria, *P. aeruginosa* can cause serious health threat, particularly in a hospital environment. It is the one of the most frequent causes of ventilator associated pneumonia and catheter related infection (Mittal et al., 2009; Ramírez-Estrada et al., 2016), and thus it has become one of the leading causes of nosocomial infections all over the world (Zavascki et al., 2006). The extraordinary ability of *P. aeruginosa* to form biofilms in different environments makes it responsible for various infections, particularly in immune-compromised persons (Rasamiravaka et al., 2015). Infections caused by this organism are often associated with high morbidity and mortality because of its intrinsic resistance to a number of drugs and its ability to gain resistance to antimicrobials from plasmids (Dwivedi et al., 2009). Centre for Disease Control reported more than 51,000 clinical *P. aeruginosa* infections with 400 deaths each year in the USA(Awan et al., 2019). In this background, we extensively studied the clinical *P. aeruginosa* strains to determine the genetic factors contributed in their biofilm forming ability.

Biofilm structures of different *P. aeruginosa* strains can show variability in biomass and morphology (B. Lee et al., 2005). In fact, a number of genes involved in *P. aeruginosa* biofilm exopolysaccharide secretion, cell to cell signaling and biofilm architecture maintenance etc (L. Zhang et al., 2013). Whole genome sequencing and subsequent bioinformatics analyses of biofilm related genes and proteins can be useful to assess the sequence and structural variabilities that may affect biofilm forming abilities of different *P. aeruginosa* strains. Determining the level of expression of biofilm related genes in related bacterial strains can also provide valuable information about the correlation between the genes and biofilm forming ability. Given that, this study focused on analyzing the sequence variation, protein structure and expression pattern of biofilm associated genes in clinical *P. aeruginosa* isolates found in Bangladesh. The findings of the study can provide more insights into the biofilm forming ability of the clinical isolates, understanding molecular basis of the variable biofilm forming abilities of *P. aeruginosa* and can support to find effective therapeutics to block the early steps involved in biofilm formation.

1.2 Review of Literature

1.2.1 Biofilms: Microbial Life on Surfaces

According to the IUPAC recommended "Terminology for bio-related polymers and applications" (Vert et al., 2012), biofilm is defined as the "Aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and/or to a surface". This type of microbial community are usually embedded in a self-produced matrix of extracellular polymeric substances (EPS).

Biofilms can be formed in diverse environment. They are frequently found on solid substrates that are immersed in or open to an aqueous solution. In high humidity climates, they can also form on the surface of plant leaves and can also be found as floating mats in liquid surfaces. They can form in both living and nonliving surfaces (Cortés et al., 2011; Kostakioti et al., 2013) and can be prevalent in natural, industrial and hospital settings (Costerton, Lewandowski *et al*. 1995, Donlan 2002). They can be polymicrobial and by including multiple microorganisms in a single community, they can obtain numerous advantages (Wolcott et al., 2013).

Physiological characteristics of biofilm communities significantly differs from that of planktonic cells (Simões et al., 2010). Biofilms support the division of labor inside the community, promote the conservation of genotypes. Inside the biofilm structures, microorganisms have metabolic collaboration, quorum sensing (QS) systems, DNA sharing and byproduct impact that can make a complex society of them and give them advantage to survive. Inside host body, they also protect microorganisms from predators and the immune system and provide a physical and structural barrier against mechanical and physical stimuli. They are more resistant to antimicrobials such as antibiotics, surfactants, disinfectants than their free floating counterparts (Kim et al., 2009; P. S. Stewart & Costerton, 2001)

1.2.2 Biofilm Structure

Biofilms are composed of Exopolymeric substances that includes extracellular polysaccharides, DNA and proteins. This substances enables the cells to be self-organized into localized communities, facilitates trapping other organics and localizing their digestion by extracellular enzymes, coordinates cell–cell chemical communication, permits redox activity supports

horizontal gene-exchange and provides a degree of physical stability (Aldecoa et al., 2017; Aminov, 2011; Mary Ellen Davey & O'toole, 2000; Gloag et al., 2013). Channels in the biofilm structure allow nutrients, water and air to enter every layers of the biofilm (Donlan, 2002). Exopolysaccharides synthesized extracellularly or intracellularly, shape the biofilm structures and are secreted into the outside environment (Nwodo et al., 2012).

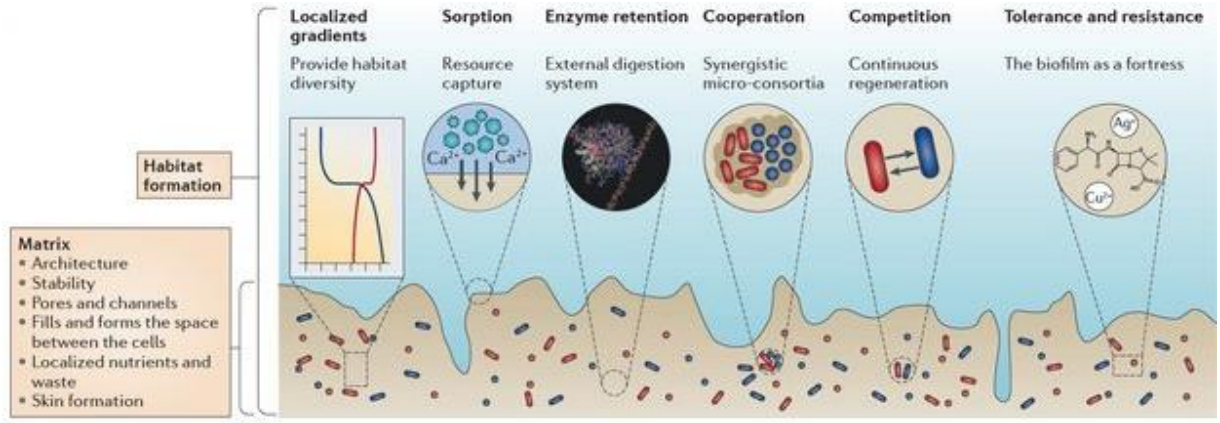


Figure 1.1: Schematic diagram showing biofilm structure and function and the biological and chemical processes that biofilms influence. (Flemming et al., 2016)

1.2.2.1 Exopolysaccharides

The exopolysaccharides synthesized by microbial cells vary greatly in their composition, chemical and physical properties. The exopolysaccharides are mostly polyanionic because of the presence of either uronic acids or ketal linked pyruvate, but some of them can be neutral macromolecules also (Sutherland, 2001). Polycationic exopolysaccharides are also rarely found in nature (Allison, 1998). Mannose, galactose and glucose are the mostly found carbohydrates, while *N*-acetylglucosamine, galacturonic acid, arabinose, fucose, rhamnose and xylose can also be found. During the switching from planktonic to biofilm state, exopolysaccharides can play a vital role in extracellular and intracellular attachment (Bales et al., 2013). Most exopolysaccharides are not known as biofilm specific, but in most of the cases, the production of exopolysaccharides increases as a stress response approach, for example- alginate synthesis in *P. aeruginosa* and colanic acid production in *Escherichia coli.*

1.2.2.2 Extracellular Proteins

Extracellular proteins are also considered as important matrix component. To form biofilm and stabilize the biofilm structure, proteins attach to cell surfaces and polysaccharides. For example, Gbps protein plays an crucial role in maintaining biofilm structure by involving bacteria and exopolysaccharides in *Streptococcus mutans* biofilm (Lynch et al., 2007). Amyloids can also play a secondary role in biofilm architecture, such as Fap amyloids in *Pseudomonas* spp. (Dueholm et al., 2013). Some extracellular enzymes can degrade biopolymers and provide energy and carbon sources to biofilm cells during starvation (X. Zhang & Bishop, 2003). These enzymes targets proteins, polysaccharides, nucleic acids, EPS matrix components, cellulose, lipids etc. that are trapped in the EPS matrix and contribute in their degradation process.

1.2.2.3 eDNA

Although, extracellular DNAs (eDNAs) were at first considered as remains of lysed bacterial cells, later it revealed that eDNA can be actively secreted by biofilms (Hamilton et al., 2005). The eDNA also play vital role in biofilm formation. DNase I treatment were found to prevent *P. aeruginosa* biofilm formation in previous reports (Whitchurch et al., 2002). eDNA therefore play a vital role for biofilm attachment. eDNA is known to interact with receptors on surface to facilitate adhesion, particularly when the space between the bacterial cell and the surface becomes as little as a few nanometers (Das et al., 2013). In addition, eDNA can play a vital role for horizontal gene transfer in microorganisms (Montanaro et al., 2011).

1.2.3 Steps of Microbial Biofilm Formation

Biofilm is closely clustered within a matrix and forms complex association between bacteria in that matrix. A number of physical, chemical and biological processes are important in biofilm growth and maturation. Microbial biofilm go through five distinct stages (Sauer et al., 2002; Stoodley et al., 2002). The steps are defined as- initial reversible attachment, irreversible attachment, maturation stage I, maturation stage II and dispersion.

Planktonic bacterial cells adhere to the surface by means of appendages such as Pilli or flagella or physical forces in the first stage of biofilm formation. A number of factors like surface texture and chemistry, pressure and temperature can affect the attachment of bacteria greatly. Van der Waals

forces, steric interactions and electrostatic interactions are the major physical forces related to bacterial adhesion. Bacterial appendages like flagella, fimbriae and pili can overcome the physical repulsive forces of the of the cell and the surface and thus strengthen the interactions between bacterial cells and the surface (Kumar & Anand, 1998). If the attachment forces are greater than the repulsive forces, some of the biofilm cells persist as immobilized cells and become irreversibly adhered. While the bacteria tend to adhere to a hydrophobic nonpolar surface, cell surface hydrophobicity can also play a vital role in forming biofilms (Hall-Stoodley et al., 2004).

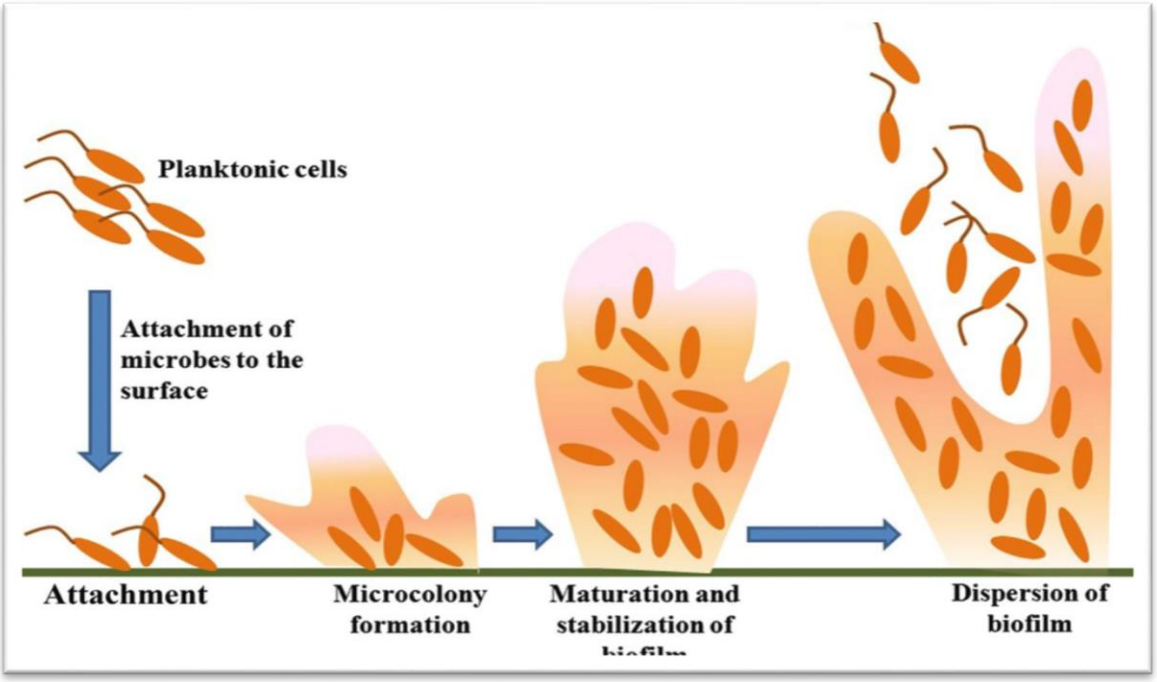


Figure 1.2: Stages of biofilm growth. Firstly, Planktonic cells attach reversibly to the surface and then become associated irreversibly to form bacterial colonies on the surface. Quorum sensing and other signaling events enables biofilm maturation and stabilization in the later stages. In the final stage, microbes inside the biofilm become dispersed by the release of surface bacteria that exist on the upper layer of biofilm structure for colonization to a new surface (Gupta et al., 2016).

During the maturation phase I, microbial communication by the production of auto-inducer signals starts. Communicating among bacterial cells inside biofilm results in expression of biofilm related

genes. At this stage, bacteria secretes extracellular polysaccharide substances (EPS). For example, *P. aeruginosa* biofilms produce and release three polysaccharides, called alginate, Pel and Psl which are important for biofilm stability and architecture. Pel and Psl act as a framework for the biofilm architecture (Franklin et al., 2011). On the other hand, Alginate is reported to interact with nutrients and water and it also supplies nutrients to the biofilm bacteria (Rasamiravaka et al., 2015). At this stage, e DNA play a vital role in cell to cell communication and stability of *P. aeruginosa* biofilm.

At the second phase of maturation, microcolonies increase by size. Microcolonies in biofilms often consist of various microbial species. This type of micro-society develops in a relatively complicated and coordinated way. Closeness of the cells in this part of biofilm growth increases substrate exchange, circulation of metabolic products and exclusion of toxic end products. At this vital stage, biofilm adapts with the environmental condition by manipulating its physiology, structure and metabolism and offers a suitable environment for the formation of mutualistic association (Mary Ellen Davey & O'toole, 2000).

The dispersion stage involves the detaching of the biofilm and reoccurrence of attached cells to its planktonic form (Hall-Stoodley et al., 2004). The microbial community of the biofilm often have different saccharolytic enzymes that can break the polysaccharide-stabilized structure of the biofilm. For example, *Streptococcus equi* uses hyaluronidase enzymes, *P. fluorescens* and *P. aeruginosa* release alginate lyase for the disintegration of the biofilm matrix. During this stage, the expression of flagella in bacteria is also upregulated to facilitate the motility and translocation to a new surface. As a result, surface bacteria that exist on the upper layer of biofilm structure become released for colonization to another surface. Other forces that are responsible for matrix disruption also important in this biofilm cycle (Otto, 2013).

1.2.4 Quorum Sensing

1.2.4.1 Quorum Sensing: Means for Cell to Cell Communication in Bacteria

The term "quorum sensing" (QS) was previously known as "autoinduction".(Turovskiy et al., 2007). QS can be defined as a bacterial cell to cell signaling process that produces, detects the signaling molecule called autoinducers (AIs) and response to those signaling molecules (Rutherford & Bassler, 2012). It is observed that, accumulation of those signaling molecules is

proportionate with the bacterial growth. If the critical threshold of population density exceeds, the signal can result in inducing a coordinated response in gene expression of the overall biofilm population (Z. Li & Nair, 2012). This QS mechanism regulates numerous activities in bacteria, including biofilm formation, virulence factor secretion, antibiotic production and resistance, competence, sporulation etc. (Novick & Geisinger, 2008; Rutherford & Bassler, 2012; Williams & Cámara, 2009).

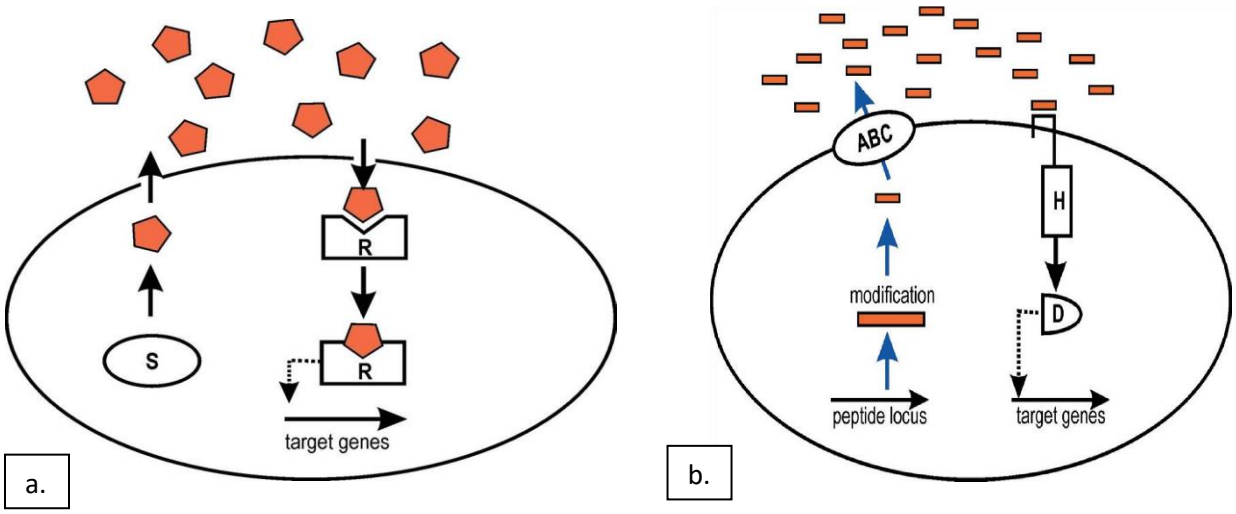


Figure 1.3: Mechanism of quorum sensing in a) Gram-negative bacteria [Pentagons = acylated homoserine lactone (acyl-HSL), S = acyl-HSL synthases, R= acyl-HSL binding protein] and in b) Gram-positive bacteria (ABC = transporter protein complex, H = histidin kinase, D = regulator protein) (Marić & Vraneš, 2007).

In gram-negative bacteria, acyl-homoserine lactones (acyl-HSL) are used as signal molecules. When the concentration of those signal molecules becomes high enough, they enter into the cell and bind to the receptor **(Figure 1.3a)**. Transcription process become activated when a complex consisting of a signal molecule and a receptor binds to suitable target genes. On contrary, oligopeptides are known as signal molecules in Gram-positive bacteria **(Figure 1.3b)**. These oligopeptides are being transported out of the cell into intercellular space by protein complex ABC. A protein system that includes protein kinase and a regulatory protein sense the signal at

sufficiently high concentration of these autoinducers. Thus the protein kinase become activated and simultaneously it activates the regulatory protein that in turn binds to specific target genes and regulates their transcription (Marić & Vraneš, 2007).

1.2.4.2 Quorum Sensing: in Biofilm

Quorum sensing affects biofilm phenotype in bacteria. QS plays very important roles in several steps of biofilm formation such as attachment, maturation, aggregation and dispersal (Parsek & Greenberg, 2005). A variety of chemical, physical, and nutritional factors can make impact on a range of process such as signal production, distribution, stability and efficiency to interact with their receptors in a biofilm (Y.-H. Li & Tian, 2012). A number of reports explain the role of quorum sensing in gram negative and gram positive bacteria. For example, the Las QS system plays a vital role for the production of mature, differentiated biofilms in *P. aeruginosa* (D. G. Davies et al., 1998). Moreover, several other groups have published findings that support the role for different QS system in *P. aeruginosa* biofilm formation (Allesen-Holm et al., 2006; Barken et al., 2008; Sakuragi & Kolter, 2007). However, in some cases a direct link could not be established between QS and biofilm formation (Heydorn et al., 2002; Purevdorj et al., 2002; Schaber et al., 2007). These contradictions in different studies indicate that different experimental parameters might have a significant impact on biofilm formation (Kievit, 2009).

It was also reported that, CSP-mediated QS system in *Streptococcus mutans* affects biological processes like genetic transformation, acidogenicity, and bacteriocin production etc. These properties are optimally expressed in biofilm cells compared to planktonic counterparts. (Senadheera & Cvitkovitch, 2008). In *Listeria monocytogenes.* AgrD-dependent QS system affects gene expression profiles, biofilm formation, invasion, virulence etc. (Riedel et al., 2009). On the other hand, *Klebsiella pneumoniae* biofilm formation is controlled by QS through the production of interspecies AI-2 autoinducers (De Araujo et al., 2010). Quorum Sensing is also reported to control biofilm formation in *Vibrio cholerae* by the modulation of Cyclic Di-GMP levels (Waters et al., 2008). Another example is, *agr* and *luxS* QS systems of staphylococci, which was reported to influence biofilm formation and virulence factors during biofilm-associated infection (Kong et al., 2006).

1.2.5 Reasons for Biofilm Formation

Bacteria has the ability to switch between planktonic and biofilm form. In environment, biofilm state seems to be comparatively predominant state of bacteria. A number of explanations can be accounted for the necessity of bacteria to form biofilms. Biofilm enables bacteria to enhance the tolerance against tough environmental conditions and thus response the environmental stress. Bacteria also form biofilm as a mechanism to remain in a favorable niche (Jefferson, 2004). By their attachment to a surface or tissue, bacteria in biofilm can avoid being carried away by water flow or blood flow in the host. The oral biofilms also can resist to repeated, shear, strong forces. Bacterial cells in biofilms are about thousand times more resistant to antimicrobial agents than their free floating state (Rasmussen & Givskov, 2006). The EPS matrix plays a vital role to protect bacteria cells, by limiting the diffusion of antimicrobials in inner layers. Biofilm immobilizes bacteria and increases cell density, that provides the bacteria an ideal condition for eDNA exchange. Some of these transfers can play vital role for antibiotic resistance. Another report suggests that, the horizontal gene transfer rate is considerably higher in biofilms than in the free floating ones (Hausner & Wuertz, 1999).

1.2.6 Clinical Importance of Biofilm

Biofilms are linked to various human disease. In medical environment, it was reported that biofilms can be involved in more than 65% of all bacterial infections (Pozo & Patel, 2007). Biofilms are responsible for numerous severe bacterial infections, such as, chronic wound, lung, and ear infections (Fux et al., 2005). Some studies indicated that over 80% of all microbial infections are somehow biofilm related (D. Davies, 2003). These infections can be very difficult to detect and treat.

Biofilms can also colonize on medical devices such as catheters, tubes and surgical implants. There are several reports on presence of biofilm forming viable multi drug resistant bacteria despite cleaning on clinical surfaces in medical ICUs (Hu et al., 2015; Vickery et al., 2012). Biofilms formed on hospital shower hoses can also be a source of nosocomial infection (Soto-Giron et al., 2016). Moreover, dry surface biofilms (DSBs) can be viable for longer periods in hospital environments, and can play a vital role in transmission of nosocomial infections, using healthcare personnel’s hands as transport means (Chowdhury et al., 2018).

1.2.6.1 Cystic Fibrosis

The formation of thick and sticky mucus in the lung, named Cystic fibrosis (CF), can block the air passage and can make it difficult for patients to breath (Lyczak et al., 2002). *P. aeruginosa* biofilms are responsible for 80% of chronically infected CF patients. *P. aeruginosa* can also contaminate medical instruments, devices and tools. A number of cases of hospital-acquired *P. aeruginosa* infections have been reported so far (Jones et al., 2001). But, there are not many good antimicrobials available for treating persistent *P. aeruginosa* infection. For patients infected with *P. aeruginosa*, antibiotic exposure is sometimes useful to get rid of the symptoms of the infection, but that does not indicate complete cure from the infection. This is because of *P. aeruginosa* biofilms which act as reservoirs for disease reappearance (J. W. Costerton et al., 1999). Development of anti-biofilm drugs is therefore necessary which can act and remove on *P. aeruginosa* biofilms.

1.2.6.2 Urinary Infection

Catheters and biomaterials in the urinary tract can increase the possibility of bacterial biofilm formation that can lead to urinary infection (Tenke et al., 2012). The biomaterials provide surfaces for bacteria to adhere. Most of the urinary catheters can be infected with bacterial biofilms. This types of biofilms, such as *P. mirabilis* biofilms, can be crystalline in nature. These biofilms can block catheters and patients often need to change the blocked catheters (Jacobsen et al., 2008).

1.2.6.3 Wounds

Biofilms are frequently found in chronic wounds (James et al., 2008). Acute wounds are not usually associated with biofilm, whereas chronic wounds are biofilm related which persists and heals slowly. Normally, biofilms occur on the outside layer of wounds. In some cases, biofilms, such as *P. aeruginosa* biofilms, can be also detected in the deep layers of a wound. These biofilms related infections are difficult to detect using a traditional wound swab culture (Hall et al., 2014).

1.2.6.4 Cardiac Valve Infection

Bacterial biofilm on cardiac valve can cause prosthetic valve endocarditis. *Staphylococcus epidermidis, S. aureus, Streptococcus* spp., *Enterococcus* spp., *Candida* spp and *Corynebacterium* spp. can be involved in endocarditis. (Trampuz & Zimmerli, 2005) . Biofilm can block or disrupt

the artificial cardiac valve. This can result in reduced flow, turbulence or sometimes leaking. Biofilm cells that are dispersed, can travel along with the blood stream and can cause infection in other areas. Biofilm remains in the blood circulation may block the blood flow at the terminal points, such as the kidney and brain.

1.2.6.5 Prosthetic Joint Infection

Gram-positive bacteria, like staphylococci can cause prosthetic joint infections. Bacteria that come from blood or the lymph can adhere to the surface of prosthetic joints and form biofilms, immediately after surgical operation. It can take some time before showing the symptoms (such as pain) of the biofilms, unlike other common bacterial infections that give symptoms like fever (Trampuz & Zimmerli, 2005).

1.2.6.6 Dental Plaque

Dental biofilms are very important in dentistry and biofilms play an important role dental infections. The arrangement and architecture of the healthy plaque biofilm is considerably dissimilar from the disease-related plaque biofilms. Unique microenvironment in the mouth is characterized by water, temperature fluctuations, carbon and nitrogen input and hard surface. The composition of bacteria in biofilms are influenced by the changes in the local environment. *Streptococcus sanguis*, *Streptococcusgordonii*, *Streptococcus oralis*, *Actinomyces* species, etc. that present in healthy biofilms often show low acid tolerance. That is why acid can play a major role in tooth decay. (Marquis, 1995).On the other hand, *S. sobrinus* and *S. mutans* , that are involved in dental caries, are normally acid tolerant. These can also produce robust biofilms which can be responsible for a number of oral cavity diseases, for example, periodontitis, dental caries, gingivitis, etc. (Sbordone & Bortolaia, 2003).

1.2.6.7 Kidney Stones

Kidney stones can also be formed by the bacterial biofilms. These stones can obstruct urine flow and produce recurrent infection and inflammation which can sometimes result in kidney failure. Roughly 20% of kidney stones are related to urinary tract infection. It was previously reported that, these stones can be formed by the interaction between bacteria and minerals derived from the urine. This type of interplay can form a complex biofilm composed of bacteria, biofilm matrix and mineralized stone (Parsek & Singh, 2003).

1.2.6.8 Device-Related Infections

Medical devices such as intravenous catheters, urinary catheter, pacemakers, electrical dialyzers and joint prosthetics are essential for the patients as there is not many alternatives against these devices. These devices are frequently related with infections caused by biofilms. In most of the cases, *Staphylococci* and *Pseudomonas* species opportunistically infect a medically intervening device and thus can enter to the host (Fedtke et al., 2004). This type of infections are called as chronic polymer associated infection in some reports (von Eiff et al., 1999). In addition, it has been observed that open wounds and implants can be infected by *Staphylococci*. *S. epidermis* has also been reported to adhere to the medical devices competently (Otto, 2009).

1.2.7 Biofilm Resistance to Antibiotics & Host Immune System

Although antibiotic therapy continues to be the most common treatment against diseases caused by bacteria, biofilms are highly resistant to antimicrobials and are often not removed effectively with mere antibiotic treatment. However, as scientists explored and still continue to explore various sources of antibiotics to build up the arsenal against bacterial infections, the seemingly innocuous targets have also been rallying to evolve mechanisms for combating them (Blair et al., 2015). Planktonic bacteria might be susceptible to specific antibiotics, but biofilm formation is an effective strategy adopted by bacteria to counter the barrage of antibiotics used against them. The minimum inhibitory concentration of antibiotics required for biofilms is much higher compared to planktonic bacteria because the biofilm matrix hinders the antibiotics physically and chemically from reaching their destination (Fux et al., 2005). A number of antibiotics can also be trapped by charged polysaccharides and eDNA. The penetration property of antibiotics into the biofilm has been measured by several groups of researchers. Ciprofloxacin concentration in *P. aeruginosa* biofilm was shown to be radically reduced, but not completely blocked (Suci et al., 1994). Another group investigated the ampicillin and ciprofloxacin penetration ability on *K. pneumoniae,* and it revealed that Ciprofloxacin have better penetration ability than Ampicillin (Anderl et al., 2000).

The frequencies of horizontal plasmid transfer are considerably higher in biofilms than between free floating cells. Previous reports revealed that *S. aureus* biofilms can enhance the spread of plasmid mediated antibiotic resistance genes by either conjugation or mobilization (Savage et al., 2013). Moreover, because of limited availability of nutrients and oxygen inside the biofilm

structure, biofilm bacteria, especially those reside in deep layers of biofilm, have slow metabolic, growth and division rate. These characteristics can make biofilm cells resistant to several antibiotic drugs that target cell division. For instance, β-lactams, which acts on cell division, has limited or no antimicrobial effect on E. coli biofilms, and their bacteriolytic activity seems to be reduced (Ashby et al., 1994).

Moreover, in biofilms, persister cells, a small subpopulation of biofilm cells, are present (Lewis, 2007). The growth rate of those persister cells is zero or exceptionally slow. Widely used antibiotics now a days target cell growth or division. These antibiotics are not effective against persister cells. Hence, these cells can act as reservoirs, and if the antibiotic stress is removed these cells can regenerate infectious particles again.

A number of studies have shown both innate and adaptive immune responses to biofilms (Jensen et al., 2010). Biofilm bacteria are also found to be more resistant against host immune system reactive molecules than their planktonic counterpart. It was speculated that, reduced adaptive immunity or memory responses may compromise the success of vaccine approaches against staphylococcal biofilms (Scherr et al., 2014). Exopolysaccharide matrix can also provide physical barrier to the aggregated biofilm cells, that can reduce the effect of immune cells. A study revealed that exopolysaccharide alginate produced by *P. aeruginosa* biofilms saved biofilm bacteria from leukocyte killing (Leid et al., 2005).

1.2.8 Disinfectant Susceptibility Biofilm Cell

Chemical agents used on nonliving surfaces to neutralize pathogenic microorganisms are called disinfectants. Antibiotics are drugs that interact with targeted cell structures or metabolic processes in microorganisms and mostly used internally to limit infections. On contrary, disinfectants have multiple targets and they act non-specifically (Meyer & Cookson, 2010). The mechanism of action of disinfectants depends on their type. These mechanisms have been extensively studied and descried in a number of reviews (McDonnell & Russell, 1999). Potential target sites of disinfectants in both Gram-positive and Gram-negative bacteria are the cell wall, cytoplasmic membrane, outer membrane, DNA, RNA, functional and structural proteins and other cytosolic components. Treatments with disinfectants are widely used in medical, industrial and domestic settings to limit the contamination of surfaces by the microorganisms. While these disinfectants

can neutralize most of the surface contaminating microorganisms, some of them may survive and can give rise to considerable public health related problems. A number of reports have emphasized on the persistence of microorganisms after disinfection in medical environments (Deva et al., 1998; Martin et al., 2008) and food (Bagge-Ravn et al., 2003; Weese & Rousseau, 2006). The disinfectant resistance of is commonly associated with the occurrence of biofilms on surfaces (Bressler et al., 2009; Vestby et al., 2009). These information are not enough to control the contamination of microorganisms in abiotic surfaces. A better understanding of the mode of action of disinfectant resistance in biofilms are now considered as a major concern for the microbiologists.

Although a number of studies have focused on the mechanisms of antibiotic resistance in biofilms (Fux et al., 2005; P. S. Stewart & Costerton, 2001; Philip S. Stewart, 2002), specific mechanisms of disinfectant resistance in biofilms are not completely known. As we know, disinfectants act non-specifically against multiple target and they interact with specific cell structures or metabolic processes in microorganisms (Meyer & Cookson, 2010). SDS is an anionic surfactant which is a common component of many domestic detergents. Many personal hygiene products, pharmaceutical, industrial and hospital cleaning products contain SDS. This surfactant affects the integrity of the cellular proteins and of the mucopolysaccharides by disrupting non-covalent bonds. These proteins and mucopolysaccharides are bacterial cell components or components of the extra cellular matrix. On contrary, Chlorine-based disinfectants act as oxidants. These disinfectants form toxic chlorocompounds by chlorinating the lipid protein components of the cell wall. They also induce the leakage of cell content to outside to destroy the bacteria (Kim et al., 2008).

Ethanol increases the solubility of outer membrane lipids and thus the structural integrity of the cell membrane become weak and vulnerable. After the disintegration of cell membrane, ethaol can enter the cell and denature the proteins inside the cell. On the other hand, Savlon is a commonly used antiseptic liquid that contain Chlorhexidine Gluconate and Cetrimide. Chlorhexidine Gluconate is known as a strong antimicrobial agent. This compound can kill bacteria and prevent infection by absorbing onto the cell wall and thereby causing leakage of the intracellular components to outside (A. Davies, 1973). Cetrimide is also considered as a powerful decontaminating agent that can play a vital role in the release of purines, pyrimidines, pentose and inorganic phosphate from microbial cells.

1.2.9 Fluorescent Microscopy and Viability Stains

Fluorescent microscopy is known as a vital and noninvasive method to evaluate antimicrobial dynamics and reactivity in biofilms and contributes to our understanding of the efficacy of disinfectants on complex biofilm structures (Bridier et al., 2011; Takenaka et al., 2008). As live and dead cells within a biofilm can be differentially visible under fluorescent microscope by the application of viability kits, they were used to observe the effect of disinfectants on biofilms in many studies (De la Fuente-Núñez et al., 2014; Korany et al., 2018). This type of Bacterial viability tests are frequently performed with two stains SYTO9 and propidium iodide (PI). These two fluorophores are found as premixed, dual staining kits, ready for use. These dye can detect membrane integrity of the cells. PI can penetrate only dead cells with disintegrated membranes. This stain is commonly used as counterstain in multicolor fluorescent techniques for identifying dead cells in a biofilm population. On the other hand, SYTO9 is the green-fluorescent nucleic acid stain. This stain can enter both live and dead bacterial cells. When SYTO9 is bound to nucleic acids inside the cell, the fluorescent signal of the fluorophore is strongly enhanced. This dye shows low intrinsic fluorescence signal when it is not bound to nucleic acid. When both SYTO9 and PI are present, PI shows a stronger affinity for nucleic acids than the other, and thus, SYTO9 is replaced by PI (Stocks, 2004). Viability tests with SYTO9 and PI have great advantages as it is a rapid procedure, allow quantitative analyses, as can be easily observed using fluorescent microscope (Auty et al., 2001; Leuko et al., 2004). But sometimes it cannot subtract background signals, which can be a limitation of using this kit (Stiefel et al., 2015).

1.2.10 Biofilm Formation by *Pseudomonas aeruginosa*

P. aeruginosa is a model organism for biofilm formation (Tolker-Nielsen & Molin, 2004). Biofil formation of this organism is a highly controlled process that have five different stages (Sauer et al., 2002). The extraordinary ability of *P. aeruginosa* to form biofilms in different environments makes it responsible for various infections, particularly in immune-compromised persons (Rasamiravaka et al., 2015). *P. aeruginosa* can cause serious health threat, particularly in a hospital environment. It is one of the common causes of ventilator associated pneumonia and catheter related infection (Mittal et al., 2009; Ramírez-Estrada et al., 2016) and thus it has become one of the prominent causes of hospital acquired infections worldwide (Zavascki et al., 2006). Infections caused by this organism are frequently associated with high morbidity and mortality because of its

characteristic resistance to many antimicrobials and its ability to acquire drug resistance (Dwivedi et al., 2009).

Recent works are focusing on the numerous aspects of biofilm formation in *P. aeruginosa* such as the role quorum sensing in biofilm structure, effect of nutrients and motility etc. An important signaling molecule, c-di-GMP, has been found to be an important regulator that regulate the synthesis of EPS components of the biofilm matrix. Some studies showed extracellular DNA as an important component of the biofilm matrix of *P. aeruginosa*. The resistance of biofilm bacteria to antimicrobials are now a prime concern and the molecular mechanisms of biofilm related antimicrobial resistance are now being studied vigorously. Understanding the environmental factors and regulatory mechanisms related to biofilm growth and dispersal have also caught the scientist's attention. Now a days, it has also become clear that formation of biofilm in *P. aeruginosa* involves interactions between subpopulations (Harmsen et al., 2010).

1.2.11 Pathway and Molecular Genetics of *Pseudomonas aeruginosa* Biofilm Formation

P. aeruginosa forms different types of biofilms depending on the environment. For example, the pellicles are known to form at the air–liquid interface of a standing culture, colonies form on agar plates and submerged solid-surface associated biofilms form in flow cells. *P. aeruginosa* biofilm is one of the most studied biofilms produced by a microorganism (J. W. Costerton et al., 1999; J. William Costerton, 2001; Gottenbos et al., 2002). But, the molecular mechanism for the formation of extracellular matrix, production of different exopolysaccharides in different *P. aeruginosa* biofilms is needed to be investigated more (Friedman & Kolter, 2004).

Several genes have been found to play important roles in biofilm formation of *P. aeruginosa*. Only 1% of genes were shown to be differentially expressed in the biofilm growth. Around 0.5% of the genes were found to be repressed and another 0.5% of the genes were found to be activated (Whiteley et al., 2001). Four major pathways (cAMP/Vfr signaling, c-di-GMP dependent Polysaccharide synthesis, quorum sensing, and the Gac/Rsm pathway) in *P. aeruginosa* are involved in biofilm formation. Those pathways play important roles in regulation of biofilms. In response to the environmental stimuli these pathways establish regulatory control at the transcriptional, translational, and post-translational level and thus regulate biofilm formation (Coggan & Wolfgang, 2012; Rasamiravaka et al., 2015) **(Figure 1.4)**. For example, Vfr is known

as a cAMP-dependent DNA-binding protein that acts as a regulator of virulence gene expression in *P. aeruginosa*. This signaling mechanism controls the transcription of several genes that express a number of important virulence factors, such as the type 2 and type 3 secretion system and their associated toxins, type IV pili, flagella etc. (Marsden et al., 2016). Accumulation of cAMP can inhibit the attachment of the bacteria to the surface.

The QS systems are arranged hierarchically with the las system and that positively regulates both the rhl and PQS systems. While las and rhl are AHL based QS system (signaling molecules are 3-oxo-C12- HSL and C4-HSL respectively), PQS involve a third signalling molecule, 2-heptyl-3-hydroxy-4(1*H*)-quinolone (Latifi et al., 1995; Passador et al., 1993; Pesci et al., 1999). These three QS systems regulates virulence factor production, biofilm maturation, and motility factors production (Kievit, 2009).

GacS/GacA is a two-component system, which promotes the expression of RsmY and RsmZ, which are two small regulatory RNAs. These RNAs influence the translational repressor RsmA. Titration of RsmA encourages the production of biofilm determining factors. On the other hand, free RsmA leads to a free floating and more virulent existence. Moreover, *P. aeruginosa* exopolysaccharide Psl is reported to be post-transcriptionally regulated by RsmA (Irie et al., 2010). That means, RsmA plays an important role as a negative translational regulator, and its effects are influenced indirectly by translation of specific regulatory factors (Brencic & Lory, 2009).

Identification and characterization of the *pel* genes, that are required for biofilm matrix formation under diverse environmental conditions in *P. aeruginosa* PA14 was first described by Lisa Friedman and Roberto Kolter (Friedman & Kolter, 2004). The *P. aeruginosa* Wsp signal transduction system is involved in cyclic-di-GMP (c-di-GMP) production. C-di-GMP is an intracellular messenger and high levels of c-di-GMP induce the processes that are responsible for production of biofilm matrix (Güvener & Harwood, 2007). Not only in *P. aeruginosa*, EPS biosynthesis regulation by c-di-GMP occurs in a variety of bacterial species (Liang, 2015). Another report showed that c-di-GMP regulation are also connected with biofilm formation, biofilm-associated motilities, and other processes in *P. aeruginosa* (Valentini & Filloux, 2016). This molecule, c-di-GMP binds to a number of effector components to control transcription, the enzymatic activity and production of larger cellular structures. In this way c-di-GMP determines the planktonic and biofilm phenotype of bacteria (Hengge, 2009).

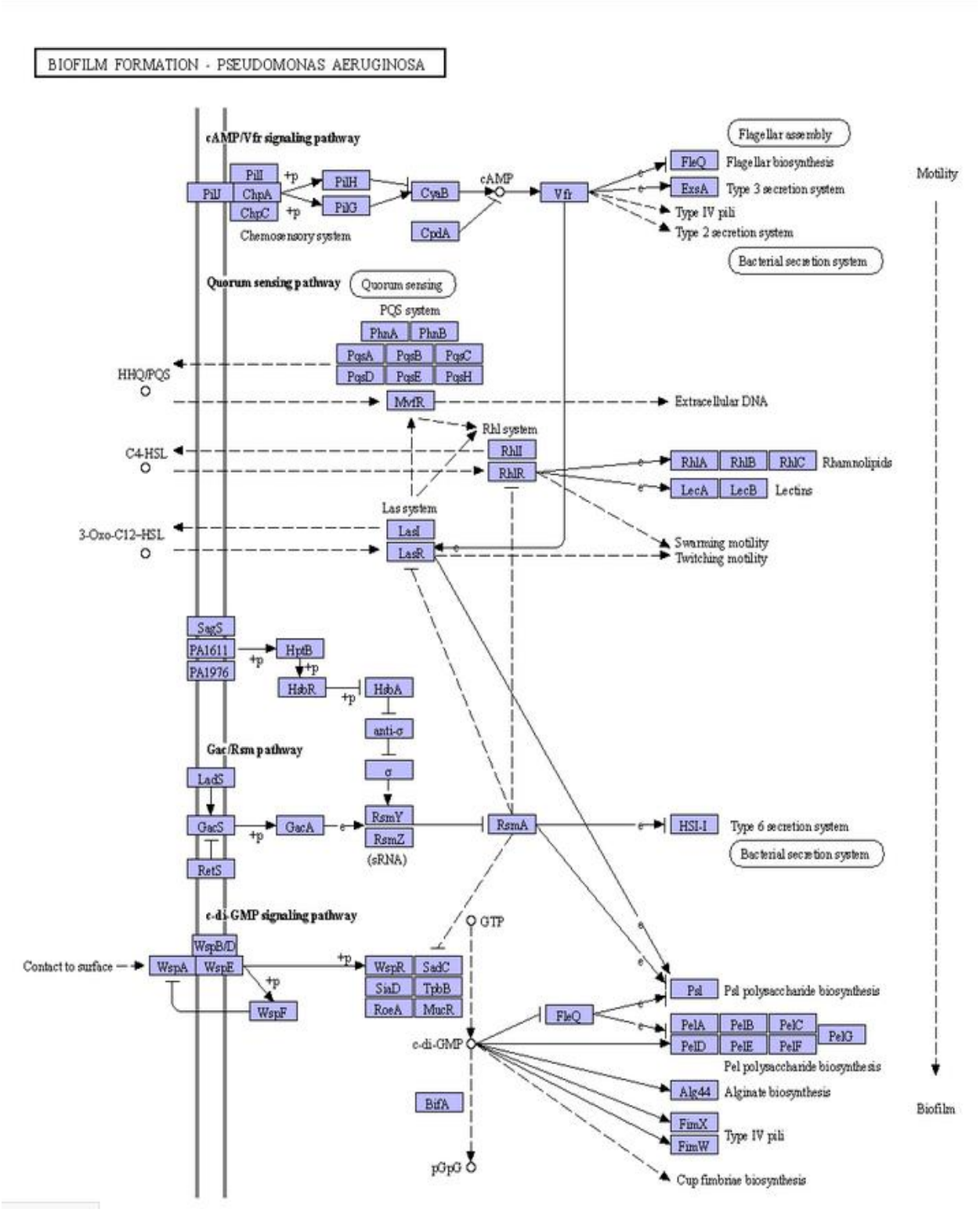


Figure 1.4: Biofilm formation pathway in *Pseudomonas aeruginosa*. (KEGG Pathway ID-ko02025)

1.2.12 Important Biofilm Related Genes and Proteins

1.2.12.1 Genes Involved in Twitching Motility

Twitching motility depends on polar type IV pili, which are also known to play a vital role in facilitating adherence to surfaces. There is a structural gene that is responsible for synthesis of type IV pili and flagella which were reported crucial for biofilm formation and host infection. *P. aeruginosa* cells that lack flagellar motility often show poor surface attachment. On the other hand, cells that lack type IV pili cannot form microcolonies (O'Toole & Kolter, 1998). In *P. aeruginosa*, *Pil*T gene is essential for twitching motility. Mutations in *PilT* result in a non-twitching hyperpiliated phenotype (Whitchurch & Mattick, 1994). In another report, Chiang & Burrows reported that, hyperpiliated, nontwitching *pil*T and *pil*U mutants of *P. aeruginosa* have thick biofilm phenotype under static growth conditions. This report shows that adhesion, not twitching motility, is necessary for biofilm initiation (Chiang & Burrows, 2003).

1.2.12.2 Rhamnolipids and Biofilm Structure

Rhamnolipids are glycolipids containing one or two l-rhamnose residues (mono-rhamnolipid or di-rhamnolipid). In *P. aeruginosa, rhlAB* operon and *rhlC* are involved in rhamnolipid production. These three of these genes are regulated by quorum sensing (Ochsner et al., 1994; Pearson et al., 1997). These glycolipids are presumably synthesized by sequential glycosyltransferase reactions involving two distinct rhamnosyltransferase enzymes. Mono-rhamnolipid synthesis is catalyzed by RhlAB (rhamnosyltransferase 1). On the other hand, *rhl*C encodes rhamnosyltransferase 2, which is involved in the production of di-rhamnolipid molecules (Rahim et al., 2001). RhlA is essential to stabilize the RhlB in the cytoplasmic membrane. RhlB is thought to be the catalytic subunit of the rhamnosyltransferase and contains two membrane-spanning domains that anchor RhlB in the inner membrane. The *rhlAB* that encodes rhamnosyltransferase I, and *rhlR–rhlI* that includes a quorum-sensing system in *P. aeruginosa* are located in the *rhl* region. All of these genes are transcribed in the same direction, but *rhlAB* transcription occurs independently of *rhlR* and *rhlI* (Ochsner et al., 1994; Pearson et al., 1997).

Rhamnolipids are produced by *P. aeruginosa* and play a vital role in the maintenance of biofilm structure. These rhamnolipids can affect cell to cell interactions and bacterial attachment to surfaces. In the later stages of biofilm development the cell concentration is high, rhamnolipid

synthesis is induced. This indicates an active mechanism of the intercellular interaction and communication in bacteria (Mary E. Davey et al., 2003). Rhamnolipids can also play an important role in the detachment or dispersion of *P. aeruginosa* biofilms (Boles et al., 2005; Wang et al., 2013).

1.2.12.3 Pel Operon: Involvement in Pellicle Formation

As we know, *P. aeruginosa* produces minimum three extracellular polysaccharides, that are known as alginate, Pel and Psl. These polysaccharides play important role in biofilm development and structure (Ryder et al., 2007). *P. aeruginosa* use either Psl or Pel as the primary biofilm matrix polysaccharide (Kelly M. Colvin et al., 2012). It is also reported that, *P. aeruginosa* PAO1 and PA14, differ in the primary polysaccharide used for biofilm development (Kelly M. Colvin et al., 2011).

Pel is an aggregative polysaccharide produced by *P. aeruginosa*. Its name come from the thick pellicle that are frequently observed in the strains that overexpress the *pel* operon. The Pel operon contain seven enzyme producing genes that have similarity other EPS synthesis proteins. It should also be noted that, not all of the genes that are predicted to be essential for Pel production are present in this particular operon (Franklin et al., 2011). Other EPS synthesis enzymes that are encoded in other locations of the chromosome are also important in Pel synthesis. The exact structure and composition of Pel is still not known perfectly, but researches are ongoing to identify the sugars and linkages present in this complex EPS. The *P. aeruginosa*. strain PA14 depends on Pel production for aggregation, as it lacks *pslABCD* genes (Friedman & Kolter, 2004). Pel production is also reported to be increased by higher amounts of cyclic diguanylate (c-di-GMP), the intracellular second messenger (V. T. Lee et al., 2007). Recent studies have indicated a role for the flagellum regulator FleQ as both a repressor and an activator to control gene expression. FleQ binds in the pel operon promoter region in response to c-di-GMP (Baraquet et al., 2012). In addition, c-di-GMP modulates the activity of PelD and thus influences Pel symthesis. Biofilms that formed on air-liquid interface are called pellicles. These pellicles can be observed in the naked eye and this can be regarded as an evidence of the contribution of Pel to biofilm structure. The Pel polysaccharide production is also strongly connected with attachment of bacterial cells to culture tubes and aggregate formation in broth culture (Franklin et al., 2011).

PelA protein resides in the periplasm and membrane. Deacetylase activity of PelA is essential for Pel polysaccharide synthesis of *P. aeruginosa* (K. M. Colvin et al., 2013). PelA and PelB proteins are reported to interact directly with each other. It was also reported that, the hydrolase activity of PelA decreases and its deacetylase activity increases during that interaction. PelB has a TPR-containing domain that localizes PelA to the Pel secretion apparatus in the periplasm. This localization is thought important for deacetylation of Pel before its excretion from the bacterial cell. The modification and secretion complex comprised of PelA and PelB is vital for Pel-dependent biofilm formation in *P. aeruginosa* (Marmont, Whitfield, et al., 2017). On the other hand, PelC plays an important role as an electronegative funnel that guides the positively charged Pel toward PelB exit channel (Marmont, Rich, et al., 2017). PelD, PelE, and PelG, reside within the inner membrane and the complex of PelD, PelE, and PelG interacts with PelF. This complex is essential for Pel polysaccharide production (Whitfield et al., 2020). Another report shows that PelF encodes a glycosyltransferase domain that interacts with UDP-glucose as the substrate for Pel production (Ghafoor et al., 2013).

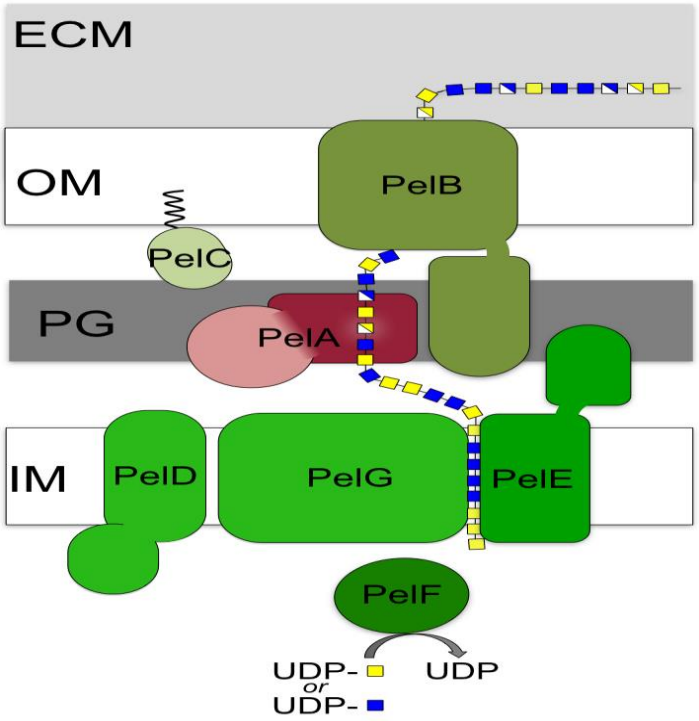


Figure 1.5- Pel polysaccharide production with Pel operon proteins (source: the Howel lab, SickKids research institute, Canada)

1.2.12.4 Lectins

Lectins are known carbohydrate-binding proteins that bind to specific sugar molecules. For example, *P. aeruginosa* is known to produce two lectins, LecA and LecB that were formerly known as PA-IL and PA-IIL, respectively. These lectins are important in biofilm formation and also related with infection process (Mitchell et al., 2002; Sabin et al., 2006). A report suggests, LecA and LecB contribute to the pathogenesis of *P. aeruginosa*-mediated lung infection (Chemani et al., 2009).

LecA is known as an adhesin and a cytotoxic lectin produced by *P. aeruginosa*. With high specificity and affinity, this protein binds with hydrophobic galactosides and thus contributes to *P. aeruginosa* biofilm structure. It also plays an important role in host cell invasion and cytotoxicity (Adam et al., 1997). On the other hand, LecB binds to fucose, mannose, and mannose-containing oligosaccharides with high specificity. It reduces ciliary beating of airway epithelium (Adam et al., 1997). Both lectins can also form biofilms on abiotic surfaces (Tielker et al., 2005). For example, LecA is reported to be required for *P. aeruginosa* biofilm formation on stainless steel and polystyrene surfaces (Diggle et al., 2006).

LecB protein is a homotetramer consisting of four monomers comprising 114 amino acids. LecB monomers require two divalent calcium ions for its function (Mitchell et al., 2002). In *P. aeruginosa*, LecB is also involved in pilus synthesis and proteolytic activity but not involved in adhesion to human tracheobronchial mucin (Sonawane et al., 2006). LecB is reported to bind with specific carbohydrate ligands in the bacterial cell surface (Tielker et al., 2005) and also binds to the exopolysaccharide Psl and stabilizes the biofilm matrix (Passos da Silva et al., 2019).

LecB from the strain PA14 and LecB from the well-studied PAO1 strain shows 13% sequence divergence between them. This divergence can also affect their ligand binding affinities and specificities. This sequence variance can also result in reduced efficacy of the LecB-directed drugs. Sommer reported this sequence variation in 2016 and suggested to use this divergence as a marker for strain family classification. (Sommer et al., 2016).

Biofilm maturation was reported to be inhibited in *P. aeruginosa* culture when the organism was grown in the presence of the monosaccharides that are the binding partner for the lectins (Tielker et al., 2005). Newer reports have shown the way to develop new therapeutic approaches by using

glycomimetics which can disrupt LecB-sugar interactions. For example, Glycopeptide Dendrimers that targets Fucose-specific LecB, is reported to be effective for inhibition and dispersion of *P. aeruginosa* biofilms (Johansson et al., 2008; Michaud et al., 2016).

1.2.13. Correlation between Genetic Makeups and Biofilm Forming Abilities

A combination of phenotypic and genotypic assays can be useful for investigating biofilm formation in bacterial isolates. As sequence divergence in LecB protein of *P. aeruginosa* can show variable ligand binding specificities (Sommer et al., 2016), genetic makeup and variance in biofilm related genes may also contribute to biofilm formation. Although many researchers have tried to establish correlation between the genetic makeups and biofilm forming abilities of bacterial strains, conflicting results between studies and experimental conditions were observed and still a lot of areas are remain to be explored.

A group of researchers tried to find the link between biofilm formation and the presence of *rpf*F, *rml*A and *spg*M genes in Cystic Fibrosis (CF) causing *Sternotrophomonas maltophilia*. They revealed that *rml*A^{-}/*spg*M^{+}/*rpf*F^{+} genotypes were significantly associated to CF causing strains, while *rml*A^{+}/*spg*M^{+}/*rpf*F^{-} genotypes were found in non-CF group. They also found a significant correlation between the detection of these genes and the biofilm expression profiles. For example they found that, presence of *spg*M genes is significantly related to strong biofilm forming ability, both in non-CF and CF strains. By analyzing other genes they concluded that biofilm formation can be differently regulated in CF and non-CF strains (Pompilio et al., 2011). On the contrary, in another study it was shown that CF causing *P. aeruginosa* isolates produce variable amounts of biofilm, although they have identical genomic profiles (Head & Yu, 2004).

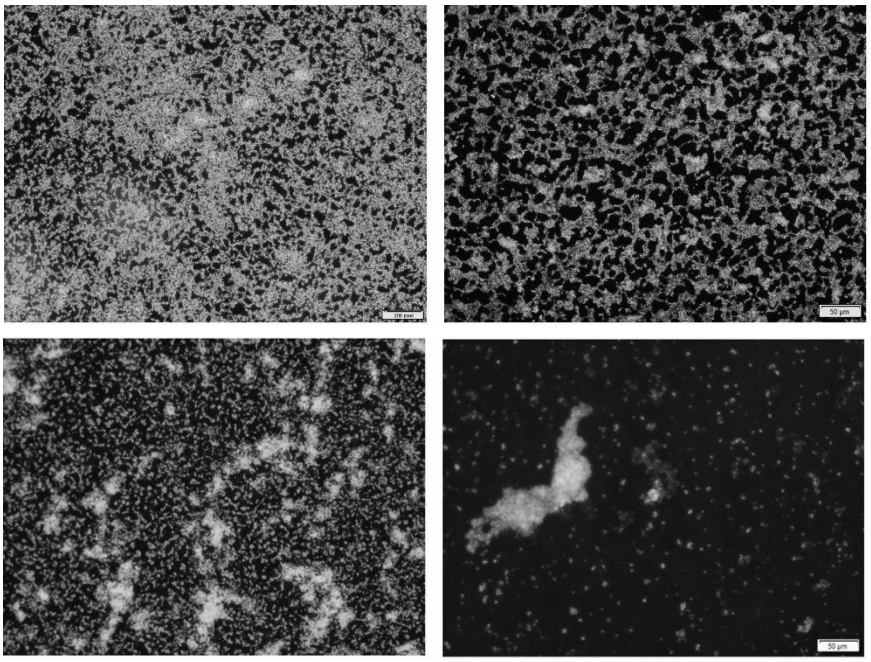
Considerable differences in biofilm forming ability were found between different phylogenic groups of *Listeria monocytogenes* strains, suggesting genomic variability contributing to biofilm forming ability (Borucki et al., 2003). Correlation between genotype and biofilm production was also observed in *L. monocytogenes* in another study (B.-H. Lee et al., 2019). In *Streptococci*, an allelic variants of *comC* that encodes the competence-stimulating peptide and extracellular DNA were shown to be crucial in biofilm maturation (Carrolo et al., 2010, 2014).

In *Campylobacter jejuni* isolates, hotspots of genetic variation in homologous sequences were identified by a group of researchers. Those variation contribute to biofilm phenotypes (Pascoe et

al., 2015). Another research group indicated a high abundance of the *ica* genes among *Staphulococcus aureus* mastitis isolates, but their presence was not always related with biofilm formation (Vasudevan et al., 2003). In case of *Vibrio parahaemolyticus*, some biofilm-associated genes were found to be present in most of the strains, but biofilm phenotypes were not similar (Mizan et al., 2016). These reports tried to focus on the relation between genomic makeups and biofilm phenotypes, but still a lot more remain unknown about the molecular mechanism of biofilm formation by different microorganisms.

Chapter 2

MATERIALS AND METHODS



2.0 Materials & Methods

The current study, conducted on multi drug resistant clinical isolates retrieved from the repository of Microbial Genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka, intends to study the biofilm forming ability, detection of biofilm genes and also to analyze differential expression of biofilm associated gene harboring these clinical isolates.

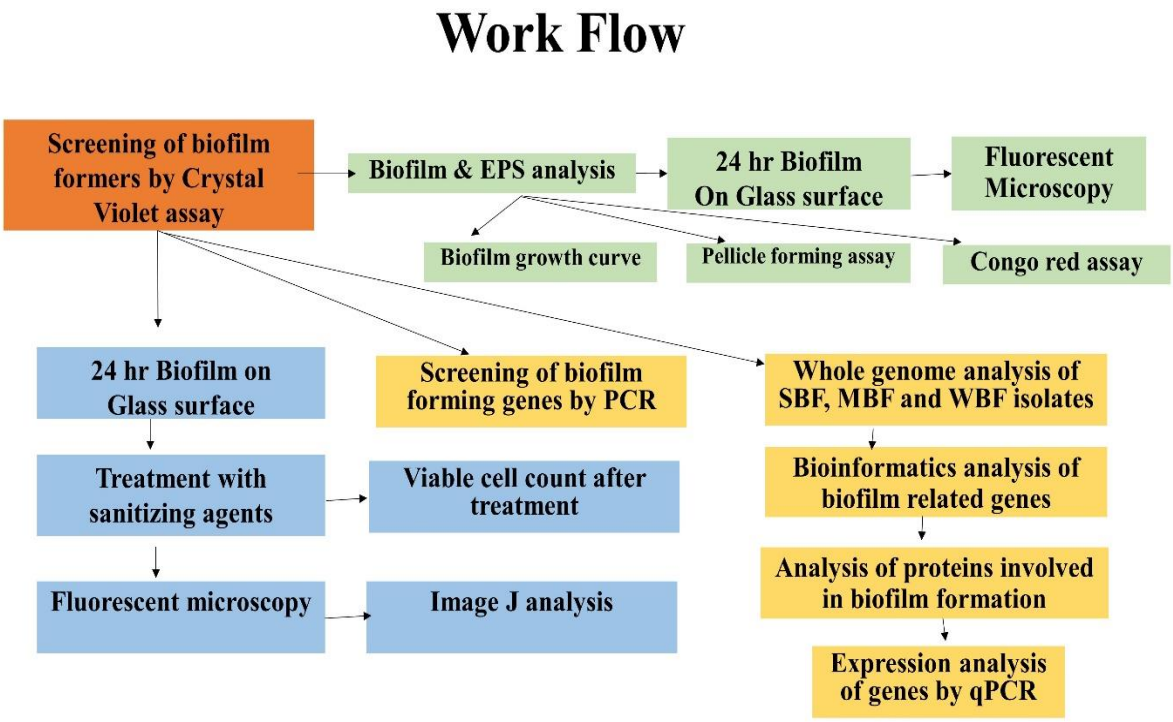


Figure 2.1 Diagrammatic representation of the research work plan *(EPS= Extracellular Polysaccharide, PCR= Polymerase Chain Reaction, SBF= Strong biofilm former, MBF= Moderate biofilm former, WBF= Weak biofilm former.)

2.1 Selection of Clinical Isolates

A total of 45 previously identified and characterized clinical isolates were retrieved from glycerol stocks preserved at -20°C in Microbial Genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka. These isolates were selected on the basis of their resistance to antibiotics and all of the selected isolates were previously found as resistant to at least 2 antibiotic groups. The isolates were isolated from 4 different sources of wound swab, urine, pus, blood and

tracheal aspirate samples that were previously collected from Dhaka Medical College Hospital during 2 different sessions- October, 2015 and March, 2016 and from Bangladesh Institute of Health Science (BIHS) during February – March, 2018. Among 45 isolates 20 were previously identified as *Pseudomonas aeruginosa*, 9 were *Klebsiella pneumoniae* and others were *P. stutzeri, P. hibiscicola, Stapylococcus aureus, Providentia stuartii, Proteus mirabilis* and *Acinetobacter baumanii.* Identification and characterization of most of these isolates were previously reported (Rakhi et al., 2019). Primarily, all the isolates were screened for biofilm formation ability and among those, selected clinical isolates were taken for further analysis on the basis of their biofilm forming abilities **(Table-2.1)**.

Table 2.1: Name, ID and source of clinical isolates used in this study

Hospital	Isolate ID	Source	Organism
DMC	27b	Urine	*Pseudomonas aeruginosa*
DMC	8b	Wound Swab	*Pseudomonas aeruginosa*
DMC	23b	Tracheal Aspirate	*Pseudomonas aeruginosa*
DMC	15b	Pus	*Pseudomonas aeruginosa*
DMC	54D	Wound Swab	*Pseudomonas aeruginosa*
DMC	20b	Wound Swab	*Pseudomonas aeruginosa*
DMC	20c	Wound Swab	*Pseudomonas aeruginosa*
DMC	7	Pus	*Pseudomonas aeruginosa*
DMC	28C	Wound Swab	*Pseudomonas aeruginosa*
DMC	30b	Pus	*Pseudomonas aeruginosa*
DMC	20D	Wound Swab	*Pseudomonas aeruginosa*
DMC	44	Pus	*Pseudomonas aeruginosa*
DMC	24	Wound Swab	*Pseudomonas aeruginosa*
BIHS	b01	Wound Swab	*Pseudomonas aeruginosa*

Hospital	Isolate ID	Source	Organism
BIHS	b02	Wound Swab	*Pseudomonas aeruginosa*
BIHS	b04	Pus	*Pseudomonas aeruginosa*
BIHS	b05	Wound Swab	*Pseudomonas aeruginosa*
BIHS	b06	Wound Swab	*Pseudomonas aeruginosa*
BIHS	b07	Wound Swab	*Pseudomonas aeruginosa*
BIHS	b08	Wound Swab	*Pseudomonas aeruginosa*
DMC	28b	Pus	*Klebsiella pneumoniae*
DMC	26	Pus	*Klebsiella pneumoniae*
DMC	17a	Urine	*Klebsiella pneumoniae*
DMC	18	Urine	*Klebsiella pneumoniae*
DMC	23a	Tracheal Aspirate	*Klebsiella pneumoniae*
DMC	19b	Wound Swab	*Klebsiella pneumoniae*
DMC	43a	Wound swab	*Klebsiella pneumoniae*
DMC	57a	Urine	*Klebsiella pneumoniae*
DMC	50D	Pus	*Pseudomonas hibiscicola*
DMC	11c	Urine	*Pseudomonas hibiscicola*
DMC	10	Tracheal Aspirate	*Pseudomonas hibiscicola*
DMC	40D	Pus	*Pseudomonas stutzeri*
BIHS	b69	Wound Swab	*Pseudomonas stutzeri*
FCH	93	Wound Swab	*Pseudomonas stutzeri*
DMC	8a	Wound Swab	*Staphylicoccus aureus*
BIHS	b69b	Wound Swab	*Staphylicoccus aureus*

Hospital	Isolate ID	Source	Organism
DMC	50C	Wound Swab	*Staphylicoccus aureus*
DMC	29b	Pus	*Staphylicoccus aureus*
DMC	28b	Wound Swab	*Providencia stuartii*
BIHS	b116	Urine	*Providencia stuartii*
DMC	15a	Blood	*Acinetobacter baumanii*
DMC	25	Wound Swab	*Acinetobacter baumanii*
DMC	27a	Urine	*Acinetobacter baumanii*
DMC	32a	Urine	*Acinetobacter baumanii*
DMC	20a	Wound Swab	*Proteus mirabilis*

2.2 Preparation of Bacterial Culture

All the isolates were revived on Tryptic Soya Agar (TSA)(**Appendix**) plate by incubation at 37 °C for 24h and the isolated single colonies were inoculated into TSB (Tryptic Soya Broth) (**Appendix**) for overnight culture.

2.3 Biofilm Production on Microtiter plate and Crystal Violet (CV) assay

2.3.1 Growing the Biofilm

Biofilm formation was performed using the methods previously described by George O'Toole (O'Toole, 2011). Overnight culture of all the isolates was diluted to 1:100 into fresh TSB medium. 100 µl of diluted culture for each isolates were inoculated into Thermo Scientific™ 96-Well Microtiter Microplates (quadruplicates) for each isolate. The plates were then incubated overnight at room temperature for biofilm production.

2.3.2 Staining the Biofilm

Staining of the biofilm was also performed as the methods described by George O'Toole (O'Toole 2011). After overnight incubation, unattached cells were removed and the plates were gently

washed 2 times by submerging in a small tub of water. This is very important to completely remove the unattached cells and the medium for staining of the biofilm. The plates were then air dried for 5-10 minutes.

The biofilms were then stained with 125 µL of a 0.1% of crystal violet in water were incubated at room temperature for 10-15 minutes. The plates were then washed 3-4 times with water. The microtiter plates were finally turned upside down and dried for a few hours or overnight.

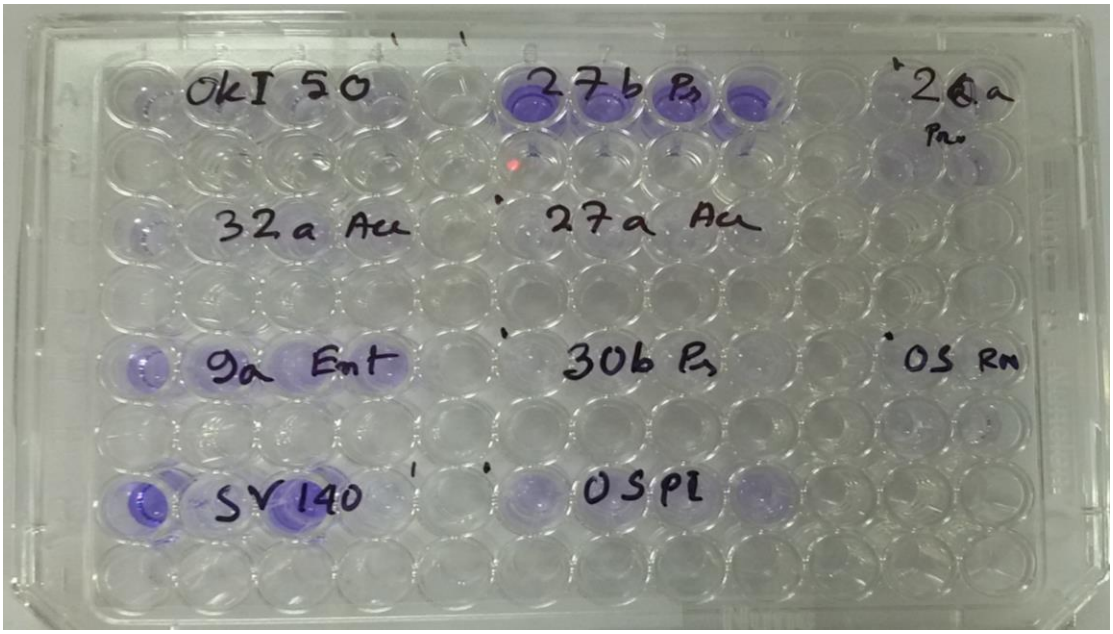


Figure 2.2: Crystal Violet biofilm assay on microtiter plate.

2.3.3 Quantification of the Biofilm

125 µl of 30% acetic acid in water was added to each well of the microtiter plates to solubilize the CV and the microtiter plates were incubated at room temperature for 10-15 minutes. 125 µl of the solubilized CV from each well was transferred to a new microtiter plate. UV absorbance was measured at 595 nm in micro plate reader (Multiskan, Thermo Labsystems) using 30% acetic acid in water as the blank. All data are expressed as mean standard deviation (SD) of the quadruple experimental data. Biofilm formation ability of isolates were determined by the standard formula ($OD \leq OD_{cut}$ = Non-biofilm-former, $OD_{cut} < OD \leq 2 \times OD_{cut}$ = Weak biofilm-former, $2 \times OD_{cut} < OD \leq 4 \times OD_{cut}$ = Moderate biofilm-former, $OD > 4 \times OD_{cut}$ = Strong biofilm-former and $OD_{cut} = OD_{avg}$ of negative control + 3 × standard deviation of ODs of negative control) (Abdi-Ali et al.,

2014; Singh et al., 2017). For biofilm growth curve, the growth was traced by measuring OD at 595 nm after 2, 4, 6, 8, 10 and 24 hours. 4 replicates were used for each isolate and for each time period (Kim & Park, 2013).

2.3.4 Biofilm Growth Pattern Analysis

Twenty clinical isolates were selected based on their ability to form biofilm. To analyze the biofilm formation ability of different isolates, diluted cultures were inoculated in separate 96-well microtiter plate (Thermo Scientific™) and incubated for 2hr, 4hr, 6hr, 8hr, 10hr and 24hr. Quantification of biofilm formation was performed by CV staining as discussed above.

2.4 Biofilm Formation on Glass Cover Slips for Microscopic Analysis

The formation of biofilm on the glass coverslips was adapted and slightly modified from a procedure as described previously by Haibo Mu and his team (Mu et al., 2016).

2.4.1 Preparation of the Bacterial Culture

All the selected isolates were sub-cultured on TSA (Tryptic Soya Agar) plate. The plates were incubated at 37 °C. After 24hr single isolated colonies for each were inoculated into 5 ml TSB to get over night culture.

2.4.2 Preparing the Cover Slips for Biofilm Production

The 18mm glass cover slips (Labtex, Bangladesh) were washed with 70% alcohol and treated by UV for 15 min. Those were then inserted into each well of 12-well plate.

2.4.3 Biofilm Production on Glass Cover Slips

50 µl of overnight bacterial culture of each isolates was added in fresh 5% TSB in 12 well culture plate (Thermo scientific) (Figure 2.3). The plate was then incubated overnight at room temperature for biofilm formation.

2.4.4 Treatment of Biofilm on Cover Slips by Different Disinfectant Agents (Savlon, 70% Ethanol, 0.1%SDS, Sodium Hypochlorite)

0.1% SDS, NaOCl (150 ppm), 70% ethanol, and Savlon solution were prepared and used to observe their effect on biofilms. We used SDS and NaOCl as they are found in common detergents

and bleaching solution in Bangladesh and their concentration were determined following previous studies (Fink et al., 2018; Melo et al., 2014). Biofilm treatment was carried out on cover slips incubated in 12-well plate. Biofilm cover slips were washed with sterile water for 3 times. Washed cover slips were placed in 50 ml Falcon tube. 15 ml of sanitizing agents was added in falcon tube. Falcon tubes were placed in an orbital shaker (Fisher scientific) at 300 rpm for 5 minutes. The cover slips were then taken out from falcon tube and washed with sterile water 3 times to remove loosely attached cells.

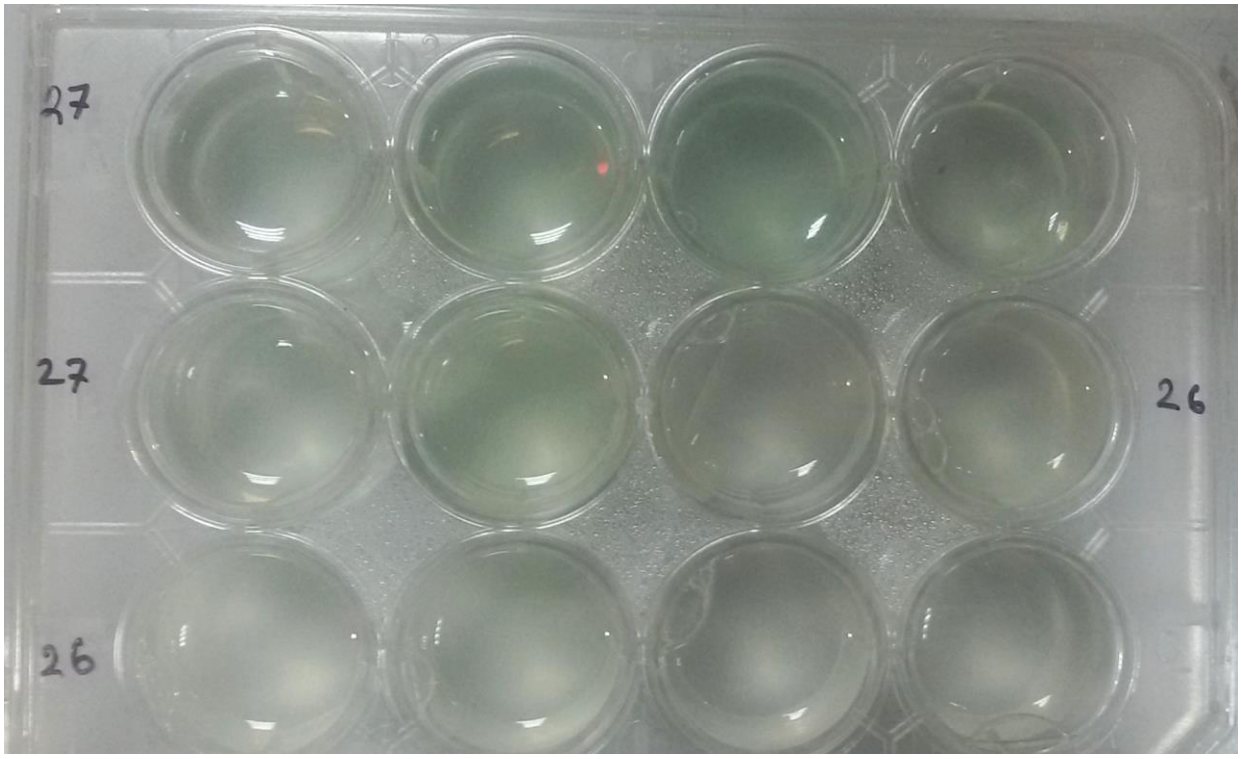


Figure 2.3: Biofilm formation on glass cover slips in 12 well plate.

2.4.5 Quantification of the Live Cells after Sanitizer Treatment

After treatment with different sanitizing agents each washed cover slips were taken in 50ml sterile falcon tube and were soaked in 10 ml 0.85% normal saline. For counting the live cells of untreated biofilm, the untreated coverslips were taken in falcon tube. These tubes were then placed in ultrasonic cleaner (Citizen Scale ultrasonic cleaner YJ5120-1) and sonication was carried out for 1 min at 25°C to remove the attached cells on the glass slides. Serial dilution was done to count

the viable cells before and after the sanitizer treatments. Drop plate techniques were used for enumeration of bacteria.

2.4.6 Microscopic Analysis of Biofilm

2.4.6.1 Staining of biofilm and Microscopy

Total cells and dead cells before and after treatment were quantified using a Neubauer chamber coupled with an Olympus BX51 fluorescent microscope equipped with a CCD color camera DP71 (OLYMPUS). Cell suspensions were stained with the commercially available LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen) following the manufacturer's instructions (Probes 2004). Two dyes were used: SYTO 9 and Propidium Iodide. SYTO 9 penetrates all cells, PI penetrates only the damaged membranes.

The biofilm formation on glass slides before and after the treatment was then observed under microscope. Viable cells would appear green, while non-viable cells would appear red.

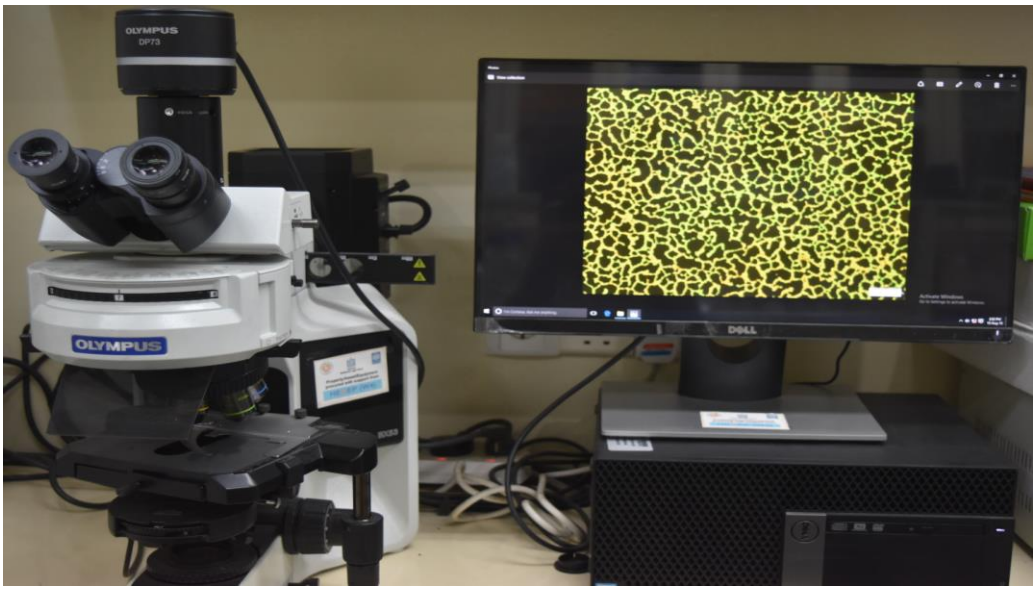


Figure 2.4: Observation of biofilm cells on glass cover slips by Epifluorescent Microscope

2.4.6.2 Microscopic Image Analysis by Image J Software

The captured Images were obtained in ×100 magnification (oil immersion). Image J software was used to generate composite (red/green) images of the stained biofilms. Brightness values were generated for each image using 'Image J color histogram analysis' software which converts RGB

pixels to brightness values (V = (R+G+B)/3). These red/green brightness values were taken for each image (e.g. untreated and treated) (Murray et al., 2017). Those red and green values for each image were then expressed in percentage value and presented in bar charts.

2.5 Molecular Analysis by PCR

2.5.1 Extraction and Purification of Chromosomal DNA

The chromosomal DNA extraction from bacterial suspension of all biofilm former isolates was performed by the standard boiling lysis method. In brief, isolated colony from the TSA plate was grown overnight in TSB at 37ºC with aeration using orbital shaker set at 120 rpm. 1.0 mL culture was taken in a 1.5 mL tube and cells were harvested by centrifugation for 5 minutes at 10,000 rpm using a centrifuge (Eppendorf, Germany). The cell pellets were washed with distilled water and 200 µl PCR water was added and pellet was suspended by pipetting. Then the cells were boiled at 100°C for 10 minutes and immediately after boiling cells were kept in ice for 10 minutes. The tubes were then centrifuged at 10,000 rpm for 10 minutes and the supernatant was collected into a fresh tube (100-150 µl).

2.5.2 Measurement of DNA Concentration

Concentration of extracted DNA was measured as ng/µl using Nanodrops at 260 nm (Thermo Scientific, USA). The ratio between the readings at 260 nm and 280 nm (A260\280) provides an estimate of the purity of the DNA. Pure DNA preparations have A260\280 value of 1.8.

2.5.3 PCR of Specific Gene

Extracted Chromosomal DNA of the selected isolates was used for the amplification of specific gene by Polymerase Chain Reaction (PCR) according to the following steps:

2.5.3.1 Primer designing

Full length sequences of target genes were obtained and then the full lenth sequences were compared to the Genbank database of the National Centre for Biotechnology Information (NCBI) (http://ncbi.nlm.nih.gov/GenBank) by means of basic local alignment search tool (BLAST) to get highly similar sequences. The sequences downloaded were analyzed in Molecular Evolutionary Genetics Analysis (MEGA, version 7) software (Tamura et al., 2007). 4 pairs of primers were

designed (**Table 2.3**) manually to get a conserved domain amplicons. Various parameters of the primers were checked by Oligo Analyzer Tool of Integrated DNA Technologies, USA (https://www.idtdna.com/calc/analyzer) **(Table 2.2)** Using the tool, Tm, GC content, length, hair pin formation, self-dimer formation, hetero-dimer formation of primers were verified.

Table 2.2: Parameters of primer designing (Primer-3 Plus)

General Settings

1. **Task:** Purpose of primers like detection, sequencing.
2. **Tm of the primer:** maximum, optimum and minimum Tm of primer according to users' need.
3. **Length of primer:** maximum, optimum and minimum length of primer.
4. **GC content of primer:** maximum, optimum and minimum GC content of primer.

Advanced Setting

1. **Product size:** maximum, optimum and minimum length of the amplicon.
2. **Threshold limit** for hairpin structure, self-dimer and heterodimer.
3. **Name of primer pair.**
4. **The number of outcome** (the number of primer offered)
5. **Need of internal primer or not.**

Table 2.3: Primers used in this study for the detection of biofilm associated genes in *Pseudomonas aeruginosa*

Primer	sequence(5'->3'	Tm	Length (bp)	Amplicon size	Reference
*PilT*_F_	TCCACGAGTCGAAGAAGTGC	60	20	145	This study
*PilT*_R	AGGCGAATGGTTTCCAGGTC	60	20		
*lecB*_F	CAAGGAGTGTTCACCCTTCC	57	20	306	This study
*lecB*_R	GTCGTTGTAGTCGTTGTCGG	57	20		
*PelB*_F	ACGCCTGCTCTGGTTCTAC	58	19	186	This study
*PelB*_R	TTGGGATTGGACTTGAGGTA	58	20		
*RhlB*_F	CGCTGCTTGTCGTAATCCAC	59	20	96	This study
*RhlB*_R	GGCCATCCAGATCCACAAGG	60	20		

2.5.3.2 Preparation of Reaction Mix

The reaction mixture for PCR was prepared by mixing the specific volume of the primers (**Table 2.3**) along with other components in an appropriately sized tube in the order provided in the **Table 2.4**. For the individual test, separate primer sets were used (**Table 2.3**). Primer designing was performed by using Primer-3 Plus software (S & H, 2000). For a number of reactions, a master mix without any template DNA was prepared and aliquoted into PCR tubes. At the end, specific template was added into a properly labeled PCR tube. In all PCR, a negative control that containing all other components of the reaction except DNA template was included as a negative control and in relevant cases, a positive control that contained known DNA template carrying known gene was also included. The PCR tubes were then placed in a thermal cycler (Veriti 96-Well Thermal Cycler, Applied Biosciences,USA) and the amplification parameters were set correctly.

Table 2.4: Components of PCR reaction mix

Ingredients	Volume added
PCR Master Mix (Go-Taq)	5.5.µl
Nuclease Free Water	2.5 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Template	1.0 µl

2.5.3.3 PCR conditions

For PCR of specific gene, the PCR reaction was set in the thermal cycler with the following program given in **Table 2.5**. After the completion of PCR, PCR tubes were stored at - 20˚C until further analysis.

Table 2.5. PCR condition for gene amplification

Segment	Condition	Cycle number
Initial Denaturation	95°C for 5 minutes	1
Extention	95°C for 1minute	35
Annealing	30seconds(temperature mentioned for each primer set in Table 2.3)	
Final extention	72°C for 7minutes	1

2.5.3.4 Analysis of the Amplicons by Agarose Gel Electrophoresis

The amplification of the desired genes was visualized by resolving the PCR products in 1% agarose gel. 1g of agarose (Sigma, USA) was added in 1X Tris-acetate EDTA (TAE) (**Appendix**) buffer to prepare a desired final concentration (1%) of agarose in a final volume of 100 mL and was heated in a microwave oven for about 2.5-3 minutes to dissolve the agarose completely. The boiled mixture was allowed to cool to about 45°C and ethidium bromide (Et-Br) was added to a final concentration of 0.5 µg/ml. The gel was poured onto gel casing preset with well former (comb) and allowed to set on a flat surface. After solidification of the gel, the comb was removed and buffer (1x TAE) was poured into tank to submerge the solidified gel. 5µl of the samples were loaded into the wells followed by electrophoresis operated at 100 volts, 200mA for 50 minutes. The gel was viewed using Alpha Imager HP Gel documentation system (Cell Bioscience, USA) and Photographs were taken using a computer attached to the machine and bands were analyzed.

2.6 Extraction of mRNA for expression analysis

In order to study the differential expression of biofilm associated gene the total mRNA was extracted by following the sequential steps,

2.6.1 Collection of cell pellets

For the purpose of growth analysis 1 ml of culture of selected isolate from log and stationary phases for all the four conditions mentioned above were collected in 1.5 ml tube and were

centrifuged to separate the bacterial cell pellets and supernatant. The cell pellets were further treated to extract the mRNAs from the cells.

2.6.2 Total bacterial RNA extraction

To extract the mRNAs from the cells, total RNA pools were collected at log and stationary phases of growth. PureLink™ RNA Mini Kit (Thermo Fisher Scientific, USA) was used for this purpose. According to kit manual, 1 ml of bacterial cells ($\leq 1 \times 10^9$) were harvested and transferred to a micro-centrifuge tube for centrifugation at 500 × *g* for 5 minutes at 4°C to pellet cells. The supernatant was discarded and 100 µL of prepared lysozyme solution was added to the cell pellet to re-suspend. Then 0.5 µL 10% SDS solution was added and vortexed to mix well. Cells were incubated for 5 minutes at room temperature. After that, 350 µl of Lysis Buffer prepared with 2-mercaptoethanol was added to the tube and vortexed to mix well. The cell lysate was homogenized using by transferring the lysate to a homogenizer inserted in an RNase-free tube, and was centrifuged at 12,000 × *g* for 2 minutes at room temperature. To bind the RNA, at first 250 µL 100% ethanol was added to each volume of bacterial cell homogenate and mixed thoroughly by vortexing to disperse any visible precipitate that may form after adding ethanol. Then the sample was transferred to a spin cartridge (with a collection tube) and centrifuged at 12,000 × *g* for 15 seconds at room temperature. The flow-through was discarded and the spin cartridge was reinserted in the same collection tube. Then, 700 µL Wash Buffer I was added to the spin cartridge and centrifuged at 12,000 × *g* for 15 seconds at room temperature. The flow-through and the collection tube were discarded. Spin cartridge was then placed into a new collection tube and 500 µL Wash Buffer II with ethanol was added. Again centrifugation was done at 12,000 × *g* for 15 seconds at room temperature. The flow-through was discarded and the spin cartridge was reinserted into the same collection tube to be centrifuged at 12,000 × *g* for 1 minute at room temperature to dry the membrane with attached RNA. The collection tube was discarded and the spin cartridge was inserted into a recovery tube. To elute the attached RNA 200 µL RNase-Free water was added to the center of the spin cartridge and incubated at room temperature for 1 minute. Then the spin cartridge and recovery tube was centrifuged for 2 minutes at ≥12,000 × *g* at room temperature. The purified RNA was then used for cDNA synthesis.

2.6.3 Measuring RNA Concentration by NanoDrop

RNA concentration was also measured using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). 2µl of buffer in which RNA was eluted was used as blank. Another 2µl of sample was loaded and RNA concentration was shown at ng/µl unit. The OD 260/280 and OD 260/230 ratios were also shown with the software indicating the purity of the sample. Concentration of single stranded RNA was calculated according to the following formula:

Concentration of ss RNA (ng/ml) = OD reading x dilution factor x 50

An OD value 1 corresponds to approximately 50 ng/ml of purified ss RNA (Sambrook *et al*., 1989). The ratio of absorbance at 260 nm and 280 nm (OD 260/0D280) provided an estimate of the purity of the RNA. Pure RNA preparations have OD 260/280 values of 1.7-2.1 (Sambrook *et al*., 1989).

2.7 Preparation of Complementary DNA (cDNA) from Extracted Total RNA Pools

The extracted total RNA pools were reverse transcribed into complementary DNA (cDNA) by using GoScript™ Reverse Transcription System (Promega, USA). According to manual, both hexameric random primers and oligo $(dT)_{15}$ primers were used to reverse transcribe the RNA. To prepare cDNA, experimental RNA was combined with the random and oligo $(dT)_{15}$ primer. The primer/template mix was thermally denatured at 70°C for 5 minutes and chilled on ice. This step was performed on heat block (Veriti 96 well Thermal cycler, Applied Biosystem, USA). A reverse transcription reaction mix was prepared on ice to contain nuclease-free water, 5X reaction buffer, ImProm-II™ reverse transcriptase, magnesium chloride, dNTPs and ribonuclease inhibitor. In experimental systems, the addition of 1unit/µl of Recombinant RNasin® Ribonuclease Inhibitor was recommended but optional. In final step, the template-primer combination was added to the reaction mix on ice and run on thermal cycler for conversion of RNA to cDNA. No cleanup or dilution was done following the cDNA synthesis, so the product was directly added to amplification reactions. The detailed method of cDNA preparation is deciphered below:

2.7.1 Target RNA and Primer Combination and Denaturation

First, sterile, thin-walled dilution tubes (ExtraGene, USA) and reaction tubes (Eppendorf, USA) were kept on ice. The extracted RNA was thawed on ice. On ice, the experimental RNA (up to 1µg) and the cDNA primer were combined in Nuclease-Free Water for a final volume of 10µl per

RT reaction. The volumes was multiplied to accommodate multiple reactions when more than one reaction was planned using a single RNA: primer combination. The target RNA/Primer combination is outlined in **Table 2.6.** Each tube of RNA was closed tightly and placed into a preheated 70°C heat block for 5 minutes. After 5 minutes heating, heat block was programmed to maintain 4o C temperature so that chilling environment can be created to incubate primer/RNA mixture. Then the tubes were spun for 10 seconds in a minicentrifuge (ExtraGene, USA) to collect the condensate and maintained the original volume. The tubes were kept closed and on ice until the reverse transcription reaction mix were added.

Table 2.6. RNA/Primer Mixture for cDNA Preparation

Ingredient	**Volume added**
Experimental RNA	5 µl
Random (Hexameric Primer)	2 µl
Oligo(dT)15 Primer (0.5µg/reaction)	2 µl
Nuclease-Free Water	1 µl
Final Volume	**10 µl**

2.7.2 Primer Designing

Primer designing was previously mentioned in the section ***2.6.3.1.***

2.7.3 Reverse Transcription

The reverse transcription reaction mix was prepared by combining the following components of the ImProm-II™ Reverse Transcription System in a sterile 1.5ml microcentrifuge tube (Eppendorf, USA) on ice. 30µl of reaction mix was prepared for each cDNA synthesis reaction to be performed. The reaction mix was vortexed gently to mix, and kept on ice prior to dispensing into the reaction tubes. The volumes needed for each component is described in **Table 2.7.** Reverse transcription reaction mix (30 µl aliquots) was then added to each reaction tube on ice. Careful handling in that step was necessary to prevent cross contamination. 10 µl of RNA and primer mix was added to each reaction for a final reaction volume of 40 µl per tube. The tubes were subsequently kept in a controlled-temperature heat block equilibrated at 25°C, and incubated for 5 minutes.The tubes

were then incubated in a controlled-temperature heat block at 42°C for up to one hour for extension of cDNA product. The extension temperature may be optimized between 37°C and 55°C as per manufacture's instruction. The reverse transcriptase was thermally inactivated prior to amplification. The reaction tubes in a controlled-temperature heat block were incubated at 70°C for 15 minutes following extension step (**Table 2.8)**

2.7.4 Measuring cDNA Concentration by NanoDrop

cDNA concentration was measured following the same procedure as RNA (described in section 2.7.3)

Table 2.7. Reaction Mixture for cDNA Preparation

Ingredient	**Volume added**
RNase Free H2O	9.6 µl
5X Reaction Buffer	8.0 µl
MgCl2 (8mM)	6.4 µl
dNTP Mix (final concentration 1.0 mM each dNTP)	2.0 µl
Recombinant RNasin® Ribonuclease Inhibitor	2.0 µl
ImPro Reverse Transcriptase	2.0 µl
Final Volume	**30 µl**

Table 2.8. Optimum reaction condition for Reverse Transcription reaction

Step	**Temperature (°C)**	**Time (minute)**
Annealing	25	5
Extention	42	60
Inactivation of Reverse Transcriptase	70	15

2.8 Biofilm Gene Expression Study Using Real Time Relative Quantitative PCR via $\Delta\Delta C_T$ Method

The Applied Biosystems 7300/7500 Real Time PCR System (7300/7500 system) uses fluorescent-based PCR chemistries to provide quantitative detection of nucleic acid sequences using real-time analysis and qualitative detection of nucleic acid sequences using end-point and dissociation-curve analysis. In this study the relative quantification (RQ) assay type was performed to analyze the differential expression of target biofilm genes in different isolets. Relative quantification determines the change in expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample.

2.8.1 RQ Experiment Designing

2.8.1.1 PCR Method Selection

Singleplex reaction was selected, where a single primer pair is present in the reaction tube or well. Only one target sequence or endogenous control was amplified per reaction.

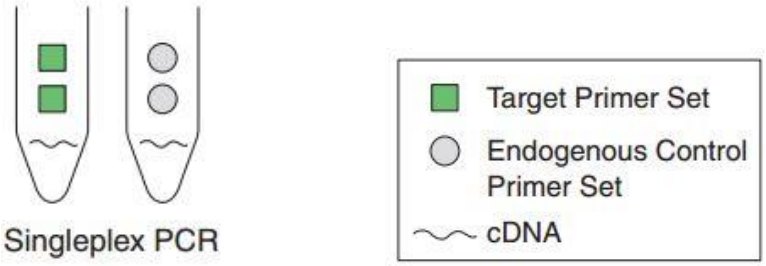


Figure 2.5 Singleplex PCR method

2.8.1.2 Selecting the PCR Chemistry

SYBR Green I dye, a double-stranded DNA binding dye, was used to detect PCR products as they accumulate during PCR cycles.

2.8.1.3 Target Genes Selection

*pel*B and *lec*B these two biofilm genes were selected as target to conduct the experiment.

2.8.1.4 Primer Designing for *pel*B and *lec*B Genes for Relative Quantification (ddCt) Study

Full length sequences of *pelB* and *lecB* were obtained and then the full lenth sequences were compared to the GenGank database of the National Centre for Biotechnology Information (NCBI) (http://ncbi.nlm.nih.gov/GenBank) by means of basic local alignment search tool (BLAST) to get highly similar sequences. The sequences downloaded were analyzed in Molecular Evolutionary Genetics Analysis (MEGA, version 7) software (Dudley et al., 2007). 2 Pairs of primers were designed (**Table 2.9**) manually to get a conserved domain amplicon of around 186bp and 306bp repectively for *lecB and pel* specific RT-qPCR. Various parameters of the primers were checked by Oligo Analyzer Tool of Integrated DNA Technologies, USA (https://www.idtdna.com/calc/analyzer). Using the tool, Tm, GC content, length, hair pin formation, self-dimer formation, hetero-dimer formation of the primers were verified.

Table 2.9: Primers used in this study for *pel*B and *lec*B genes for Relative Quantification (ddCt) Study

Primer	Sequence (5'->3')	Tm	Length (bp)	Amplicon size	Reference
*lecB*_F	CAAGGAGTGTTCACCCTTCC	57	20	306	This study
*lecB*_R	GTCGTTGTAGTCGTTGTCGG	57	20		
*PelB*_F	ACGCCTGCTCTGGTTCTAC	58	19	186	This study
*PelB*_R	TTGGGATTGGACTTGAGGTA	58	20		

2.8.1.5 Endogenous Control Gene Selection

Gyrase gene was selected as an endogenous control gene depending on its consistent level of expression in all experimental samples. By using an endogenous control as an active reference, quantification of a cDNA target for differences in the amount of cDNA added to each reaction was normalized.

2.8.1.6 Preparation of RQ Plate Reaction Mixture

For the detection of differential expression of *pelB* and *lecB* genes in biofilm growth of selected isolets, Relative quantification (ddct) was done using GoTaq® qPCR Master Mix. The Master Mix formulation includes a proprietary dsDNA-binding dye, GoTaq® Hot Start Polymerase, $MgCl_2$, dNTPs and a proprietary reaction buffer. So, the stabilized 2X formulation includes all components for qPCR except sample DNA, primers and water. The samples used in this case were the cDNAs mentioned above, hence the name RT-qPCR. The reaction setup and the cycling parameters are mentioned in **Table 2.10** and **2.11** respectively. This step was performed on Applied Biosystems™ 7500 Real-Time PCR System.

Table 2.10. Reaction set up for qPCR carried out in 25µl reaction volume

Ingredients	Volume added
GoTaq® qPCR Master Mix, 2X	12.5µl
PCR grade water	9.5µl
Forward primer	0.25µl
Reverse primer	0.25µl
Template	2.5µl

Table 2.11.Cycling parameters for qPCR

PCR stages	Number of cycles	Temperature (℃)	Time
Hot-Start Activation	1	95	2 minutes
Denaturation	40	95	15 seconds
Annealing/Extension		55	60 seconds
Dissociation	1	60-95	

The thermal cycler was programmed using the SYBR® as the detection dye for the entire plate. A standard, two-step, 40-cycle qPCR and dissociation program was selected and the data were designated to be collected during the annealing step of each cycle. When the run was complete, the data were analyzed from the amplification plots, normalized by housekeeping gene and RQ data was generated by the software.

2.9 Genome Sequencing

2.9.1 Whole Genome Sequencing and Assembly

2.9.1.1 DNA Extraction and Purification

DNA from the pure culture of the isolate DMC-27b, DMC-20C and DMC-30b were extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and quantity of the extracted genomic DNA were assured by Nanodrop ND-200 (Thermo Fisher, Waltham, MA, USA) and the integrity was assured by agarose gel electrophoresis.

2.9.1.2 Whole Genome Sequencing

Whole genome sequencing was performed by Ion-Torrent High Throughput Sequencing technology. Machine generated data was transferred to the Ion Torrent server where data was processed through signal processing, base calling algorithms and adapter trimming to produce mate pair reads in FASTQ format.

2.9.1.3 Retrieval of Sequence Data and Genome Assembly

The FASTQ reads quality was assessed by the FastQC tool (Babraham Bioinformatics - FastQC A Quality Control Tool for High Throughput Sequence Data, n.d.) followed by trimming of low quality reads and reads less than 200 bp using the Trimmomatic tool (Bolger et al., 2014), where quality cut off value was Phred-20. De novo assembly of the reads was performed using SPAdes, (version 3.5.0) genome assembler (Bankevich et al., 2012). Generated assembled reads were mapped and reordered according to a reference sequence of *Pseudomonas aeruginosa* PAO1 genome from NCBI (accession number: NC_002516.2) by progressive Mauve algorithm in Mauve software (Darling et al., 2004).

2.9.2 Partial Genome Sequencing of the Selected Genes

2.9.2.1 PCR Amplification of the Selected Genes

PelB and LecB protein coding region of 10 *Pseudomonas aeruginosa* isolates were amplified by primer pairs following the procedure described in **sub-section 2.6.3**. PCR negative control was o included to validate the reaction. PCR product was visualized by Agarose gel electrophoresis according to protocol mentioned in section **2.6.3.4**.

2.9.2.2 Purification of Amplicons

After Agarose gel electrophoresis, PCR positives samples were purified using The Wizard® SV Gel and PCR Clean-Up System (Promega, USA; Appendix I) according to the manufacturer's instructions. The Wizard® SV Gel and PCR Clean-Up System is based on ability of DNA to bind to silica membranes in the presence of chaotropic salts (guanidine isothiocyanate). After amplification, an aliquot of the PCR product is added to the equal volume of guanidine isothiocyanate containing Membrane Binding Solution (MBS) and directly purified.

In case of gel purification, an equal amount of gel cut containing the intended band was mixed with equal volume of MBS in a micro centrifuge tube and incubated at 65^{o}C in a heat block until completely dissolved. In both the cases, the mixture was transferred to the mini column pre-set with a collection tube (SV mini column assembly). After short incubation (2 minutes) at room temperature, SV mini column was centrifuged at 16,000×g for 1 minute. Then the flow-through was discarded and SV mini column was washed for two times with Membrane Wash Solution (Supplied in the kit, ethanol added). After washing the SV mini column, DNA was eluted in Nuclease Free Water (Supplied in the kit).

2.9.2.3 Measurement of the Concentration of Amplicons

The concentration of amplicons were measured using a NanoDropTM spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). PCR product was measured as ng/ µl. The purified PCR product was stored at -20^{o}C until further processing.

2.9.2.4 Sequencing of PCR products

After purification of the PCR products, purified products and respective primers were sent to Genome Centre of Jashore University of Science and Technology for single-pass DNA sequencing by Sanger method. The sequencing reaction was done both for forward and reverse primers (**Table 2.9**). The sequences (tracer files) were viewed using sequence viewer software like chromas. Both forward and reverse sequences were assembled into a single contig using SeqMan version 7.0.0 (Lasergene, DNASTAR, USA).

2.10 Analysis of the Sequenced Genomes

2.10.1 Identification of Bacterial Species

Assembled contigs of whole genome sequences were analyzed by BLAST and the k-mer algorithm in the KmerFinder 2.0 tool to identify the bacterium at species level (Hasman et al., 2014). Whole genome based phylogenetic analysis was performed using REALPHY (Bertels et al., 2014).

2.10.2 Comparison of the Sequenced Genome with Reference Strain

To comparte the sequenced genomes of DMC-27b, DMC-20c and DMC-30b with the reference genome of *Pseudomonas aeruginosa* PA01, Blast Ring Image Generator (BRIG) software (Alikhan et al., 2011) was used. The circular image of the sequenced genomes were constructed and these three genomes were marked with different colors. Some important biofilm related genes and operons were also marked using the software. With the color gradient the similarity of the genomic regions were indicated.

2.10.3 Genome Annotation

The assembled draft genome of the isolate DMC-27b, DMC-20C and DMC-30b were annotated by multiple annotation schemes to improve accuracy. Used software includes NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016), PROKKA (e= 0.000001) and RAST (e= 0.000001) (Aziz et al., 2008). Annotated genes by each software were then cross checked for each tool.

2.10.4 Comparative Analysis of Annotated Genes and Proteins

The SEED viewer (Aziz et al., 2012) was used for the exploration and comparative analysis of annotated genes. From *Pseudomonas* genome database (www.pseudomonas.com) (Winsor et al., 2005), the nucleic acid and protein sequences of the biofilm related genes/proteins of *Pseudomonas aeruginosa* PAO1(reference genome) were retrieved. The nucleic acid and protein sequences were compared with the annotated genomes in the SEED viewer to determine the homology and variation in the genes/proteins among the studied isolates. This server was also used to perform Synteny analysis of the desired genes/proteins.

2.10.5 Other Analyses of Whole Genomes

Pathogenic profile of the sequenced genomes were determined by PathogenFinder (Cosentino et al., 2013). Secondary metabolite gene clusters were identified by anti-SMASH version 4.0.2 software (Medema et al., 2011). KEGG MG Mapper tool (Kanehisa & Goto, 2000) was used for metabolic pathway reconstruction.

2.11 Analysis of the Proteins and Protein Structures

2.11.1 Secondary Structure Prediction

Secondary structure of LecB and Pel operon proteins of sequenced isolates were predicted by PredictProtein (Rost et al., 2004) web tool.

2.11.2 Interactive 3D Modelling and Visualization of Proteins

The 3D structure of the LecB and Pel operon proteins of the sequenced genomes were constructed using Phyre 2 (Kelley et al., 2015) and the variation in the protein structures among those isolates were visualized with PyMOL(TM) 2.4.0 software (Schrodinger, 2017).

2.11.3 Protein-Ligand Docking

Protein- Ligand docking was performed by HADDOCK (High Ambiguity Driven protein-protein DOCKing) webtool (van Zundert et al., 2016). Protein-ligand binding affinities were measured by PRODIGY-LIGAND web server (Vangone et al., 2019).

2.11.4 Homo Oligomer Structure Prediction

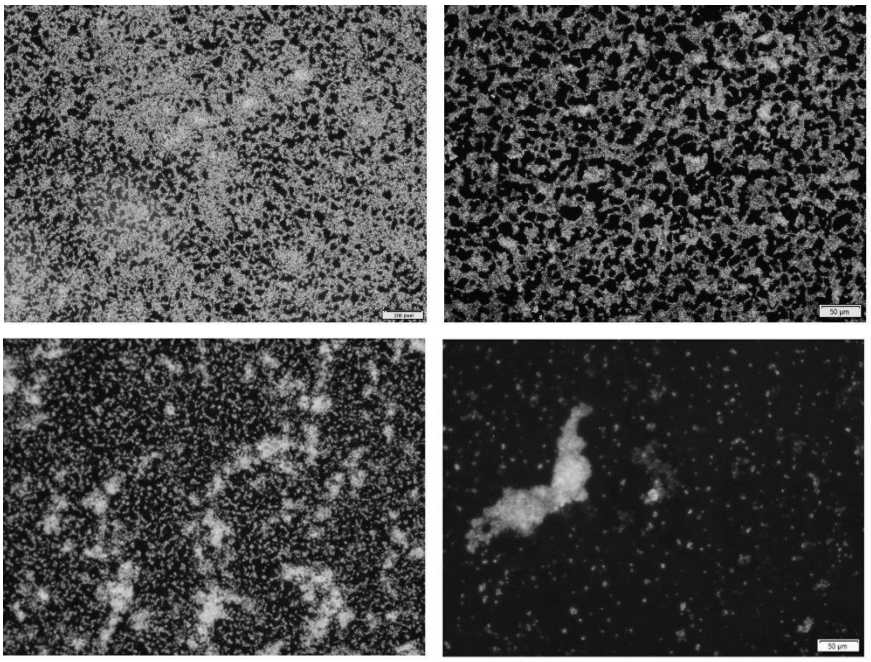
For PelC dodecamer structure prediction GalaxyHomomer webserver were used. This server predicts homo oligomer structure of protein from a monomer sequence or structure (Baek et al., 2017).

2.11.5 Protein Tree Construction

The aligned protein sequences were used for the construction of protein tree using the maximum-likelihood method in Molecular Evolutionary Genetics Analysis (MEGA X) software (Kumar et al., 2018). Interactive Tree Of Life (iTOL) v5 (Letunic & Bork, 2019) was used to adjust the branch and label color of the phylogenic tree.

Chapter 3

RESULTS



3.0 Results

Biofilm former Bacteria have an enormous impact in clinical microbiology, because this is associated with owning of large number of pathogenesis and antimicrobial resistant properties of the bacteria. Furthermore, biofilm former bacteria play important role in animal and public one health. Therefore, categorization of clinically associated biofilm former bacteria, their response to disinfectants and understanding of genetic determinants that contribute biofilm formation of 45 clinical isolates have been focused in the current thesis/dissertation. The findings of the study can be highlighted as-

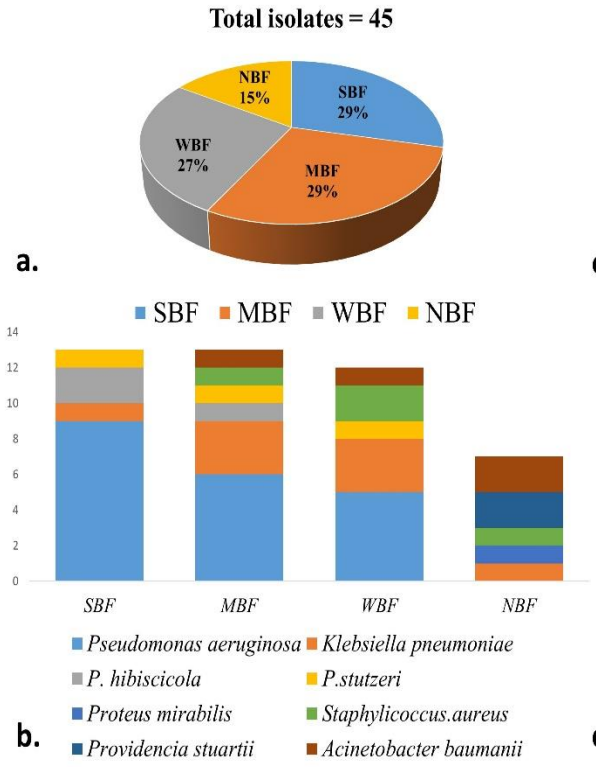
- Categorization of the clinical isolates on the basis of their biofilm forming ability;
- Susceptibility of the biofilm cells to different commonly used disinfectants;
- Molecular detection of the biofilm associated genes and studying the patterns of biofilm genes expression; and
- Functional implications of biofilm associated genes and proteins among different groups of biofilm formers.

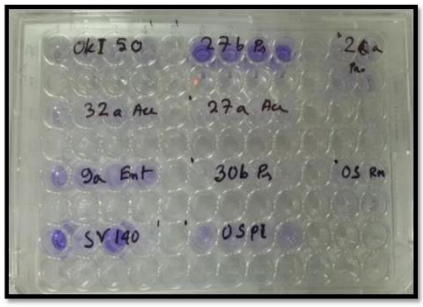
3.1 Evaluation of Biofilm Forming Ability by Crystal Violet Microtiter Plate Assay

3.1.1 Screening the Biofilm Formation Abilities of Different Clinical Isolates

A total of 45 previously isolated and characterized clinical isolates (see section 2.1, **Table 2.1** and Rakhi et al. 2019) were revived and sub-cultured on Tryptic Soya Agar (TSA). Later, these isolates were screened for biofilm formation ability by Crystal violet (CV) microtiter assay. Among the 45 isolates analyzed, 38 (85%) isolates formed biofilm, while 7 (15%) of the isolates were found to be non-biofilm former (NBF). Among the biofilm formers 13 (29%) isolates produced strong biofilms and characterized as strong biofilm formers (SBF), while 13 (29%) others were moderate biofilm formers (MBF) and 12 (27%) were weak biofilm formers (**Figure 3.1**).

Among the 45 clinical isolates, 26 were characterized as *Pseudomonas* spp, and all of the *Pseudomonas* spp isolates produced biofilms. Among them 9 *P. aeruginosa*, two *P. hibiscicola* and one *P. stutzeri* isolates were strong biofilm formers. The rest of the isolates were characterized as *Klebsiella pneumoniae, Proteus mirabilis, Staphylococcus auerus, providencia stuartii and Acinetobacter baumanii. Among those isolates* only 1 of the *Klebsiella pneumoniae* isolates were found to be strong biofilm former.





d.

Bacteria	SBF	MBF	WBF	NBF	Total
Pseudomonas aeruginosa	9	6	5	0	20
P. hibiscicola	2	1	0	0	3
P. stutzeri	1	1	1	0	3
Klebsiella pneumoniae	1	3	3	1	8
Proteus mirabilis	0	0	0	1	1
Staphylicoccus.aureus	0	1	2	1	4
Providencia stuartii	0	0	0	2	2
Acinetobacter baumanii	0	1	1	2	4

Figure 3.1: a. Percentage of Strong (SBF), Moderate (MBF), Weak (WBF), Non (NBF) biofilm formers among the 45 clinical isolates. b. Distribution of different bacterial species with their variable biofilm forming ability. c. Crystal Violet Biofilm forming assay. d. Chart showing the total number of tested isolates and numbers of SBF, MBF, WBF, NBF isolates from each bacterial species.

3.1.2 Biofilm Growth over Time

Biofilm formation pattern of representative SBF (DMC-27b, DMC-8b)), MBF (DMC-20c, DMC-20b) and WBF (DMC-24, DMC-30b) *P. aeruginosa* isolates over time shows that after 6 to 8 hours SBF and MBF biofilm formers attach significantly on the microtiter plate surface and CV assay could distinguish them from the WBF biofilm formers at that point (**Figure 3.2**).

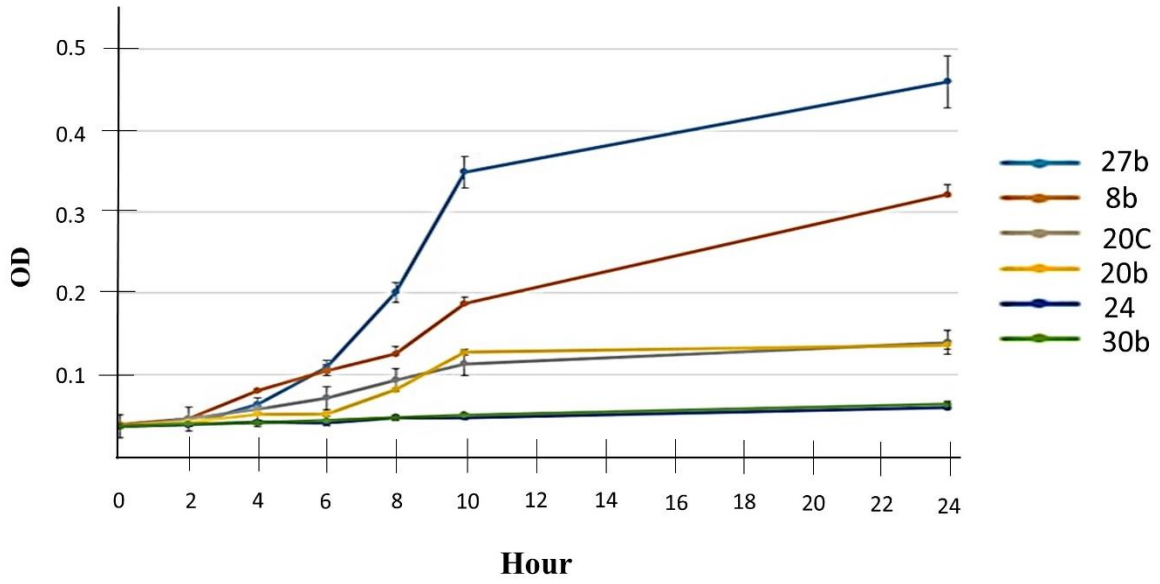


Figure 3.2: Biofilm formation pattern of Strong (27b, 8b), Moderate (20c, 20b) and Weak (24, 30b) biofilm forming isolates after 2, 4, 6, 8, 10 and 24 hours. The experiment was performed three times and the error bars indicates the standard deviation from the average values of OD for 0 hr, 2hr, 4hr, 6hr, 8hr, 10hr and 24hr time period.

3.2 Observation of Biofilms Using Fluorescent Microscopy

The biofilm surface was stained by Film tracer LIVE/DEAD biofilm viability kit (Thermofisher, USA) to visualize biofilms and microclonies attach to glass surfaces. Attached cells were distinguishable between the live or active cells (fluorescent green) and dead or inactive cells (fluorescent red) under Olympus BX53 upright fluorescence microscope (Olympus, Japan) (Figure 3.3, 3.4, 3.5).

3.2.1 Biofilm Formation and Visualization with Reference Strain

P. fluorescens Migula 1895 (ATCC 13525) strain was used to validate the method and to determine the minimal media concentration and initial inoculum size for biofilm formation after 24 hours. TSB concentration of 5% with 5×10^7 cfu/ml initial inoculum showed good attachment of the strain on glass surface (**Figure 3.3**). Later on, we used that media concentration and inoculum size for observing the biofilms of other tested isolates.

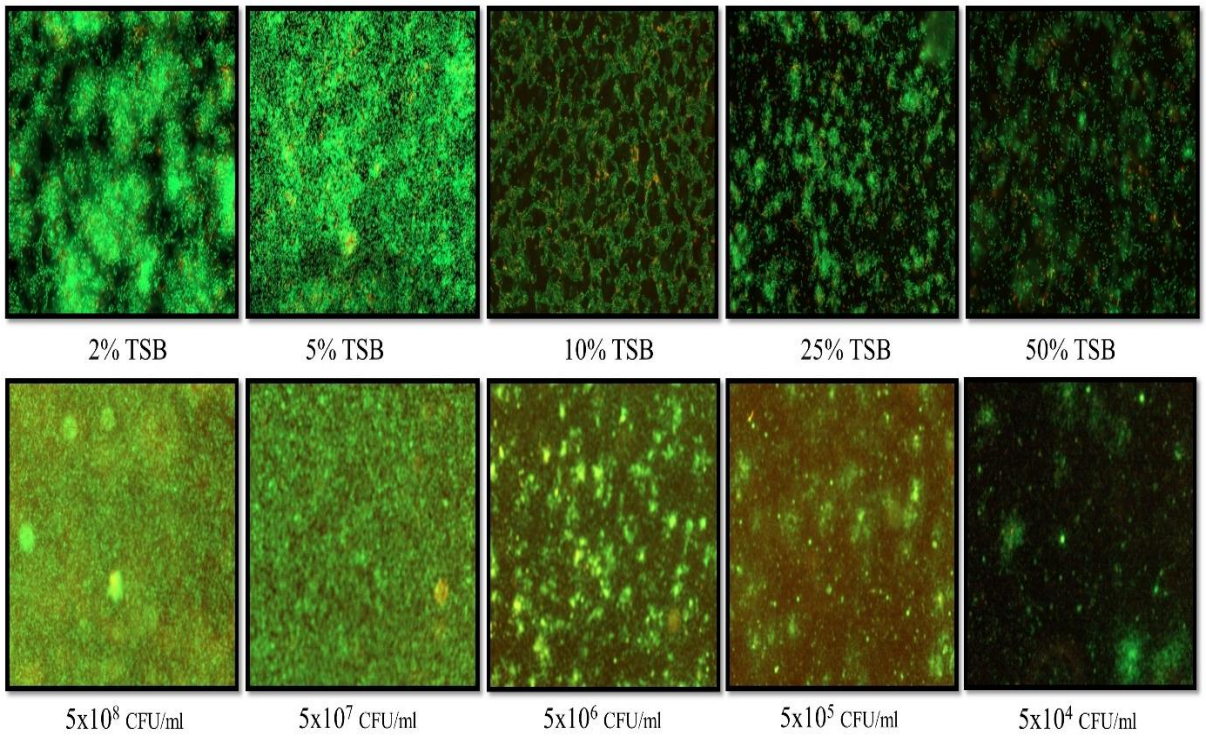


Figure 3.3: Fluorescent microscopic image of 24 hour biofilm produced by *Pseudomonas fluorescens* Migula 1895 (ATCC 13525) strain with different media concentration and inoculam size.

3.2.2. Observation of Strong Biofilm Formers under Fluorescent Microscope

Strong biofilm former isolates from different species were grown on glass surface with 5% TSB and the produced biofilm stained with Film tracer LIVE/DEAD biofilm viability kit was observed under microscope. From the microscopic images, we can see that, *P. aeruginosa* DMC-27b isolate attached more efficiently on the glass surface and produced dense biofilm all over the surface. Other isolates from *Pseudomonas* spp (*P. stutzeri* and *P. hibiscicola*) also produced dense biofilms while *Klebsiellia pneumoniae* showed microcolonies on the surface (**Figure 3.4**).

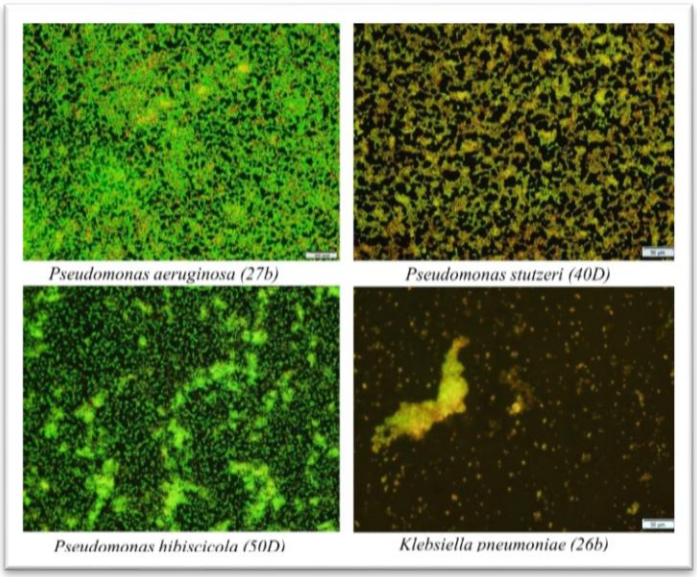


Figure 3.4: Fluorescent microscopic images of 24 hour biofilm produced by *Pseudomonas aeruginosa* (27b), *Pseudomonas stutzeri* (40D), *Pseudomonas hibiscicola* (50D) and *Klebsiellia pneumoniae* (26b) isolates.

3.3 Efficacy of Sanitizing Agents on Strong Biofilm Formers

Twenty four hours biofilms of 5 strong biofilm former isolates of *P. aeruginosa* were treated with 0.1% SDS, NaOCl (150 ppm), 70% Ethanol and Savlon solution for 10 minutes and observed under fluorescent microscope after staining with Film tracer LIVE/DEAD biofilm viability kit. The microscopic images were investigated with the image J software to observe the efficacy of different sanitizers on the biofilms (**Figure 3.5 and 3.6A**).

3.3.1. Microscopic Observation after Treatment

Microscopic observations and 3D image analysis showed reduced number of green cells and increased after treatment with sanitizing agents and disinfectants. The thickness of the biofilm was also reduced after treatment (**Figure 3.5**). Treatment with Ethanol and Savlon increased the amoun of red cells but the green fluorescence indicates that a number of cells are still viable after treatment with those disinfectants. Dense microcolnies were observed on the surface which protects the bacteria that reside on the inner part of the biofilm. Lethal disinfectants like Ethanol and Savlon could kill the bacteria on the upper layer of biofilms but showed little success in penetrating the lower parts and kill the bacteria efficiently.

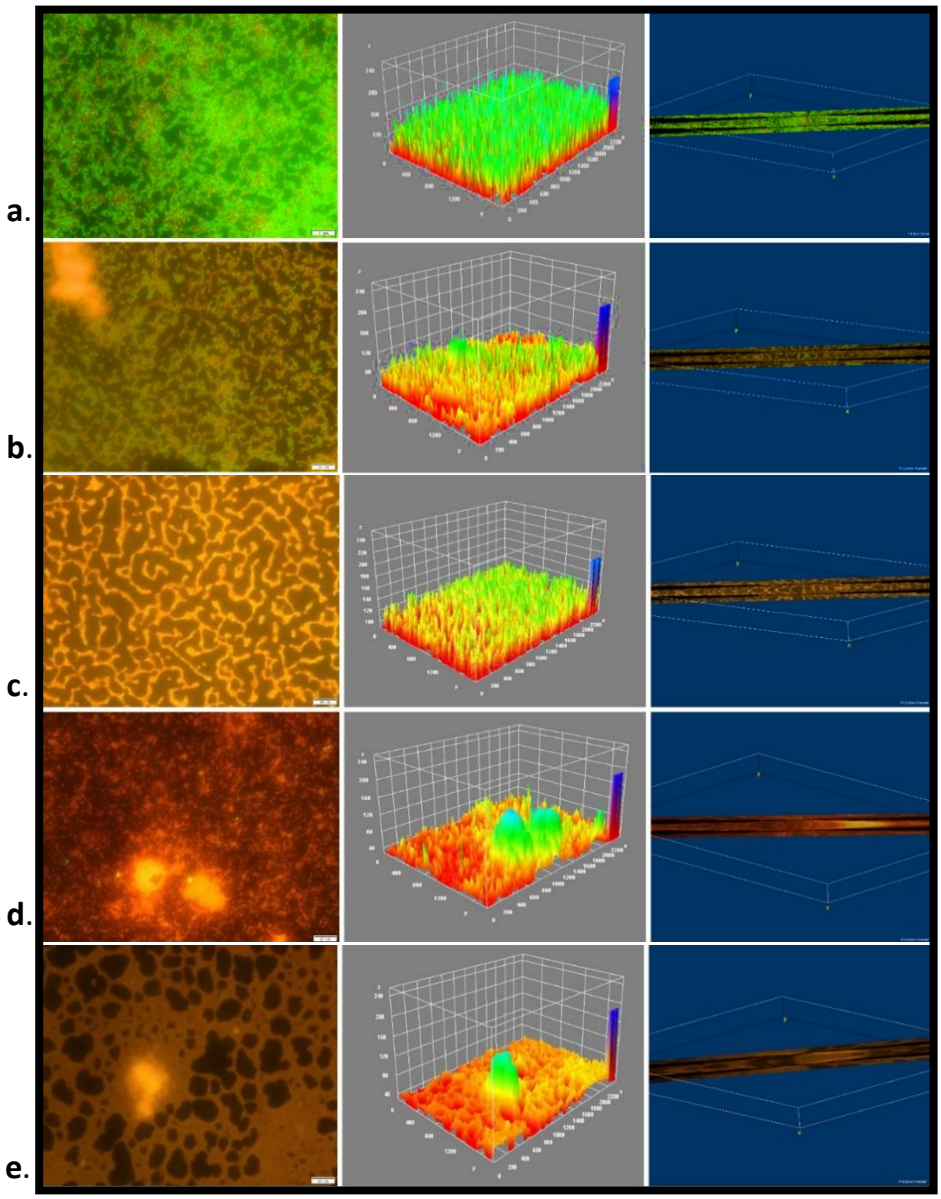


Figure 3.5: Fluoroscence microscopy images of (a) untreated; (b) Sodium Hypochlorite treated; (c) 0.1% SDS treated; (d) 70% Ethanol treated; (e) Savlon treated 24 h biofilm formed by *Pseudomonas aeruginosa* 27b under 20x magnification. Live or active cells are fluorescent green and dead or inactive cells are fluorescent red. 2D view (left side image), surface plot of 3D volume image (center image) and cross section of 3D volume image (right side image) show the distribution of live and dead cells throughout biofilm layers.

3.3.2. Microscopic Image Analysis of Treated Biofilms

Twenty-four hours biofilms of 5 strong biofilm former *P. aeruginosa* isolates (b01, b07, DMC-27b, DMC-23b and DMC-8b) along with positive control *P. fluorescens* Migula 1895 (ATCC 13525) strain were tested for disinfectant susceptibility. For the microscopic images of the biofilms treated with sanitizing agents and disinfectants, brightness values were generated using 'Image J color histogram analysis' software that converts RBG pixels to brightness values (V = (R+G+B)/3).

These red/green brightness values and their ratio shows that all of the disinfectants increased the amount of red fluorescence. Ethanol and Savlon treated biofilms have comparatively higher amounts of red values, indicating that these two disinfectants could kill more bacterial cells than 0.1% SDS and NaOCl. In case of *P. fluorescens* Migula 1895 (ATCC 13525), the green value decreased from 59.89% in untreated biofilm to 34.94% in Ethanol treated biofilm and 36.24% in Savlon treated biofilm. In case of *P. aeruginosa* isolate DMC-27b, Savlon reduced the green value from 53.1% to as low as 24.6%. These results indicate the reduction of live cells in the biofilms treated with disinfectants, and also indicate that no disinfectant could completely eradicate the viable cells.

3.3.3. Viable Cell Count of the Treated Biofilms

Viable cell count of the treated biofilm also shows that Ethanol and Savlon could reduce the number of viable cells attached in the surface more efficiently than the other two disinfectants. SDS and NaOCl were not effective on killing the bacteria on the biofilm. While Ethanol and Savlon showed 2 to 4.5 log cycle reduction of viable cells, SDS and NaOCl showed 1 or less than 1 log cycle reduction. Despite having a lethal impact, Ethanol and Savlon treated biofilms still contain 10^2-10^4 cells per millimeter of the glass surface (**Figure 3.6 B and 3.6C**).

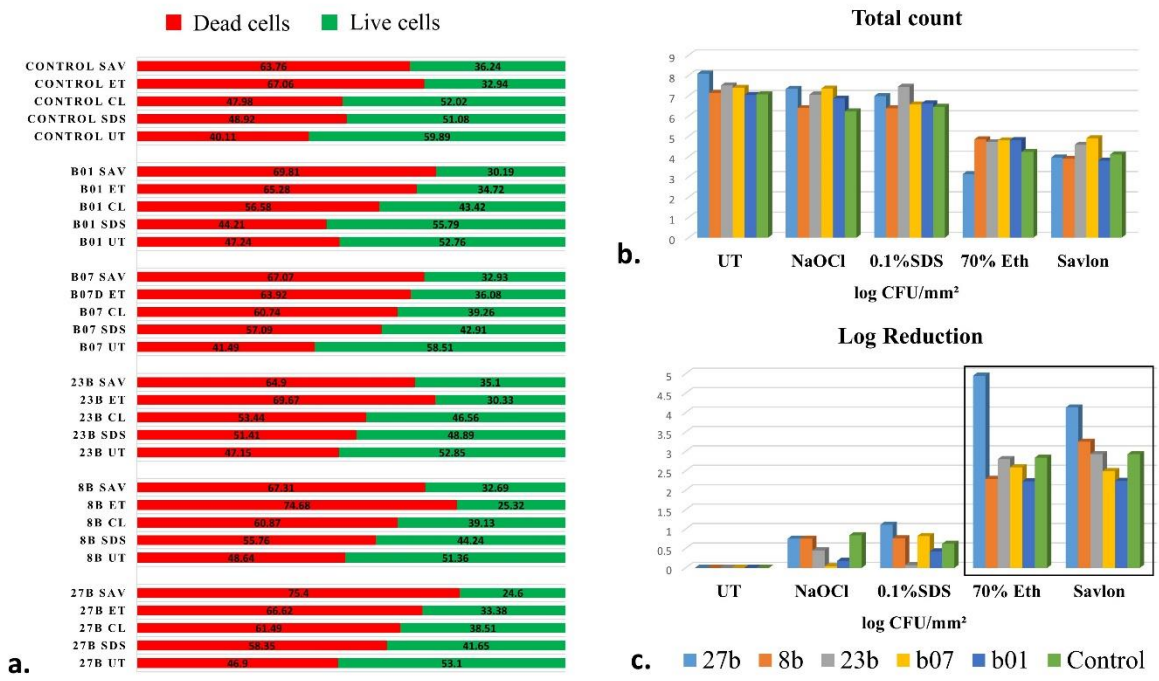


Figure 3.6: Effect of disinfectants on biofilms a. Bar chart showing the percentage of red and green value of the fluorescent images of the biofilms produced after treatment with those sanitizers and disinfectants. 24 hour biofilms of *Pseudomonas aeruginosa* isolates (marked with B01, B07, 27B, 23B and 8B) along with positive control *Pseudomonas fluorescens* Migula 1895 (ATCC 13525) strain (marked as CONTROL) were treated with 0.1% SDS (SDS), NaOCl (CL), 70% Ethanol (ET) and Savlon (SAV). Untreated biofilms were designated as UT. b. Viable cell count after treatment. c. log reduction after treatment.

3.4 Isolates of *Pseudomonas aeruginosa* Show Variability in Their Biofilm Forming Ability

Not all of the *P. aeruginosa* isolates formed robust biofilms. Microscopic analysis showed varied biofilm architecture from strain to strain. Among the 20 *P. aeruginosa* isolates, we have found 8 are very strong biofilm formers, 6 of them produce moderate biofilm and 6 have weak attachment tendency to the surface.

3.4.1 Microscopic Observation of Strong, Moderate and Weak Biofilm Former *Pseudomonas aeruginosa*

Representative 2D and 3D images of a strong (DMC-27b), moderate (DMC-20C) and weak (DMC-30b) biofilm produced by fluorescent microscope using Live and Dead Biofilm staining kit showed difference in attachment pattern to the surface (**Figure 3.7**).

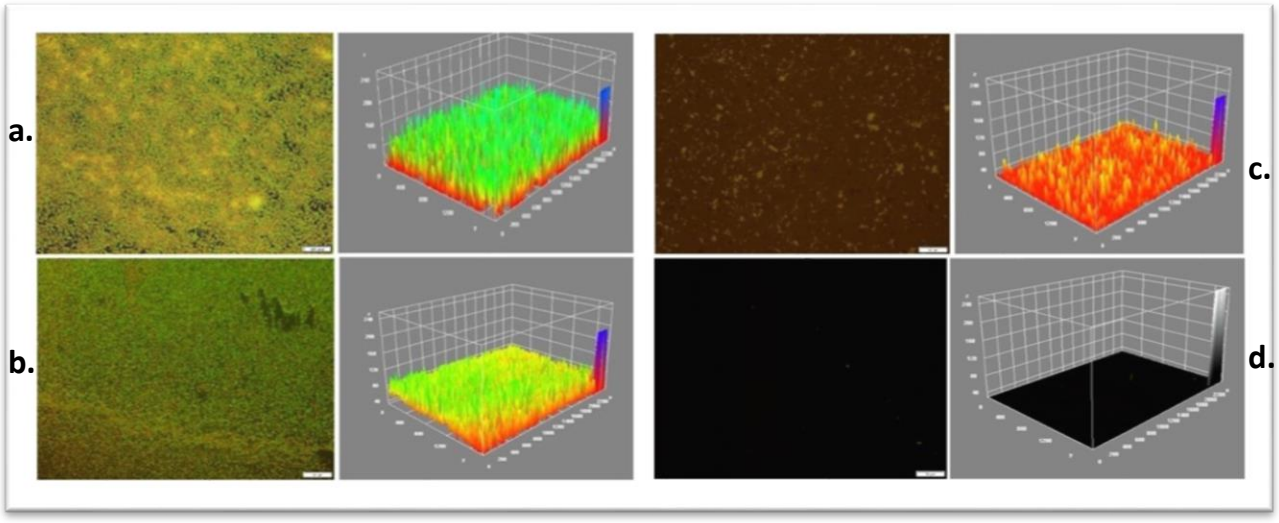


Figure 3.7: Microscopic images with 3D view of 24 hour biofilms of a. strong (DMC-27b), b. moderate (DMC-20C) and c. weak (DMC-30b) biofilm former *Pseudomonas aeruginosa*. d. Negative control with no bacterial inoculum.

3.4.2: Biofilm Assays of *Pseudomonas aeruginosa* Isolates

Several biofilm assays (CV assay, Pellicle formation assay, CR assay, CR release assay) also show difference between *P. aeruginosa* isolates.

3.4.2.1 Crystal Violet Assay and Attached Cell Count of *Pseudomonas aeruginosa* Isolates

CV biofilm formation assay clearly distinguish between strong, moderate and weak biofilm formers. Viable cell counts also showed that large number of cells attach to the surface in case of strong biofilm former isolate 27b. The log cfu/mm^2 count of viable cells attached in glass surface are 8.09, 4.96 and 3.38 for strong, moderate and weak biofilm former respectively (**Figure 3.8**).

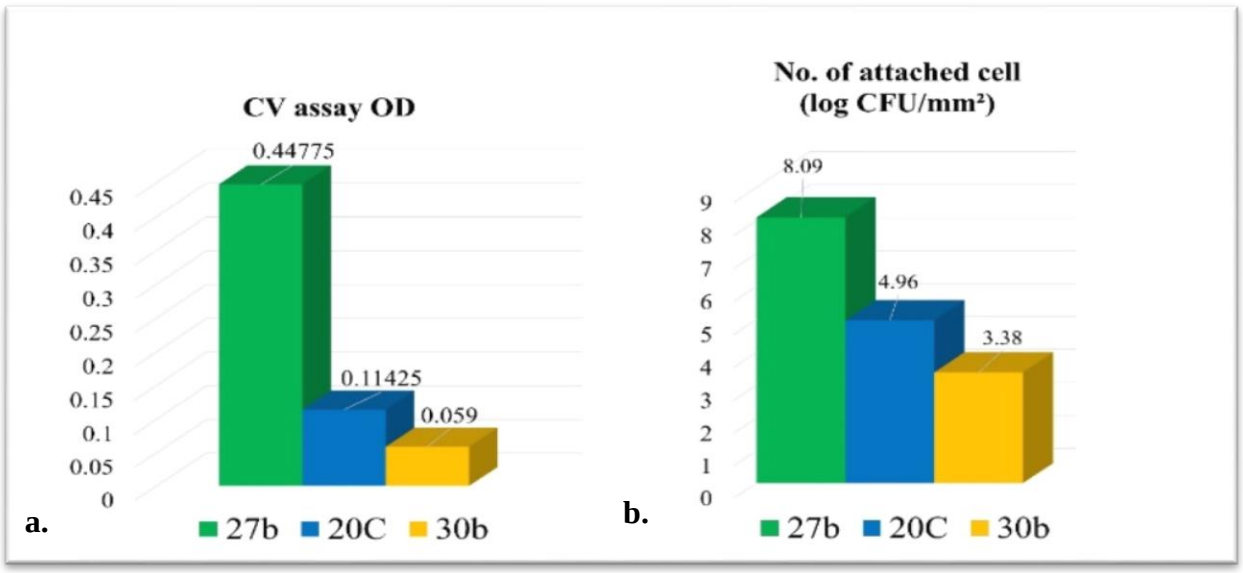


Figure 3.8: a. Bar chart showing the optical Density measured after Crystal Violet biofilm formation assay for strong (DMC-27b), moderate (DMC-20C) and weak (DMC-30b) biofilm former *Pseudomonas aeruginosa* isolates. b. Viable cell counts after 24 hours of biofilm growth in glass surface of those isolates.

3.4.2.2 Pellicle Formation Assay

After 2 days of incubation at room temperature distinct thick pellicle was observed in case of 27b and it becomes more pronounced over time. When 30b produce less or no and 20c produces moderate pellicle over time. In case of 27b, after inoculation of standing LB broth at low cell densities (OD600 = 0.0025), bacterial cells showed exponential growth in the non-biofilm free floating phase of the culture. After 24 hour, a thin pellicle started to form at the air–liquid interface of the tube. After 2 days, the pellicle showed a characteristic structure with bacterial cells and opaque matrix visible with the naked eye. After 120 hours, the pellicle had acquired extremely rigid structure and extensive vortexing and boiling could not disperse that pellicle (**Figure 3.9a**).

3.4.2.3 Congo Red (CR) Assay

We investigated the role of Pel polysaccharide in biofilm formation and EPS production by comparing three strains, i.e. 27b, 20c and 30b. On LB agar plates with CR, colonies of strain 27b showed a wrinkled or ‘rugose’ morphology, whereas 20c colonies were moderately wrinkled and 30b colonies were smooth (**Figure 3.9b**). Rugose colonies in 27b suggest the overproduction of

Pel exopolysaccharide and smooth morphology suggest absence of Pel. Dark red colonies of 27b, pink and pale pink colonies of 20C and 30b (**Figure 3.9b**) indicated the relative amount of Congo red absorption.

When these strains were grown in liquid LB medium, CR staining showed the presence of cell aggregates and slime in the air liquid interfaces. (**Figure 3.9c**). CR release assay also revealed that 27b absorbed more CR than others, thus releasing less CR in the liquid media (**Figure. 3.9d**). The amount of CR released in media is higher for 20c and highest for 30b.

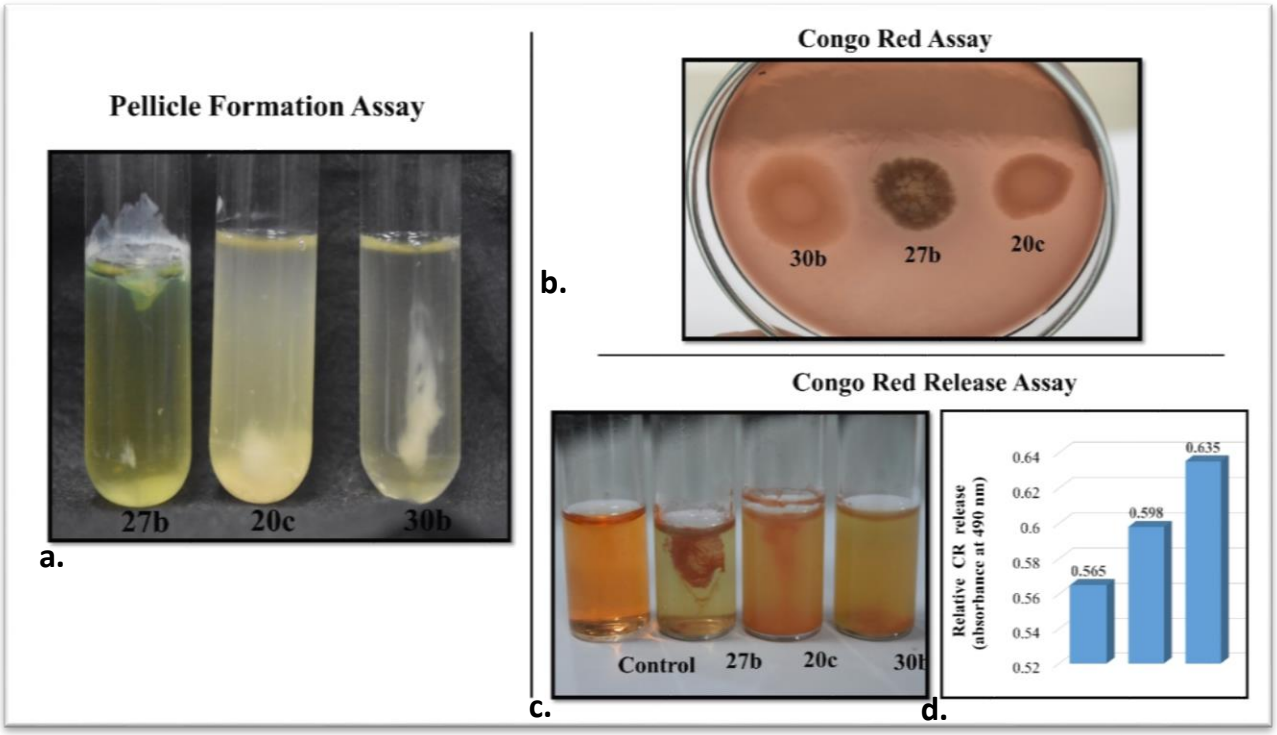


Figure 3.9: Pellicle formation assay. a. Pellicle formation assay on LB broth. 27b forms a thick pellicle at the air–liquid interface of the standing culture. 20c forms moderate and 30b forms no pellicle in air-water interface. b. Colony morphology observed on Congo red (CR) containing LB agar plates. c. 27b, 20c, and 30b were grown in CR containing LB for 24 h at 37◦C, showing amount of CR dye absorbed by the Pellicles formed. d. Relative exopolysaccharide production by 27b, 20c and 30b, using the CR release assay. (Absorbance was taken at 490nm and the error bar shows the standard deviation.

3.5 Molecular Analysis of Biofilm Former Isolates

3.5.1 Detection of Important Genes Responsible for Biofilm Formation

We screened for genes (*pel*B, *lec*B, *rhl*B, *pil*T) that are reported to be involved in biofilm formation. In pre-described PCR condition with defined annealing temperature, four primer sets were used to detect the biofilm formation ability. 10 isolates (5 SBF, 3 MBF, 2 WBF) were selected from different sources to observe if the 4 tested genes (*pel*B, *lec*B, *rhl*B and *pil*T*)* are present in all three types of biofilm formers. Regardless of their variability in biofilm forming ability, 4 biofilm related genes were detected in all the tested isolates (**table 3.1**). PCR results obtained using gene specific primers of representative isolates 27b (SBF), 20c (MBF) and 30b (WBF) were shown in **figure 3.10.**

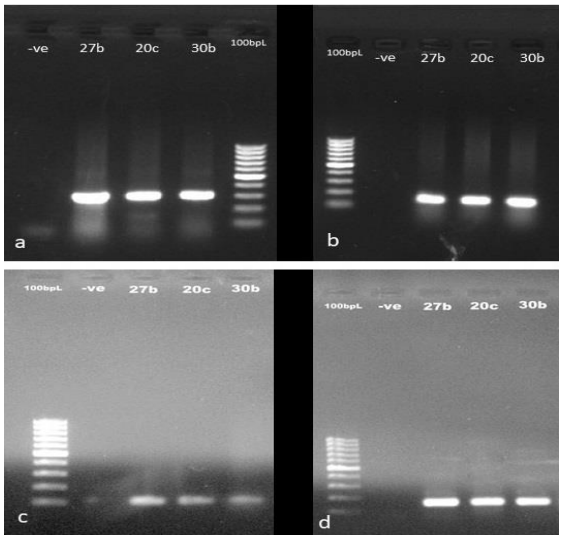


Figure 3.10: PCR results for the detection of biofilm associated genes in *Pseudomonas aeruginosa* isolate 27b (SBF), isolate 20c (MBF) and isolate 30b (SBF) strong biofilm former. PCR products were run on 1% Agarose gel with 100bp marker (Promega, USA) a) (*lec*B : 306 bp) Lane 1 is negative blank control and lane 5 is molecular ladders (100 bp). Lanes 2–4 are strain 27b, 20c and 30b. b) (*pel*B*:* 186 bp) Lanes 3–5 are strain 27b, 20c and 30b. c) (*rhl*B : 96bp) Lanes 3–5 are strain 27b, 20c and 30b. d) (*pil*T:145 bp) Lanes 3–5 are strain 27b, 20c and 30b. For b,c and d Lane 2 is negative blank control and lane 1 is molecular ladders (100 bp). DNA bands at the appropriate position was observed in all three *Pseudomonas aeruginosa* isolates (27b, 20c and 30b).

ID	Source	Biofilm forming ability	Presence of genes			
			*pil*T	*rhl*B	*pel*B	*lec*B
DMC 27b	DMC, Urine	SBF	+	+	+	+
DMC-8b	DMC, Ws	SBF	+	+	+	+
DMC-23b	DMC, Ta	SBF	+	+	+	+
b01	BIHS, Ws	SBF	+	+	+	+
b07	BIHS, Ws	SBF	+	+	+	+
DMC-20b	DMC, Ws	MBF	+	+	+	+
DMC-20c	DMC, Ws	MBF	+	+	+	+
DMC-7	DMC, Pus	MBF	+	+	+	+
b03	BIHS, Ws	WBF	+	+	+	+
DMC-30b	DMC, Ws	WBF	+	+	+	+

Table 3.1: Overall chart showing isolate ID, source of the isolates, presence of biofilm genes, DMC indicates Dhaka medical college hospital, ta indicates tracheal aspirate and Ws indicates wound swab. ND means not done. SBF, MBF and WBF denotes, strong, moderate and weak biofilm former respectively.

3.5.2 Whole Genome Analysis of *Pseudomonas aeruginosa* Isolates Having Variable Biofilm Forming Ability

A total of 3 *P. aeruginosa* isolates from three categories (SBF, DMC-27b; MBF, DMC-20c; and WBF, DMC-30b) that differ in biofilm forming ability were selected for whole genome sequencing.

3.5.2.1 Genomic Overview

The whole genome sequences were annotated by the RAST server. The GC content and number of coding sequences differ slightly for these 3 isolates according to the server (**Table 3.2**). All three isolates possess 72-75% chance of being human pathogen according to the pathogen predictor.

	27b (SBF)	20C (MBF)	30b(WBF)
Size	6,952,319	7,018,827	7,211,991
GC Content	65.8	65.9	65.7
Number of Subsystems	432	429	432
Number of Coding Sequences	7552	7112	7565
Number of RNAs	65	66	66
Pathogen Probability	0.727	0.751	0.749
MLST ST	New	664	244
Predicted strain	E6130952	PABL012	W16401

Table 3.2- Overview of the genomic profile of 3 *Pseudomonas aeruginosa* isolates with pathogen probality, MLST profiling and Kmer analysis.

3.5.2.2 MLST Profiling

MLST profiling indicates that DMC-27b has a unique sequence type while DMC-20c and DMC-30b are similar to number 664 and 244 sequence types respectively.

3.5.2.3 Kmer Analysis

Kmer analysis showed that DMC-27b, DMC-20c and DMC-30b isolates are closely related to the *P. aeruginosa* strain E6130952, PABL012 and W16401 respectively.

3.5.2.4 Secondary Metabolite Profile

Secondary metabolite profile of the isolates were predicted by anti-SMASH server. The analysis showed all the 3 isolates have genes that produce important secondary metabolites like Homoserine lactone and Phenazine that can contribute to biofilm formation (**Figure 3.11**).

Metabolites	27b	20c	30b
Homoserine lactone	+	+	+
Phenazine	+	+	+
Bacteriocin	+	+	+
Non ribosomal peptides	+	+	+
Arylpoleyene	-	-	+
Mangotoxin	-	-	71%
Pyocyanin	-	-	42%
Pyochelin	40%	40%	-

15

Figure 3.11 : Secondary metabolite profile of the 3 isolates.

3.5.2.5 Comparison of the Sequenced Genome with Reference Strain

The sequenced genome of DMC-27b, DMC-20c and DMC-30b were compared with a reference genome of *P. aeruginosa* PA01. With the help of BRIG software the circular genomes of the isolates were constructed and were marked with different colors (**Figure 3.12**). Some important biofilm genes and operons were also marked. With the color gradient the similarity of the genomic regions were indicated. The figure indicates that the Pel operon genes of DMC-30b isolate are less identical in comparison with reference strain and while Pel operon genes of other two sequenced isolates (DMC-27b, DMC-20) have higher identity.

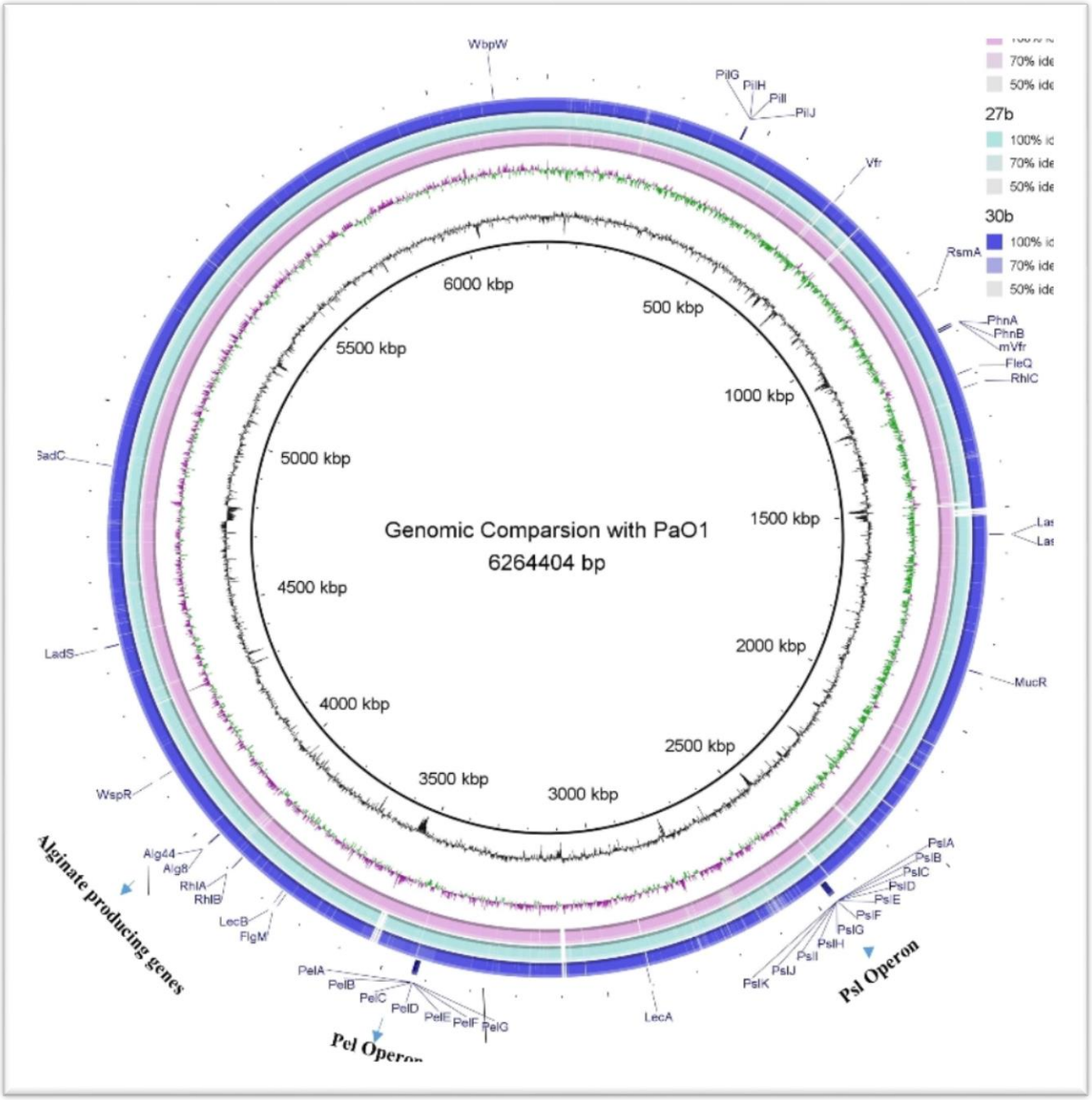


Figure 3.12: Genomic comparison of 3 isolates with reference strain PAO1. The genomes of the isolates are marked with different colors. Color gradient in genomic locations shows the percentage of identity with reference sequence. The genome location of some important biofilm associated genes are also marked.

3.5.3 Analysis of Biofilm Related Genes and Gene Products

Amino acid sequences of biofilm 88 related genes and gene products were compared with reference sequences and the percentage of identity was plotted in a circos plot (For detailed data see Appendix I). The circos plot showing significant variation in the *lec*B gene of DMC-27b and Pel operon genes of DMC-30b in comparison with the reference sequences (**Figure 3.13**). The amino acid identities in percentage for LecB protein and Pel operon proteins are shown in **table 3.3**. LecB protein of DMC-27 showed 13% sequence divergence from the reference protein while Pel operon proteins showed 5% to 13% sequence divergence.

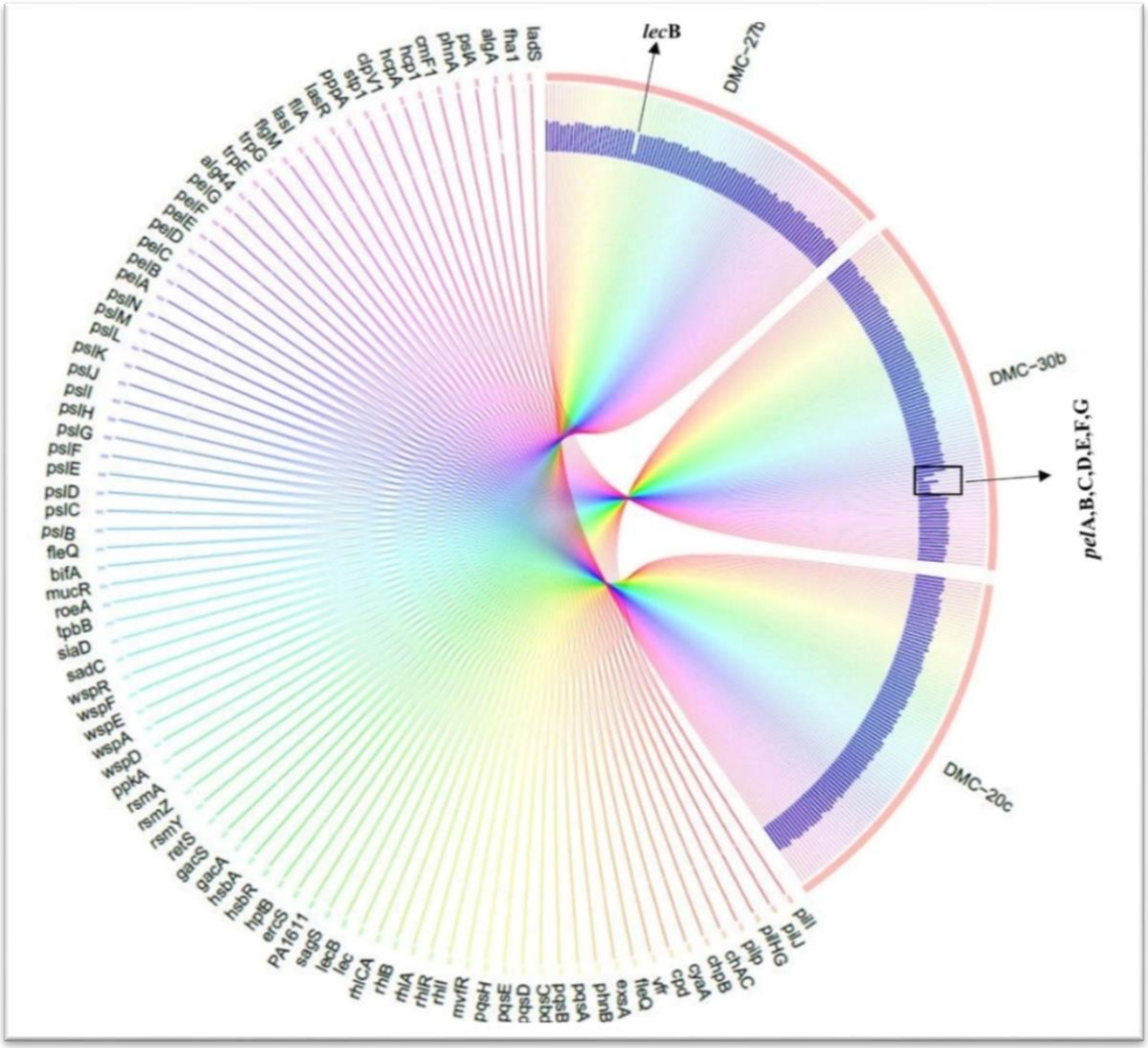


Figure 3.13: Circos plot showing the percentage of sequence homology of proteins related to biofilm formation

Name of Genes/ Products	Location	Number of Amino acids	aa sequence variation (compared with *P.aeruginosa* PA01)		
			27b	20C	30b
fucose-binding lectin PA-IIL, LecB	Unknown	115	**99/115 (87%)**	115/115 (100%)	115/115 (100%)
PA3064 (pelA)	Periplasmic space	948	946/467 (99%)	946/467 (99%)	**884/948 (93%)**
PA3063 (pelB)	Outer Membrane	1193	1180/1193 (98%)	1188/1193 (99%)	**1042/1193 (87%)**
PA3062 (pelC)	Outer Membrane	172	172/172 (100%)	172/172 (100%)	**165/172 (95%)**
PA3061 (pelD)	Cell Membrane	455	450/455 (98%)	449/455 (98%)	**410/455 (90%)**
PA3060 (pelE)	Cell Membrane	329	320/329 (97%)	327/329 (99%)	**298/329 (90%)**
PA3059 (pelF)	Cytoplasmic	507	505/507 (99%)	506/507 (99%)	**459/507 (90%)**
PA3058 (pelG)	Cell Membrane	456	456/456 (100%)	456/456 (100%)	**440/456 (96%)**

Table-3.3: Comparison of amino acid sequences of LecB protein and Pel operon proteins in sequenced strong, moderate and weak biofilm former isolates (27b, 20C and 30b).

3.6 Analysis of LecB Protein

3.6.1: Sequence Analysis of *lec*B Genes and LecB Proteins of Isolate 27b, 20c and 30b

Nucleotide sequences of *lec*B gene from three *P. aeruginosa* isolates (30b, 20c, and 27b.) were retrieved from their whole genome sequences. When compared to reference sequences, 28 nucleotide variation results in change in 16 amino acid positions in isolate 27b **(Figure 3.14a and 3.14b)** . The results also suggest that, 27b possses13% amino acid sequence variance from the other 2 isolates. Isolates 30b and 20C have reference PA01 like sequence which is known to be less virulent model strain of *P. aeruginosa* but 27b has PA14 like sequence which is highly virulent model strain of *P. aeruginosa*.

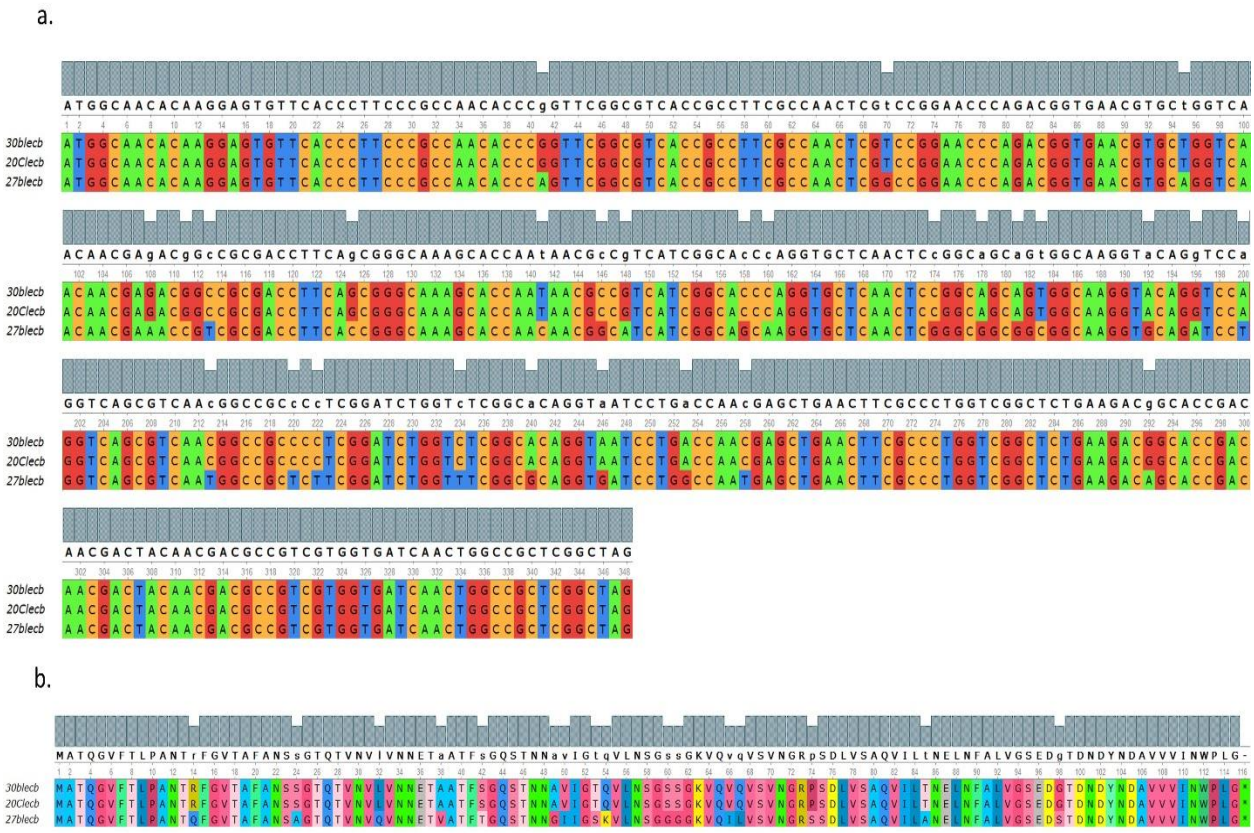


Figure 3.14: *lec*B sequence comparison: (a) multiple alignment of *lec*B sequences from 30b, 20c and 27b and (b) corresponding protein sequence alignment of LecB from 30b (weak), 20c (moderate), 27b (strong) biofilm former.

3.6.2 Phylogeny of LecB protein

A total of 30 LecB like protein sequences were collected from NCBI protein database and a maximum likelihood protein tree were constructed using those sequences along with sequenced LecB of 27b, 20c and 30b. Phylogenetic analysis revealed that LecB protein of 27b is phylogenetically distant from 30b and 20c and it resides with the PA14 like sequences of LecB. 30b LecB and 20c LecB that possess similar aa sequences like P.*aeruginosa* PA01, are located in same branch (**Figure 3.15**).

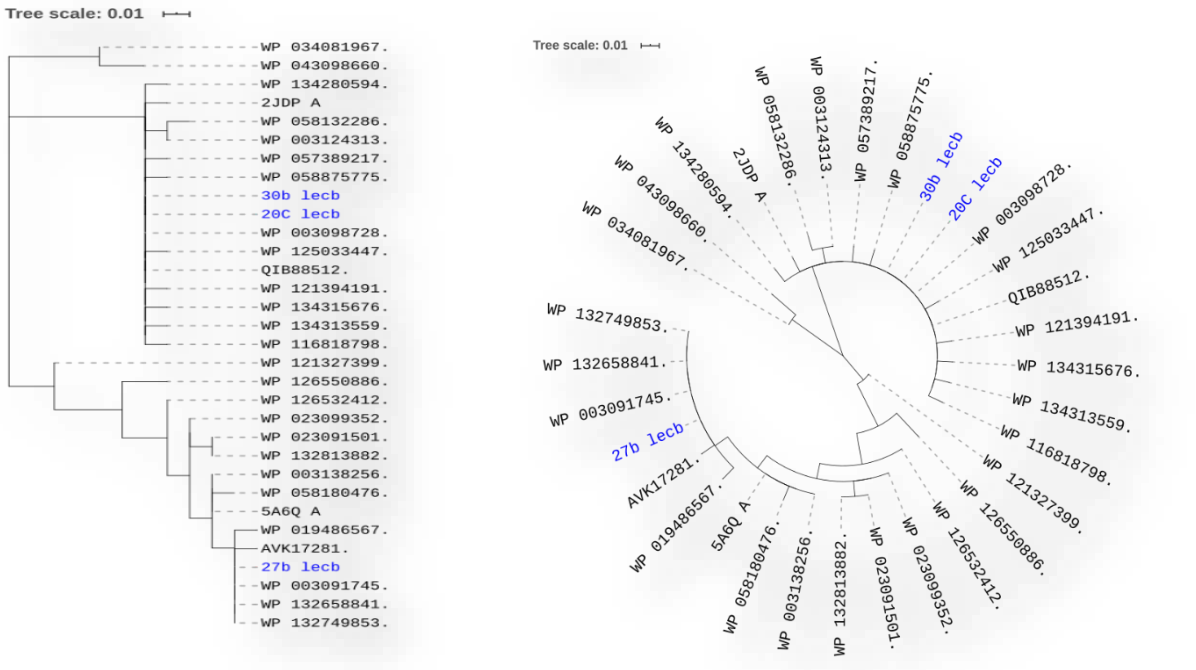
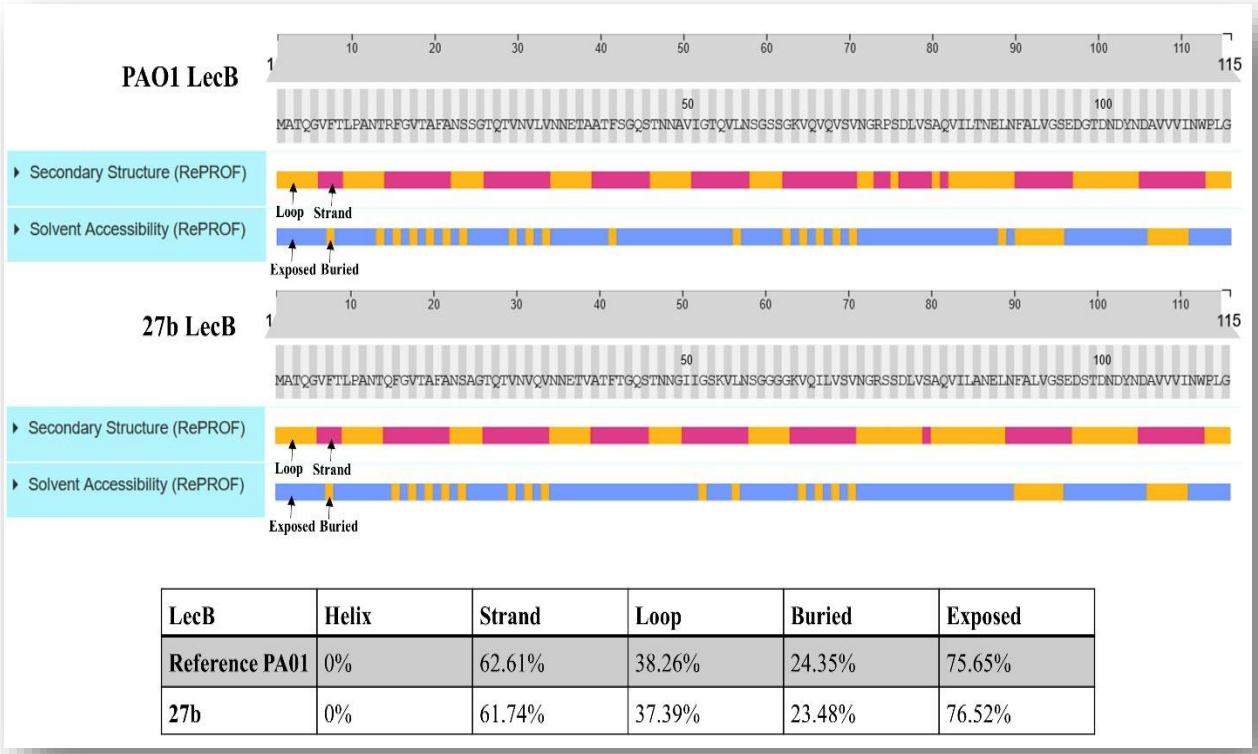


Figure 3.15: Phylogenetic analysis of LecB protein in linear and circular view. LecB proteins of our sequenced isolates marked with blue. Other LecB proteins were designated with their NCBI accession numbers.

3.6.3 Secondary Structure of LecB Protein

The Secondary stucture analysis performed by PredictProtein showed that strand (Beta sheet) portion of 27b LecB protein is slightly lower (61.74%) than reference LecB of PA01 (62.61%). Solvent accessibility analysis by the same webtool also showed that, exposed proportion of the 27b LecB protein is slightly higher (76.52%) and buried proportion is slightly lower (23.48%) than that of PAO1 (75.65% and 24.35% respectively) (**Figure 3.16**). This results predicts the probable change in overall protein structure and composition between two forms of LecB protein.



LecB	Helix	Strand	Loop	Buried	Exposed
Reference PA01	0%	62.61%	38.26%	24.35%	75.65%
27b	0%	61.74%	37.39%	23.48%	76.52%

Figure 3.16- Secondary structure and Solvent Accessibility analysis of 27b LecB protein and Reference PAO1 LecB protein.

3.6.4 3D Structure Prediction of LecB Protein

3D structure of LecB protein was cereated by PHYRE 2 software and visualized by PyMol software (**Figure 3.17)**. 3D crystal structure of 27b LecB is compared with reference PA01 structure and amino acid variations were marked to visualize the position of amino acid variations. The structural alignment of 27b LecB and PAO1 LecB showed that all of the amino acid variations found in 27b LecB are resided solely on the outer surface of the LecB tetramer.

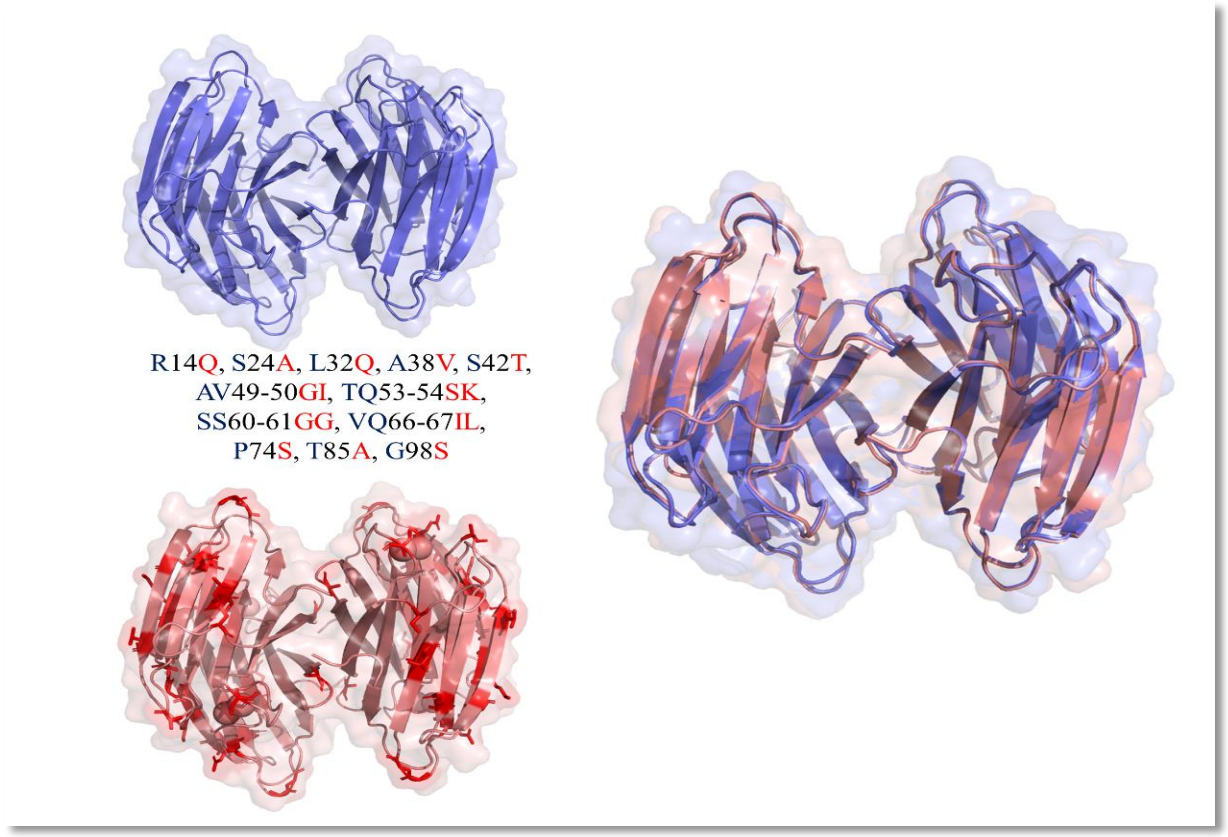


Figure 3.17: 3D structure of the LecB protein visualized by PyMol. Upper left structure shows LecB of PAO1 (slate blue), Lower Left structure shows LecB of 27b (salmon red) with mutation sites (red). On the right, 27b LecB and PAO1 LecB structures were superimposed on each other.

3.6.5 Partial Sequence Analysis of LecB protein

A number of 10 *P. aeruginosa* isolates were selected from our study (5 SBF, 3 MBF and 2 WBF) and amplified with designed primers. The amplified region was sequenced by Sanger sequencing. Partial sequence of LecB proteins was derived from the gene sequences. Partial protein sequences revealed that, DMC-30b, DMC- 20C, DMC-20b, DMC-7 and b03 have PA01 like amino acid sequence and DMC-27b, DMC-23b, DMC-8b, b07 have PA14 like sequence (**Figure 3.18a**). All of the isolates containing PA14 like LecB sequence showed strong biofilm forming ability according to the CV assay, while PAO1 like sequences showed moderate of weak biofilm forming ability (**Figure 3.18b**).

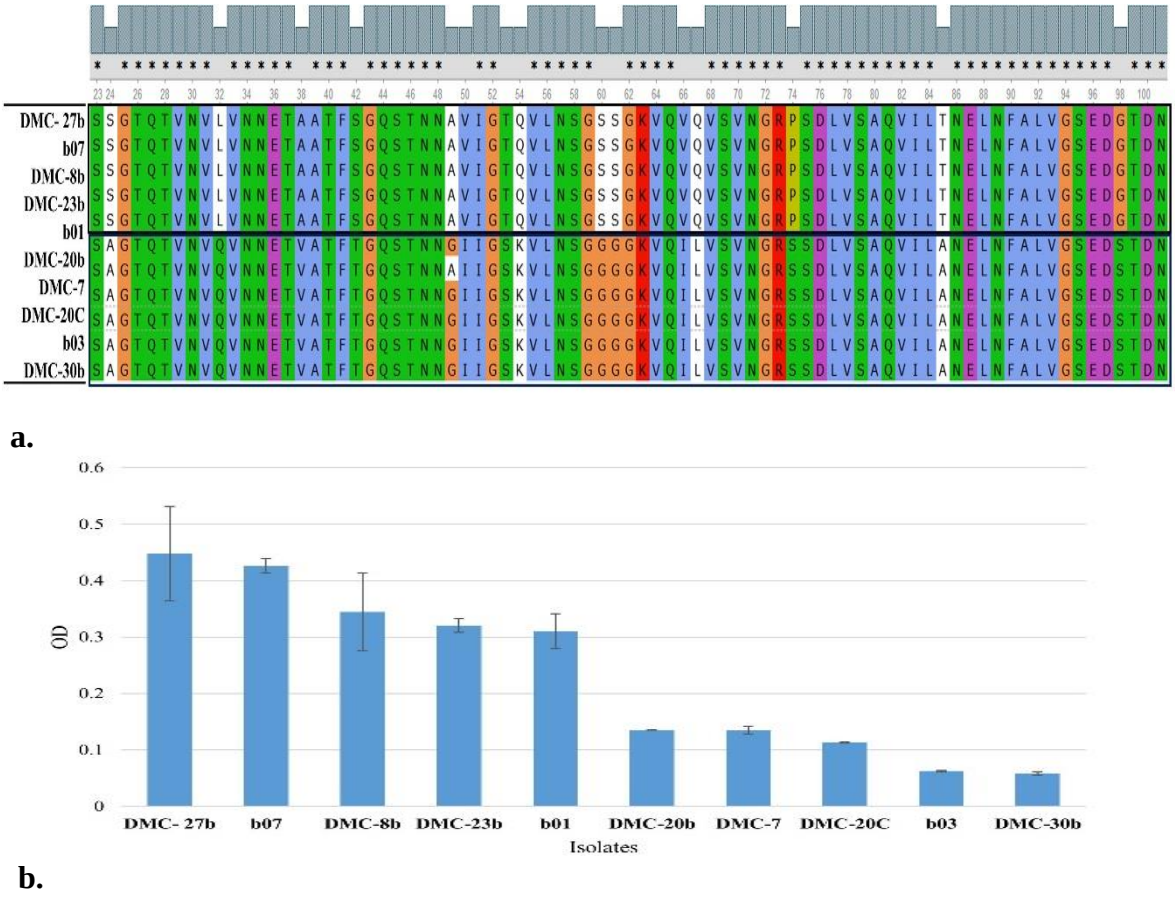


Figure 3.18: LecB variance. a. Partial sequence of LecB (23-101) protein from 10 clinical isolates. b. CV assay OD value of 10 clinical *Pseudomonas aeruginosa* isolates. Error bar indicates the standard deviation from the mean value for triplicated assay.

3.7 Analysis of Pel operon Genes and Proteins

3.7.1 Upstream Sequence Analysis of Pel operon

Analysis of sequences upstream of Pel operon genes transcription start point showed that isolate 27b and 20c have similar upstream sequences like PAO1. Both of 27b and 20c isolates have two FleQ binding region in the promoter region of the *Pel operon* (**Figure 3.19**). The upstream sequence of 30b Pel operon does not contain any of the FleQ binding sites.

Figure 3.19: Two FleQ binding sites in the promoter region of 27b *Pel*B and 20c *Pel*B.

3.7.2. Synteny Analysis

Synteny analysis showed that the genomic organization of the Pel operon genes of isolate 27b, 20C, 30b have similarity with *P. aeruginosa* PAO1 in genomic size and orientation despite of their sequence variation (**Figure 3.20**). Other bacterial species also possess this type of gene cluster with variation in gene size and content.

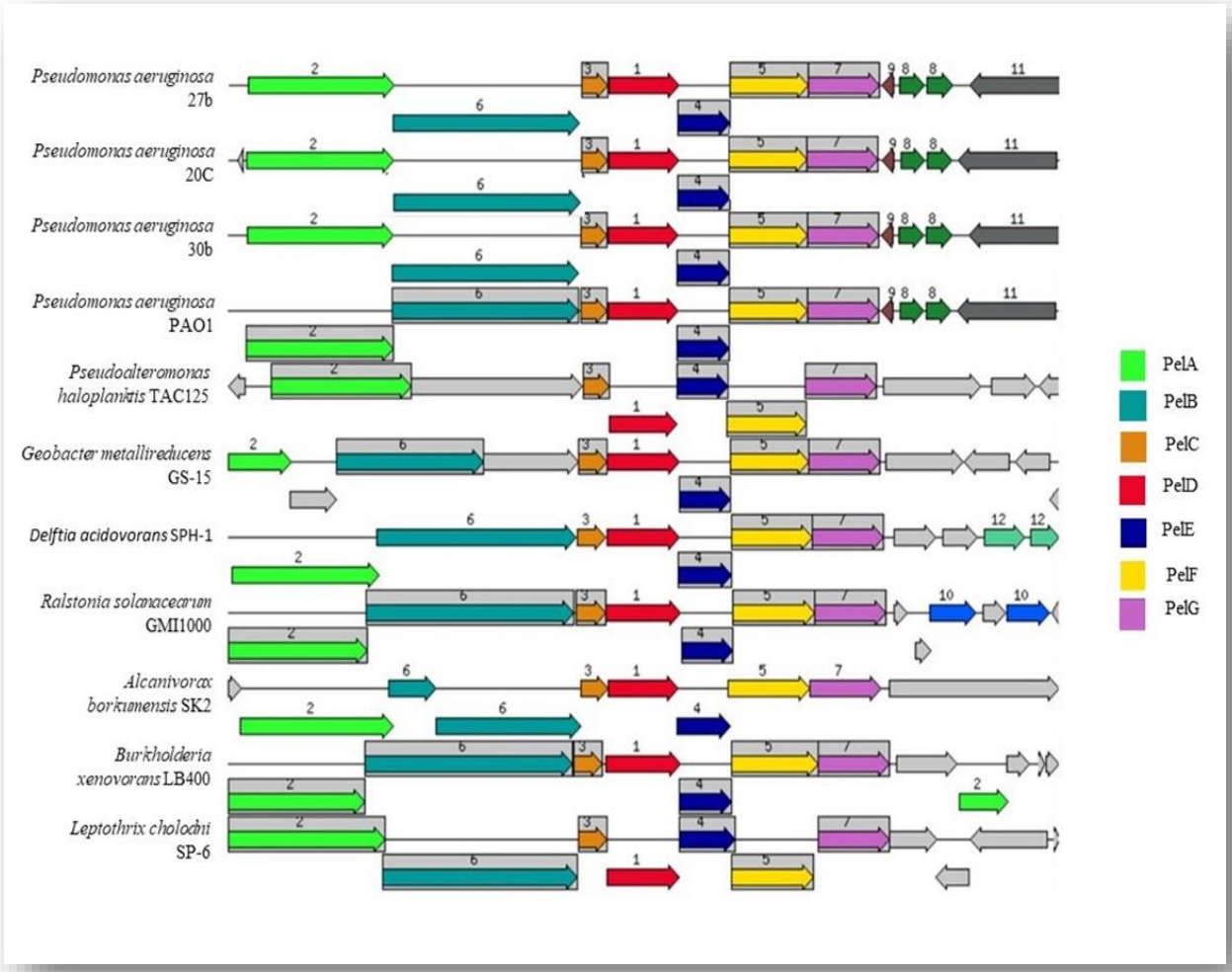


Figure 3.20 : Synteny analysis and gene organization of Pel operon gene clusters. Isolate 27b, 20c, 30b Pel operon gene clusters were compared with *Pseudomonas aeruginosa* PAO1 and other bacterial species containing Pel operon like genes.

3.7.3 Sequence Diversity in Pel operon Genes

BlastN search of individual Pel operon genes of isolate 27b, 20c and 30b in NCBI database revealed that, all of the Pel operon genes of 30b possess certain amount of sequence variation. Only 6 *P. aeruginosa* strains in the database showed similar amount of nucleotide variation in the Pel operon genes. All of the Pel operon genes (*pel*A, *pel*B, *pel*C, *pel*D, *pel*E, *pel*F, *pel*G) showed 100% sequence identity to only one strain in the database, that is *P. aeruginosa* FDAARGOS_570. Five other strains showed 98-99% sequence similarity with Pel operon genes of 30b (**Figure 3.21** and **Table 3.4**). All of the Pel operon genes of those 6 strains and 30b showed significant amount of neucleotide divergence (5-13%) from the other *P.aeruginosa* strains in the database (data not shown). This analysis also revealed that, if any strain show significant neucleotide sequence variation in one of the Pel operon genes, other six genes should also contain significant divergence. This data suggests that, all of the seven Pel operon genes may have evolved together (See Appendix III).

30b *pel*A Description	Query Cover	**E value**	Per. Ident	Accession
Pseudomonas aeruginosa strain FDAARGOS_570 chromosome, complete...	100%	0.0	100.00%	CP033835.1
Pseudomonas aeruginosa PA7, complete genome	100%	0.0	99.82%	CP000744.1
Pseudomonas aeruginosa strain CR1 chromosome, complete genome	100%	0.0	99.36%	CP020560.1
Pseudomonas aeruginosa strain AR_0356 chromosome, complete genome	100%	0.0	99.36%	CP027169.1
Pseudomonas aeruginosa strain AR441 chromosome, complete genome	100%	0.0	99.33%	CP029093.1
Pseudomonas aeruginosa strain AZPAE15042 chromosome, complete gen...	100%	0.0	99.26%	CP041354.1
Pseudomonas aeruginosa strain PA-3 chromosome, complete genome	96%	0.0	92.83%	CP033084.1
Pseudomonas aeruginosa strain LW chromosome, complete genome	96%	0.0	92.79%	CP022478.1
Pseudomonas aeruginosa PA96 genome	96%	0.0	92.79%	CP007224.1
Pseudomonas aeruginosa strain FDAARGOS_505 chromosome, complete...	95%	0.0	92.93%	CP033832.1

Figure 3.21: NCBI nucleotide blast result for 30b *pel*A. The blue color box shows the strains that have 99-100% sequence homology with 30b *pel*A.

Organism	Accession	*pel*A identity	*pel*B identity	*pel*C identity	*pel*D identity	*pel*E identity	*pel*F identity	*pel*G identity
***Pseudomonas aeruginosa* strain FDAARGOS_570**	CP033835.1	100	100	100	100	100	100	100
***Pseudomonas aeruginosa* PA7**	CP000744.1	99.82	99.78	99.81	99.85	99.8	99.93	99.85
***Pseudomonas aeruginosa* strain CR1**	CP020560.1	99.36	99.58	99.61	98.46	99.7	98.56	99.49
***Pseudomonas aeruginosa* strain AR_0356**	CP027169.1	99.36	99.53	99.61	98.61	99.6	98.56	99.2
***Pseudomonas aeruginosa* strain AR441**	CP029093.1	99.33	99.53	99.61	98.61	99.6	98.49	99.2
***Pseudomonas aeruginosa* strain AZPAE15042**	CP041354.1	99.26	99.64	99.81	98.54	99.6	98.69	99.49

Table 3.4 : Seven Pel operon genes of six *Pseudomonas aeruginosa* strains were compared with Pel operon genes of isolate 30b. The percentages of sequence identities are shown in the table.

3.7.4 Partial Sequence Analysis of PelB Protein

Analysis of biofilm related genes of 3 sequenced isolates in our study also showed that, all of the Pel operon proteins of isolate 30b have 5-13% sequence divergence from 27b and 20c **(Table 3.3)**. To understand the frequency of this variation, and to double check the Pel operon protein sequences of those isolates, a portion of *pel*B gene from 10 clinical isolates including isolate 27b, 20c and 30b were amplified and sequenced. Partial aa sequence of PelB protein (649-692) was retrieved from those nucleotide sequences and aligned to observe the variation among the aa sequences. Three amino acid variations (A651T, L658T, S671N) were observed in 30bPelB sequence only **(Figure 3.22).** Other isolates have almost similar type of amino acid sequence in that region of PelB. BlastP search in NCBI database showed that, 30bPelB protein have similar sequence homology with PelB of *P. aeruginosa* PA7 (data not shown). While most of the *P. aeruginosa* strain including PAO1 and PA14 PelB have similar type of PelB protein sequences (98-100% identity) in the NCBI database, 30bPelB and PA7PelB showed aproximately 13% sequence divergence from them (87% identity compared to PAO1).

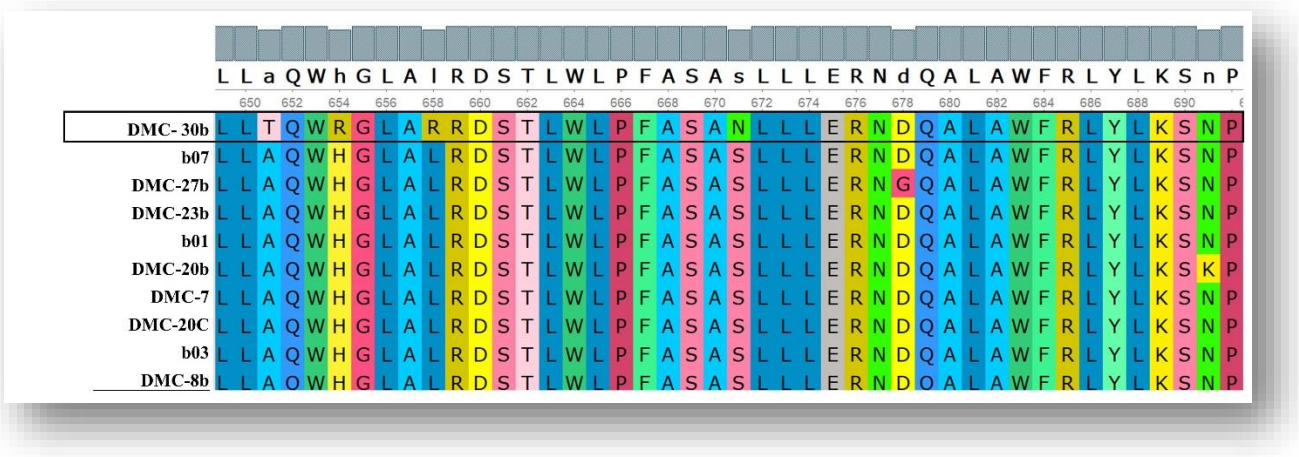


Figure 3.22: Partial sequence of PelB (649-692) protein from 10 clinical isolates.

3.7.5 Phylogenetic Analysis of PelB Protein

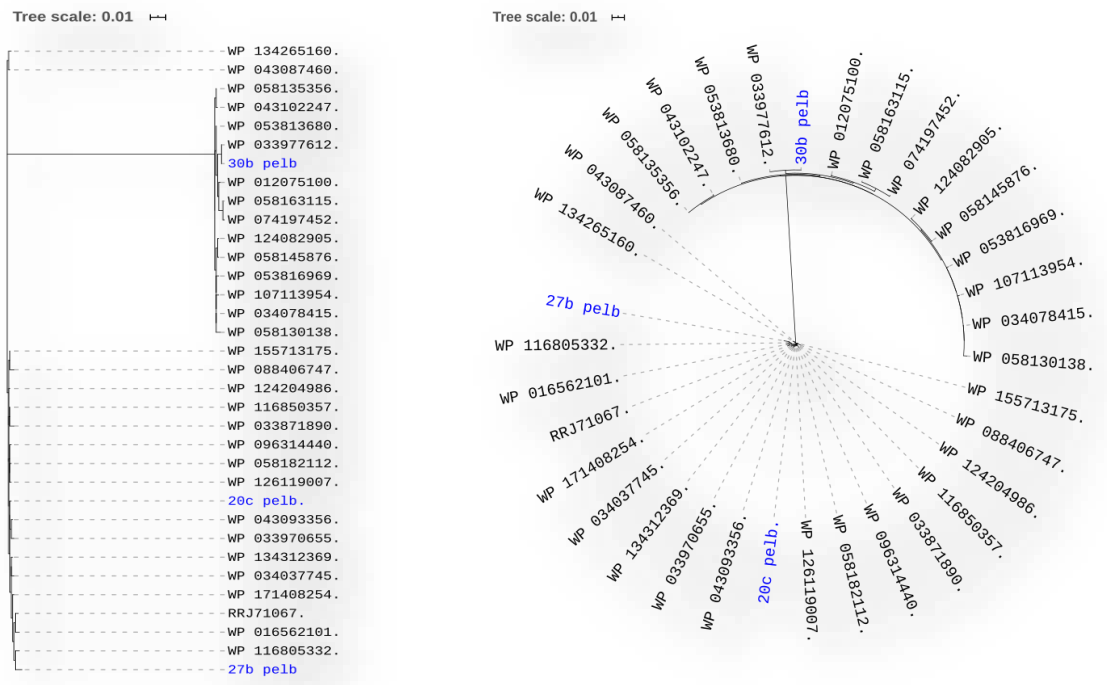


Figure 3.23: Phylogenetic analysis of PelB protein in linear and circular view. PelB proteins of our sequenced isolates marked with blue. Other PelB proteins were designated with their NCBI accession numbers.

A total of 30 PelB like protein sequences were collected from NCBI protein database and a maximum likelihood protein tree were constructed using those sequences along with sequenced PelB of 27b, 20c and 30b. Phylogenetic analysis revealed that PelB protein of 30b is phylogenetically distant from 27b and 20c. (**Figure 3.23**).

3.8 3D Structure Analysis of Pel Operon Proteins

3.8.1. PelA

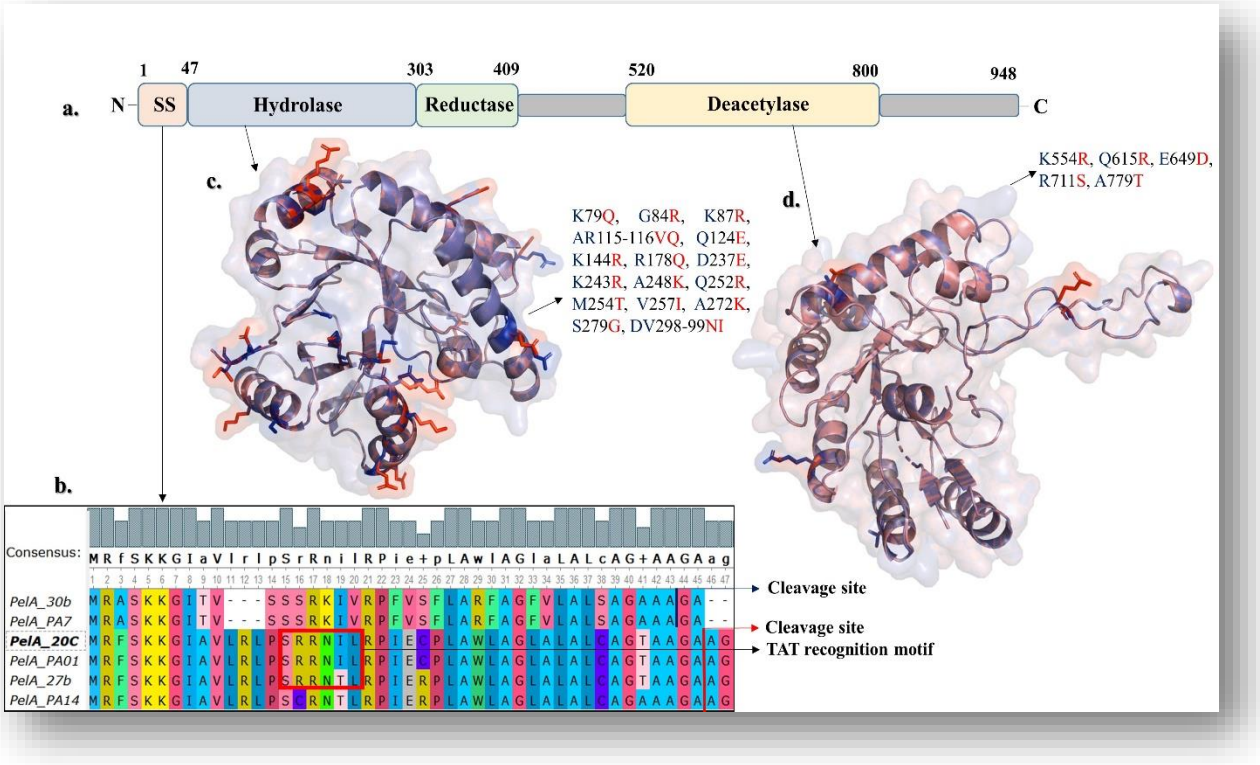


Figure 3.24: PelA structure. a. Important enzymatic domains and signal sequence of PelA. b. N terminal sequence comparison of PelA in different isolates and strains. c. Cartoon and surface representation of the PelA Hydolase domain (47-303) of 30b and PAO1, superimposed on each other. Amino acid variation sites in 30b PelA are marked with red sticks. d. Superimposed cartoon and surface representation of PelA deacetylase domain (520-800) of PAO1 and 30b, with aa variation sites marked with red sticks.

Bioinformatics analysis of PelA using *Phyre*[2] predicts at least four, and possibly five, distinct domains, three of which have structural similarities to proteins with hydrolase, reductase and deacetylase activity (**Figure 3.24a**). A signal sequence (SS) with tat recognition motif containing twin arginine residue was found in the N-terminal region of *P. aeruginosa* PAO1. Similar tat recognition motif was also found in isolate 20c and 27b, but not in PA7, PA14 and 30b (**Figure 3.24b**). The N terminal region of isolate 30b and *P. aeruginosa* PA7 are different from PAO1 with 3 aa deletion in 11-13 position. N terminal sequence of PA14 PelA have 4 aa variation from PA01 PelA (R16C, C25R, I19T and T41A), while PelA of 27b have only two (C25R and I19T) (**Figure 3.24b**). 18 aa acid variation was observed between 30b PelA and reference PA01 PelA hydrolase domain, while 5 aa variation was found in the deacetylase domain (**Figure 3.24c&d**).

3.8.2 PelB

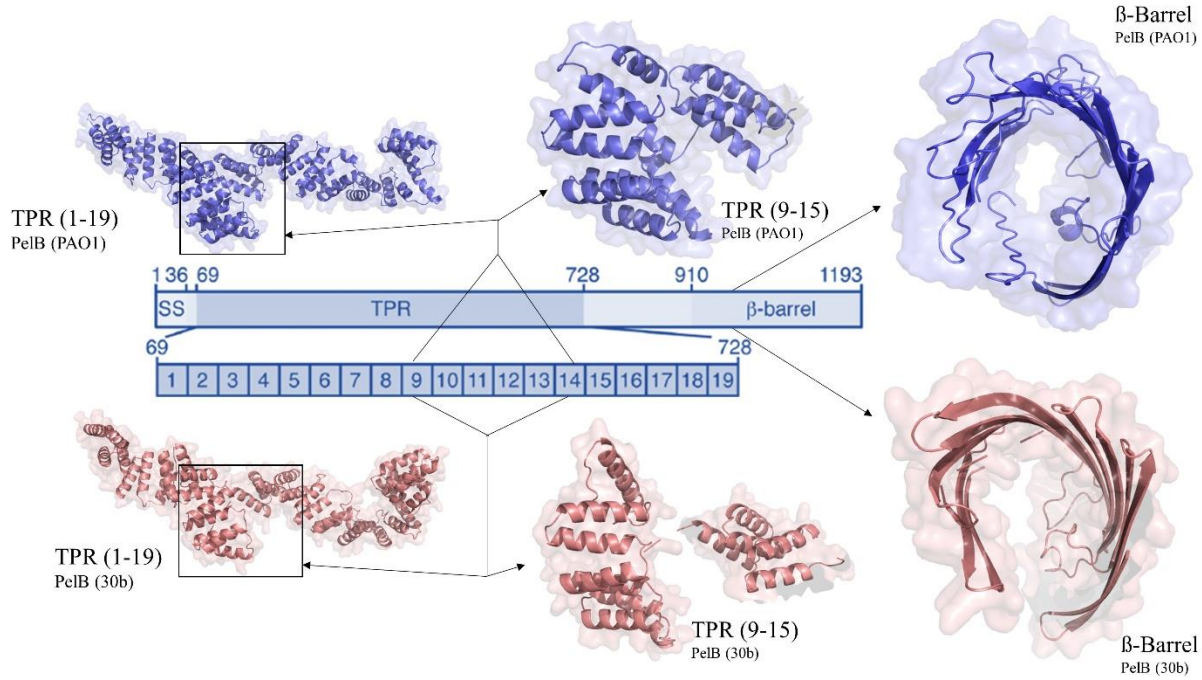


Figure 3.25: 3D structure prediction of TPR motif region and ß-barrel domain of PAO1 PelB (top) and 30b PelB (bottom).

The 19 Tetratricopeptide repeat (TPR) motif containing region of 30b PelB shows significant variation in 3D structure in comparison with PAO1. TPR 9-15 region of PelB that interacts with PelA, contain 27 aa variation between PAO1 PelB and 30b PelB. 3D modelling also shows

significant structure alteration of TPR9-15 motifs (351-588) in 30b PelB (**Figure 3.25**). ß-barrel structure of PelB which is proposed to interact with PelC dodecamer to form a outer membrane Pel polysaccharide secrteion complex, was also found to be distorted in 30b PelB according to the *Phyre*[2] prediction.

3.8.3 PelC

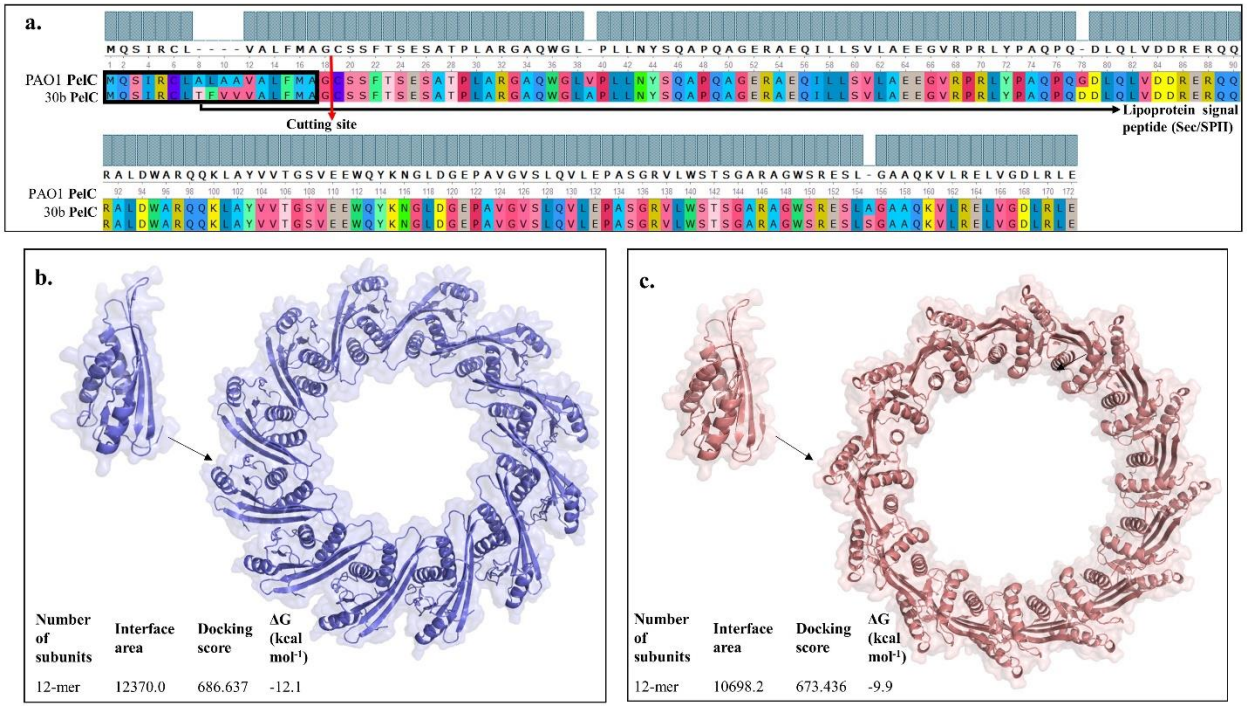


Figure 3.26: PelC structure. a. Amino acid sequences of PAO1 PelC and 30b PelC with Lipoprotein signal sequence Sec/SPII (1-17) and cutting site (18). b. Cartoon and surface representation of mature PA01 PelC monomer (19-172) and deodecamer ring structure with homomer docking results. c. Cartoon and surface representation of mature 30b PelC monomer (19-172) and deodecamer ring structure with homomer docking results.

A total of 7 amino acid variation was observed between PAO1 PelC and 30b PelC. 4 consecutive amino acid variations occurred from 8th to 11th position in the N terminal signal sequence of PelC (**Figure 3.26a**). Amino acid variations in 30b PelC seems to alter the conformation of the monomer (**Figure 3.26c),** which interfere with the formation of dodecamer ring. Homomer docking data and ΔG value suggests stronger association between the monomers of PAO1 PelC to form deodecamer ring than 30b PelC.

3.8.4 PelD,E,F,G

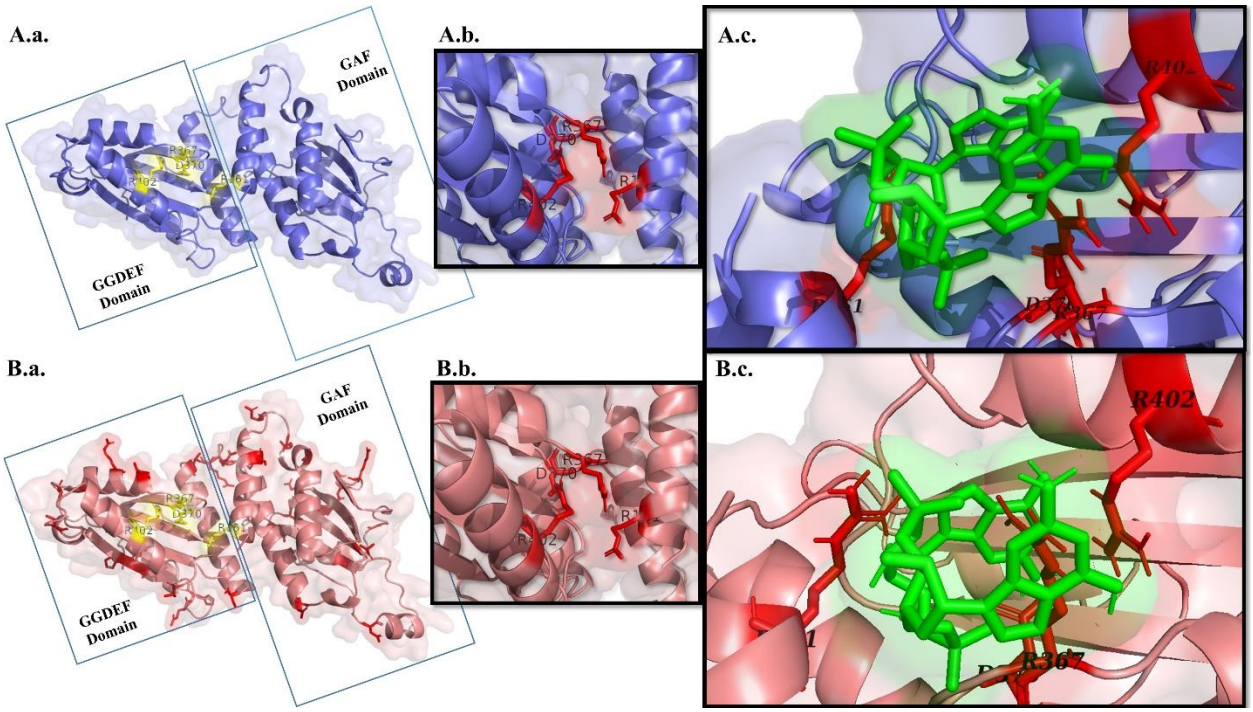


Figure 3.27: PelD structure. A.a. Predicted cartoon and surface structure of GGDEF and GAF domain of reference PAO1 PelD (158-455) with c-di-GMP binding sites marked with yellow sticks. A.b. Closer view of c-di-GMP binding sites containing 3 amino acids from GGDEF domain and 1 amino acid from GAF domain of PAO1 PelD that are marked with red sticks. A.c. C-di-GMP binding with binding sites in PAO1 PelD. C-di-GMP is marked with green and amino acids involved in binding are shown with red sticks. B.a. Predicted cartoon and surface structure of GGDEF and GAF domain of reference 30b PelD (158-455) with c-di-GMP binding sites marked with yellow sticks. Amino acid variation sites in 30b PelD are shown with red sticks. B.b. Closer view of c-di-GMP binding sites containing 3 amino acids from GGDEF domain and 1 amino acid from GAF domain of 30b PelD that are marked with red sticks. B.c. C-di-GMP binding with binding sites in 30b PelD. C-di-GMP is marked with green and amino acids involved in binding are shown with red sticks.

Protein sequence analysis revealed PelD, PelE, PelF, and PelG proteins of 30b have 45, 31, 48 and 16 amino acid variation from similar PAO1proteins respectively, while 20c and 27b have only 0-9 aa variations in those proteins **(Table 3.3)**.

In PelD, R161, R367, D370, and R402 are the 4 amino acids that interacts with c-di-GMP (marked with yellow stick in **Figure 3.27 A.a. & B.a**) are present in all of our sequenced isolates (27b, 20c and 30b). In 30b PelD, c-di-GMP binding sites do not show any changes in conformation (**Figure 3.27 A.b. and B.b)** , but other amino acid variations (marked with red sticks) in both cytoplasmic domains alter the predicted 3D structure in 30bPelD (**Figure 3.27 B.a**). Molecular Docking reveals that, c-di-GMP binds with both PA01 PelD and 30b PelD with almost similar binding affinity.

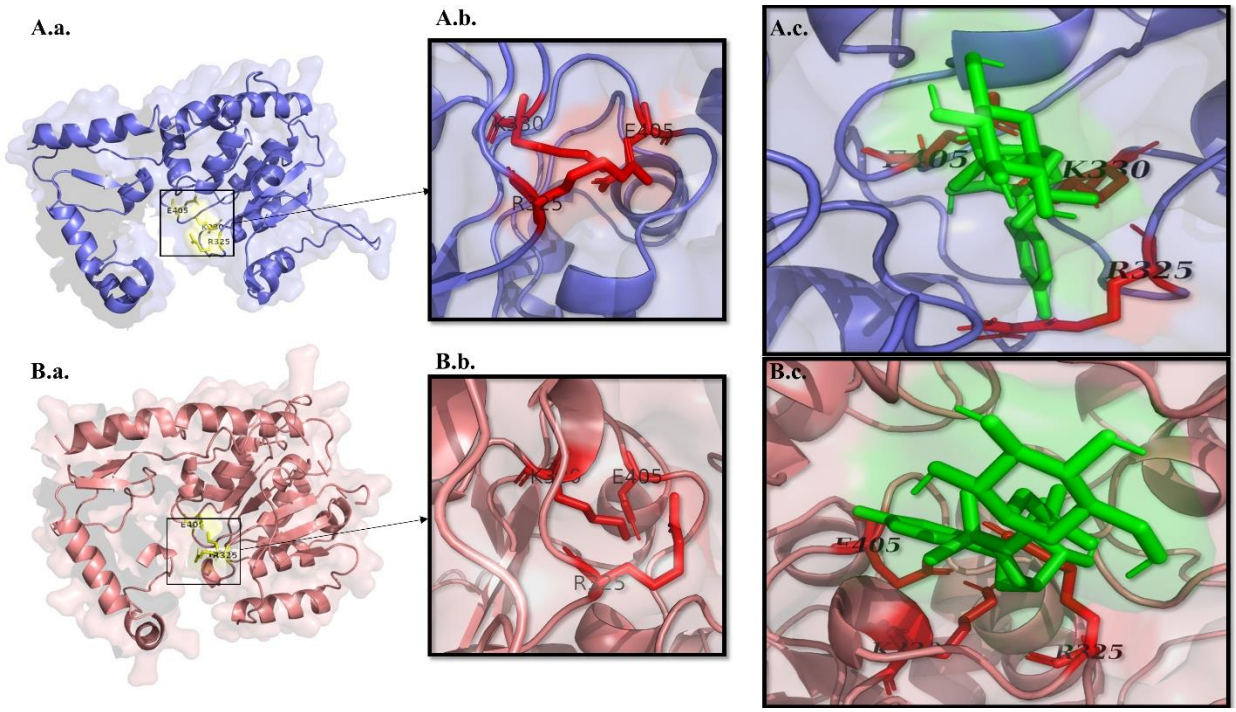


Figure 3.28 : PelF structure A.a. Predicted cartoon and surface structure of of reference PAO1 PelF (13-506) with UDP-glucose catalytic sites marked with yellow sticks. A.b. Closer view of UDP-glucose catalytic sites of PAO1 PelF containing 3 amino acids that are marked with red sticks. A.c. UDP-glucose binding with catalytic in PAO1 PelF. UDP-Glucose is marked with green and amino acids involved in binding are shown with red sticks. B.a. Predicted cartoon and surface structure of of 30b PelF (13-506) with UDP-glucose catalytic sites marked with yellow sticks. B.b. Closer view of UDP-glucose catalytic sites of 30b PelF containing 3 amino acids that are marked with red sticks. A.c. UDP-glucose binding with catalytic in 30b PelF. UDP-Glucose is marked with green and amino acids involved in binding are shown with red sticks.

No amino acid variation was seen at the catalytic region (E405, R325 and K330) of PelF in case of our sequenced isolates (**Figure 3.28 A.a, A.b, B.a and B.b**). Due to the other amino acid changes in the protein sequence, 30bPelF shows conformational changes on that catalytic site (**Figure 3.28 B.b**). Molecular docking suggests that these structural changes in the conformation of UDP-Glucose binding sites affects UDP-glucose binding (**Figure 3.28 A.c and B.c**). Binding affinity of reference PAO1 PelF with UDP-glucose is higher ($\Delta G = -8.1$) than that of 30b PelF ($\Delta G = -7.2$).

3.9 Gene Expression Study by Relative Quantitative Real Time PCR

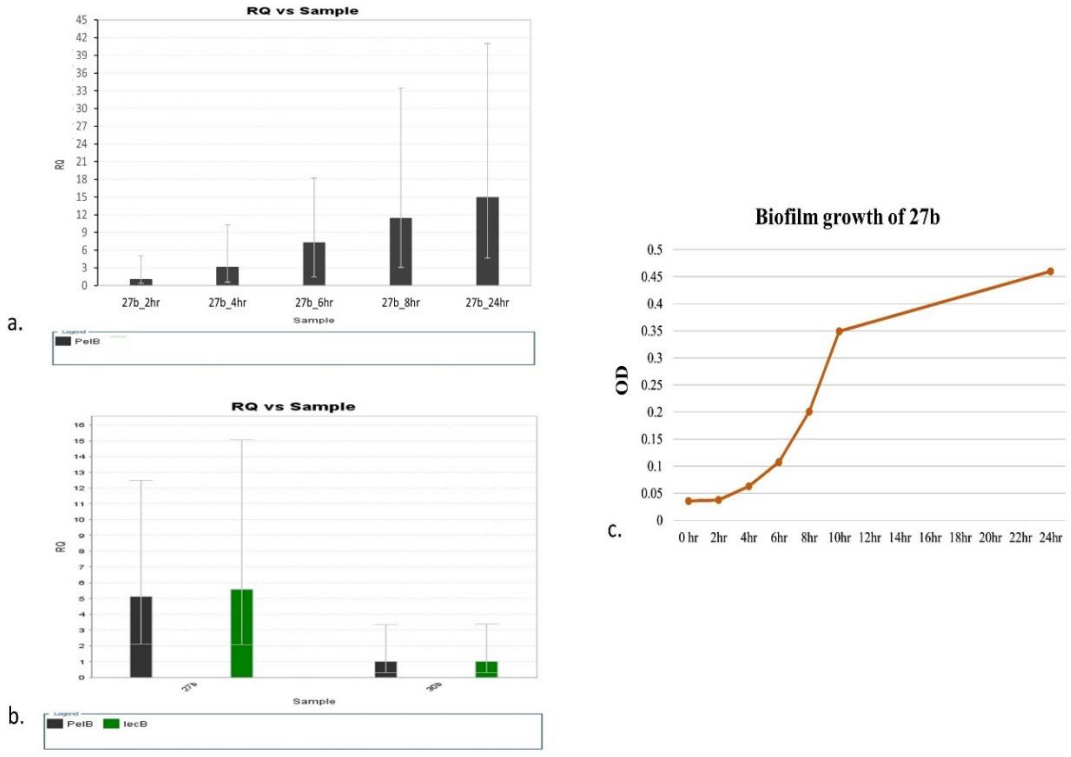


Fig 3.29: Relative expression *pel*B and *lec*B. of a. Relative amount of *pel*B transcripts after 4, 6, 8 and 24 hour of biofilm growth compared with 2 hour growth in isolate 27b quantified by ΔΔCt method. b. Relative quantification of *pel*B *(black bars) and lec*B *(*green bars) transcript levels after 24 hours in 27b and 30b. c. Biofilm growth curve of 27b after 2hr, 4hr, 6hr, 8hr, 10hr and 24hr time period.

Table 3.5: RQ data table representing targets, ΔΔCt and RQ values

Sample	*Target*	ΔΔ*Ct*	*RQ value*
27b_24hr	*pel*B	-2.355	5.116
30b_24hr	*pel*B	0	1
27b_24hr	*lec*B	-2.483	5.59
30b_24hr	*lec*B	0	1

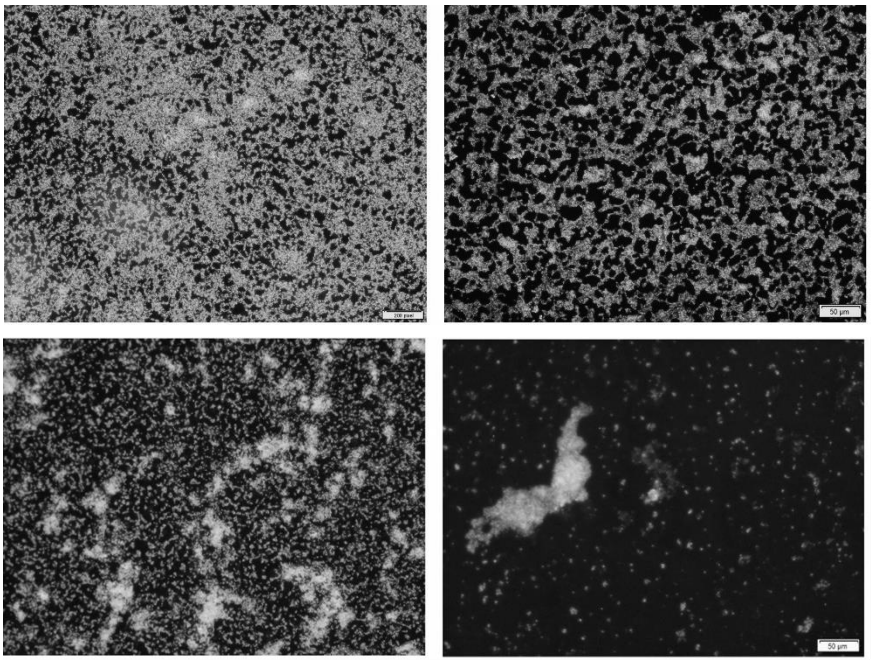
Sample	*Target*	ΔΔ*Ct*	*RQ value*
27b_2hr	*pel*B	0	1
27b_4hr	*pel*B	-1.612	3.057
27b_6hr	*pel*B	-2.849	7.205
27b_8hr	*pel*B	-3.512	11.408
27b_24hr	*pel*B	-3.896	14.887

The relative levels of *pel*B transcripts for isolate 27b were measured after 2hr. 4hr. 6hr. 8hr and 24hr of biofilm growth by qRT-PCR. RQ analysis revealed that *pel*B transcripts levels increased gradually from 2 to 8 hours, where we found that the transcript level increased about 3, 7 and 11.5 folds after 4, 6 and 8 hours compared to the transcript level at 2 hour growth **(Figure 3.29a & Table 3.5).** After 24 hour of biofilm growth the level of *pel*B gene transcripts is about 14 folds higher. This results correlates with the biofilm hourly growth curve of 27b where biofilm biomass was increased with time from 2hr to 24hr **(Figure 3.28c)**. Pellicle formation assay also showed that 27b form thick pellicle in air liquid interface, and the thickness of pellicle increased over time. High amount of PelB expression over time indicates the increased production of pel polysaccharide in the exponential phase of biofilm growth.

The relative levels of *pel*B and *lec*B transcripts were also quantified by qRT-PCR for both 27b and 30b isolates. This analysis revealed that in a matured biofilm of 24 hour, the levels of transcripts of *pel*B and *lec*B were present in higher amount for strong biofilm former 27b than weak biofilm former 30b **(Figure: 3.29b & Table 3.5).** In strong biofilm former isolate 27b, the *pel*B and *lec*B genes were found to be about 5 and 6 folds up-regulated than that of weak biofilm former 30b. This results also correlate with the phenotypic assays including CV assay, pellicle formation assay and CR assay**.**

Chapter 4

DISCUSSION



4.0 Discussion

Biofilms formation by microorganisms have become an emerging health problem globally because microbes in biofilms are more resistant to antimicrobial agents, associate with chronic infections and contaminate surfaces of medical and sanitary equipment (Stewart and Costerton 2001, Kim J. *et al.* 2009, Jamal M. *et al.*, 2018). As a result, biofilms former pathogens persist as reservoirs to spread pathogens from patients to healthy person and environments (Maria Kostakioti et al 2013). Therefore, a suitable strategy to eradicate this reservoir is highly needed. Findings discussed in this dissertation-

I. characterizes multidrug resistant (MDR) clinical isolates from Bangladeshi hospitals as non, weak, moderate and strong biofilm formers (NBF, WBF, MBF and SBF);
II. reveals that Savlon and 70% alcohol solution serve comparatively as better disinfectant but cannot kill all the bacteria in a mature biofilm; and
III. explains the genomic variation in biofilm related genes that affect biofilm forming abilities.

4.1 Characterization of MDR clinical isolates based on their biofilm forming abilities

Biofilms are more resistant to antimicrobial agents, i.e. antibiotics, surfactants, disinfectants than their planktonic counterparts (Kim et al., 2009; Stewart & Costerton, 2001). But, the mechanism of biofilm-associated antimicrobial resistance are dependent on multiple factors and these factors may vary from organism to organism (Patel, 2005). It was also reported that, gaining of antimicrobial resistance can reduce or increase biofilm forming ability in a number of species of Gram-negative bacteria (Cepas et al., 2018). To assess the biofilm forming ability in MDR clinical isolates of Bangladesh, we selected 45 previously identified and characterized clinical isolates which are resistant to at least 2 antibiotic groups. For the assessment of the early stages in biofilm formation, the Crystal violet microtiter plate assay (CV assay) is known as an important tool and has been applied primarily for the study of bacterial biofilms in numerous studies (O'Toole 2011). In our study, CV assay of 45 clinical isolates showed that, most of the MDR clinical isolates (85%) formed biofilms and strong biofilm formers were frequently found among the isolates that were identified as *Pseudomonas* spp (*P. aeruginosa, P. stutzeri, P.hibiscicola)* (**Figure 3.1**). Other isolates did not show strong biofilm forming ability in our study except only one isolate of *K. pneumoniae*. *Proteus mirabilis* (n=1) and *Providencia stuartii* (n=2) isolates did not show biofilm

formation. As the isolate selection criteria in our study was based on multi drug resistance and we have witnessed an increasing occurrence of multi drug resistant (MDR) and extensively drug resistant (XDR) *P. aeruginosa* strains due to their outstanding capacity of carrying antimicrobial genes, we found more *P. aeruginosa* than any other isolates (Breidenstein et al., 2011; Horcajada et al., 2019). The predominance of biofilm former *P. aeruginosa* in our study is also not surprising as it was previously reported that, *P. aeruginosa* have extraordinary ability to form biofilms in different environments and that makes it responsible for various infections (Rasamiravaka et al., 2015) . Prevalence of the strong biofilm former MDR *P. aeruginosa* isolates in our study indicates the importance of eradicating *P. aeruginosa* biofilms from hospital environment.

Different studies on biofilm formation of clinical *P. aeruginosa* isolates showed variability in the biofilm forming ability around the world. A research group from India showed that among 80 *P. aeruginosa* isolated from lower respiratory tract, 20% were found to be SBF (Saxena et al., 2014), while another group of researchers from Pakistan showed 52% of the tested clinical *P. aeruginosa* isolates (n=25) were SBF (Samad et al., 2019). In the other hand, Brazilian researches did not find any SBF isolates from 35 *P. aeruginosa* isolated from cystic fibrosis patient (Perez et al., 2011). In our study, we found 45% of *P. aeruginosa* isolates (n=20) from wound swab, pus, urine and tracheal aspirate showed strong adherence to the surface, which is similar to the finding of Samad et al., 2019.

4.2 Savlon and Ethanol perform better as disinfectant against biofilms

In Bangladeshi hospitals, alcohols, commercially available bleaching solutions, SDS containing detergents and disinfection liquids are commonly being applied to limit the risk of nosocomial infection. For efficacy study of disinfectants against biofilms this dissertation used 70% alcohol, 0.1% SDS, 150 ppm NaOCl and Savlon (ACI, Bangladesh). Working concentration of those disinfectants were in accordance of previous studies and manufacturer's instruction (Rok fink 2018, MELO, Poliana de casto *et al.* 2014).

In this study, 3D volume fluorescent images showed that SDS and NaOCl reduced the surface attachment and volume of the biofilm, but the number of green cells (Live) remain high **(Figure 3.5)**. The RGB brightness analysis of fluorescent image **(Figure 3.6)** indicated that, SDS and NaOCl had little lethal activity on biofilm cells. It is known that SDS is an anionic surfactant that

works by disrupting non-covalent bonds in the proteins, while chlorine-based biocides act as oxidants which destroy cells by chlorinating the lipid protein substances and by inducing the leakage cell content outside the cell (Kim et al., 2008). The reduced efficacy of SDS can be caused because of the electrostatic repulsion between the anionic biofilm matrix and the negatively charged SDS as explained by Simões (Simões et al., 2008). Previous studies also indicated poor penetration of hypochlorite into biofilm due to reactive neutralization of the active chlorine in the outer regions of biofilms (Anderl et al., 2000; Xu et al., 1996).

On the other hand, treatment of the biofilm with ethanol and Savlon showed higher red intensity and a significant log reduction than the untreated ones. We observed 2 to 3 log cycle reduction in case of ethanol and Savlon treatment, but the viable bacterial counts were as high as approximately 10^3 to 10^4 CFU per mm^2 of glass surface that survived these treatments **(Figure 3.6)**. Microscopic images showed the red cells on the upper layer of the biofilm after these treatments, but 3D volume images showed green signals from the inner layers suggesting these antimicrobials could not efficiently penetrate the inner layers of the biofilm structure **(Figure 3.5)**. These results are indicating that, although these agents performed better that SDS and NaOCl, they could not kill all the bacteria present in biofilm. Ethanol kills bacteria by increasing the solubility of outer membrane lipids and thus the structural integrity of the cell membrane become weak and vulnerable. After the disintegration of cell membrane, ethaol can enter the cell and denature the proteins inside the cell. On the other hand, On the other hand, Savlon is a commonly used antiseptic liquid that contain Chlorhexidine Gluconate and Cetrimide. can kill bacteria and prevent infection by absorbing onto the cell wall and thereby causing leakage of the intracellular components to outside (A. Davies, 1973). Cetrimide can play a vital role in the release of purines, pyrimidines, pentose and inorganic phosphate from microbial cells.

This study suggests that commonly used disinfectants have variable effects on their biofilms but none possessing complete biofilm eradication capability. Due to the increased resistance and protective mechanisms against disinfectants and antimicrobials, biofilms have become an important target for therapeutics now a days. Till date several approaches have been used to block the early step of biofilm formation or to destroy the already formed biofilms (Sharma et al., 2014; Sigurdsson et al., 2012). To make those therapeutics more useful and effective, intensive study to understand the molecular basis of the steps involved in biofilm formation is very important.

4.3 Genomic properties of the biofilm formers relate the *P. aeruginosa* isolates as strong, moderate and weak biofilm former

In this study, the presence of four previously reported biofilm associated genes (*rhl*B, *pil*T, *lec*B and *pelB*) were primarily screened to assess the molecular basis of biofilm formation in the *P. aeruginosa* isolates. These genes *(rhlB, pilT, lecB* and *pelB)* were detected in all of the ten isolates tested in our study (**Figure 3.10** and **Table 3.1**), despite of having variable biofilm forming ability. To have more insights into the molecular basis of these variation we selected 1 SBF, 1 MBF and 1 WBF isolate (27b, 20c and 30b) of *P. aeruginosa* and sequenced their genomes. Primary analysis and comparison of whole genome revealed that, these isolates have variation in some regions of their genomes where biofilm related genes are present (**Figure 3.12).** To assess the genomic basis of diverse biofilm forming ability in these isolates we analyzed the amino acid(aa) sequences of 85 proteins and nucleotide (nt) sequences of 3 regulatory RNAs by comparing them with reference *P. aeruginosa* PAO1 (**Figure 3.12)**. These proteins and regulatory RNAs are involved in four major pathways (cAMP/Vfr signaling, c-di-GMP dependent Polysaccharide synthesis, quorum sensing, and the Gac/Rsm pathway) in *P. aeruginosa*, that are responsible for regulating biofilm formation and producing biofilm matrix (Coggan & Wolfgang, 2012; Rasamiravaka et al., 2015).Comparison of 88 proteins and regulatory RNA sequences revealed that, most of the proteins and regulatory RNAs have 98-100% sequence homology with the reference strain, except LecB protein that have 13% sequence variation in SBF isolate 27b and 7 Pel operon proteins that have 5-13% sequence variation in WBF isolate 30b **(Table 3.3, Figure 3.13 & Appendices 1)**.

In SBF isolate 27b, 28 nucleotide variation results in 16 aa substitutions in LecB protein in comparison with PAO1 LecB, butMBF 20c and WBF 30b possess PAO1 like LecB sequences **(Figure 3.14a and 3.14b).** The Secondary stucture analysis showed that this divergence affects alpha-helix and beta-sheet composition and solvent accessibility (**Figure 3.16)** and the 3D structural alignment of 27b LecB and PAO1 LecB revealed that, all amino acid variations in 27b LecB are located exclusively on the outer surface of the tetramer. This sequence divergence in LecB protein has already been proposed as a marker for strain family classification and these differences can affect the ligand binding specificities of this protein (Sommer et al., 2016). Given that, no previous reports directly mentioned about the relation between this type of sequence divergence and biofilm forming ability. To establish the relation between the LecB sequence

divergence and biofilm forming ability, we further analyzed the partial LecB protein sequence of 10 clinical isolates that have variable biofilm forming ability. This study revealed that, 5 of these isolates have PAO1 like sequence and 5 others have PA14 like sequence (**Figure 3.18a**). Isolates that have PAO1 like LecB sequence were found to be strong biofilm former. Five isolates that contain PAO1 like LecB sequence were characterized as moderate or weak biofilm former (**Figure 3.18b**). Moreover, the relative quantification of *lecB* transcripts revealed that, in SBF isolate 27b (containing PA14 like *lec*B sequence), *lec*B genes were about 5 folds up-regulated than that of weak biofilm former 30b (containing PA01 like *lec*B sequence). These data suggests that PA14 like LecB protein protein contributed in formation of stronger biofilms of the clinical *P. aeruginosa* isolates reported in this thesis. .

Molecular analysis of Pel operon also showed sequence divergence between the SBF, MBF and WBF isolates. BlastN search of individual Pel operon genes of isolate 27b, 20c and 30b in NCBI database revealed that, all of the pel operon genes of 30b possess certain amount (5-13%) of sequence variation from the most other pel operon genes in the database. Only 6 strains including reference strain PA7 share 98-100% sequence homolgy with Pel operon genes of 30b (**Figure 3.21** and **Table 3.4**). If any strain show significant neucleotide sequence variation in one of the pel operon genes (for example, *pel*B) , other six genes (*pel*A, *pel*C, *pel*D, *pel*E, *pel*F, *pel*G) also contain significant divergence (5-13%) from reference Pel operon genes. In this background, to determine the PelB type based on sequence divergence we amplified and partially sequenced a small portion of PelB protein (649-692) from 10 clinical isolates as amino acid variations are spreaded all over the protein. . The analysis of aligned partial protein sequences revealed that, 30bPelB sequence contain three amino acid variations (A651T, L658T, S671N), which are not present in other tested isolates **(Figure 3.22).** These data suggest that all the Pel operon protein may have evolved together and 30b have a very rare Pel operon type with significant sequence variations. Based on this result, we propose here two types of Pel operon proteins, PAO1 like (reference) and PA7 like (variant) Pel operon.

The RQ analysis of *pel*B revealed that in a 24 hour biofilm, the levels of *pel*B transcripts of SBF 27b was found to be about 5 and 6 folds up-regulated than that of WBF 30b . **(Figure. 3.29b & Table 3.5).** Bioinformatic analysis of sequences upstream of Pel operon genes transcription start point revealed that both of 27b and 20c have two FleQ binding region in the promoter region of

the Pel operon, while 30b (PA7 like) does not contain any of the FleQ binding sites(**Figure 3.19**). Recent studies have described a role for the flagellum regulator FleQ as both repressor and activator to control Pel operon expression by binding to promoter region in response to c-di-GMP (Baraquet et al., 2012). Absence of these FleQ binding sites may play vital role in the expression of Pel operon proteins. Although, despite the absence of FleQ binding site, we could detect the presence of *pel*B transcripts with universal *pel*B primer.

Bioinformatics analysis of Pel Operon proteins give us more insights about the Pel operon protein structure and function. It was previously reported that PelA have a tat-dependent signal sequence, suggesting the protein is localized to the periplasm (Colvin et al., 2013). In our study, the tat recognition motif containing twin arginine residue was found in the N-terminal region of *P. aeruginosa* PAO1, 20c and 27b, but not in PA7 and 30b (**Figure 3.24b**). This finding indicates that, PelB of 30b (PA7 like) cannot betransported across the cytoplasmic membrane in its folded state via the Tat secretion machinery. We also found 18 aa acid variation between 30b PelA (PA7 like) and reference PA01 PelA hydrolase domain, while 5 aa variation was found in the deacetylase domain (**Figure 3.24c&d**), which may affect PelA function. It was reported that, PelA and PelB directly interacts with each other to form a secretion and modification complex and this is essential for Pel -dependent biofilm formation (Marmont, Whitfield, et al., 2017). We observed that, TPR 9-15 region of PelB that interacts with PelA, contain 27 aa variation between PAO1 PelB and 30b PelB (PA7 like). 3D modelling also shows significant structure alteration of TPR9-15 motifs (351-588) in 30b PelB (**Figure 3.25**). Moreover, the ß-barrel structure of 30b PelB which interact with PelC dodecamer, was also found to be distorted in 30b PelB (PA7 like) according to the *Phyre*[2] prediction. PelC dodecamer functions as an electronegative funnel around the ß-barrel domain of PelB, that guides the positively charged Pel toward PelB exit channel. (Marmont, Rich, et al., 2017). We observed 7 amino acid variation between PAO1 PelC and 30b PelC (PA7 like), 4 among them occurred in the consecutive amino acids in signal sequence (8^{th} to 11^{th} position) of PelC (**Figure 3.26a**). Amino acid variations in PA7 like PelC seems to alter the conformation of the monomer**,** which interferes with the formation of PelC dodecamer ring (**Figure 3.26c)**.

On the other hand, an inner membrane complex of PelD, PelE, and PelG is formed that interacts with PelF in order to facilitate its localization of Pel to the inner membrane. (Ghafoor et al., 2013; Whitfield et al., 2020). PelD, PelE, PelF, and PelG proteins of 30b (PA7 like) have 16-45amino

acid variation from similar PAO1 proteins, while 20c and 27b (PAO1 like) have only 0-9 aa variations in those proteins, which may affect their interactions **(Table 3.3)**. c-di-GMP interaction sites of PelD (R161, D367, R370, and R402) (Whitfield et al., 2020; Whitney et al., 2012) are present in all of our sequenced isolates (27b, 20c and 30b) (**Figure 3.27 A.a, A.b, B.a. B.b**). In 30b PelD, c-di-GMP binding sites do not show any changes in conformation, but other amino acid variations in both cytoplasmic domains show distinguishable changes in the predicted 3D structure in 30bPelD (**Figure 3.27 B.a**). E405, R325 and K330 amino acids of PelF are essential for the activity of PelF as glycosyltransferase and these sites are proposed to be its catalytic site (Ghafoor et al., 2013). Although no sequence variation was seen at this catalytic region in case of our sequenced isolates, due to the other amino acid changes in the protein sequence, PA7 like PelF (30b) shows conformational changes on that catalytic region (**Figure 3.28 B.a, B.b)**. Molecular docking suggests these conformational changes affect the binding UDP-glucose to PA7 like PelF (**Figure 3.28 A.c and B.c)**. These data also correlate with the pellicle formation assay and CR assay, where 30b that contain PA7 like pel operon sequence showed absence of pellicle (**Figure 3.9)**. From the expression and structural analysis of Pel Operon proteins, we propose that organisms that have PA7 like Pel Operon, cannot produce pellicle due to the absence of regulatory protein binding site in the upstream and because of significant structural variation in the Pel operon proteins. Pel production mechanism in PAO1 and probable affects caused by Pel operon sequence variation in PA7 are discussed in the following figure (**Figure 4.1).**

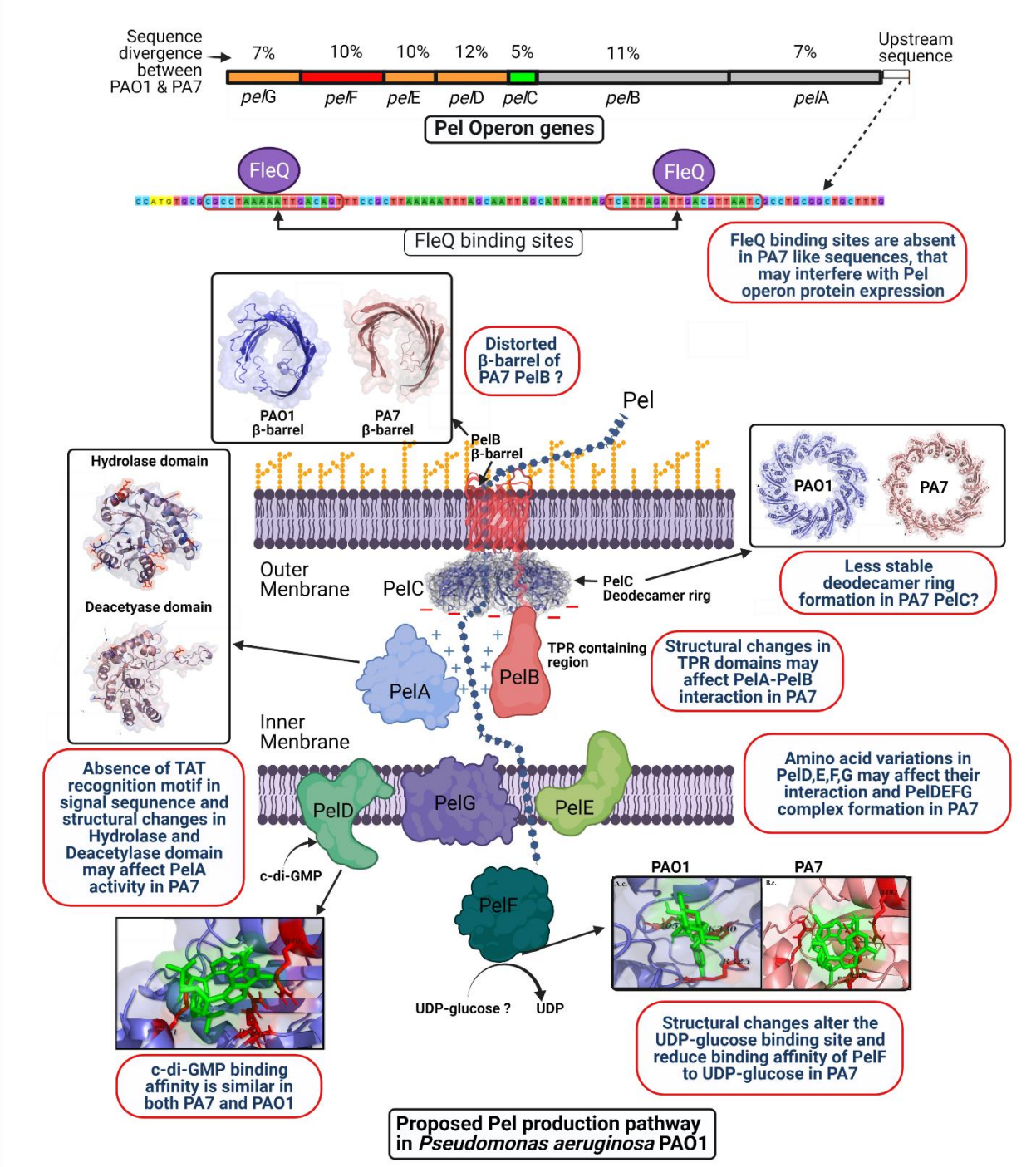
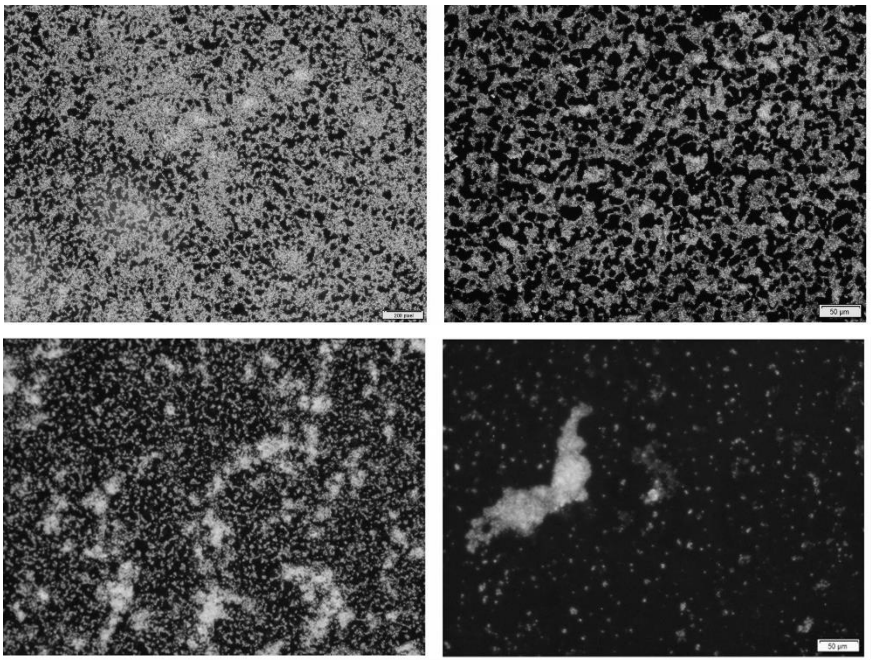


Figure 4.1 Schematic diagram showing proposed Pel production pathway in *Pseudomonas aeruginosa* PAO1. This figure also illustrates the sequence variation between PAO1 and PA7 strains and probable effects of the sequence divergence on Pel operon proteins.

Chapter 5

CONCLUSION

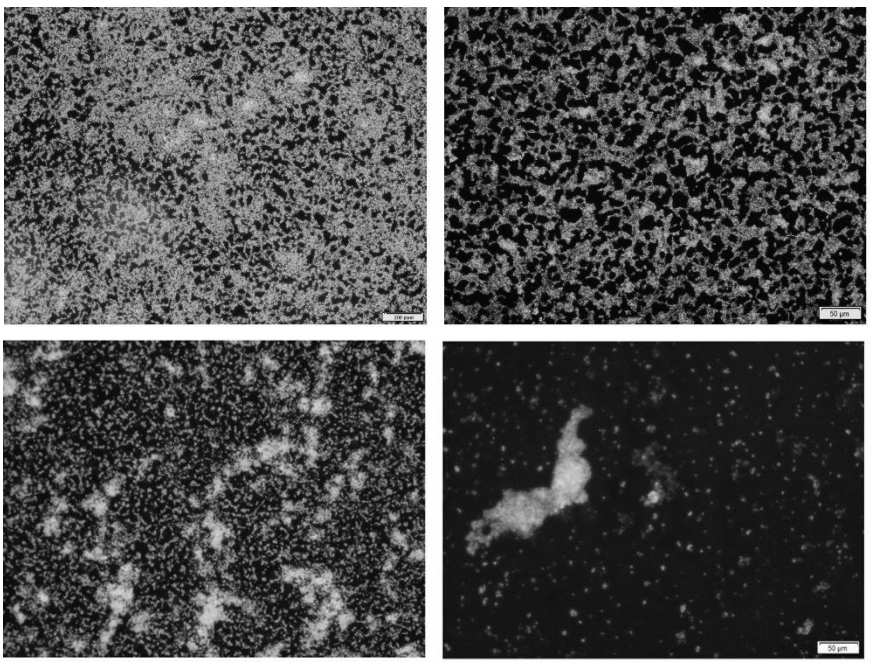


5.0 Conclusion

Clinical isolates from Bangladesh have variable biofilm forming abilities. Among the strong biofilm formers, *P. aeruginosa* isolates are prevalent. Treatment with commonly used disinfectants (0.1% SDS, NaOCl, 70% ethanol and Savlon) reduced cell number and biomass of *P. aeruginosa* biofilms, but none of them were capable to completely eradicate the biofilm. Despite of having variable biofilm forming ability, common biofilm related genes are present in all of the *P. aeruginosa* isolates. Comparison of whole genomes of strong, moderate and weak biofilm former isolates revealed that, 77 of the 85 biofilm related proteins have 98-100% sequence similarity. Strong biofilm former isolate 27b possesses PA14 like *Lec*B sequence and have 13% sequence divergence with PAO1 like *Lec*B of 20c and 30b. Further analysis revealed that, PA14 like *Lec*B containing isolates have strong biofilm forming ability, while PAO1 like *Lec*B containing isolates are either moderate or weak biofilm formers. *lec*B transcripts is also found to be upregulated in strong biofilm former isolates. Moreover, all of the 7 Pel operon genes in weak biofilm former isolate 30b show significant sequence variation with other isolates, and have similarity with the Pel operon proteins of PA7. PA7 like pel operon do not have FleQ binding sites in their promoter region, suggesting that this FleQ protein do not act as activator or repressor in PA7 like Pel operon expression. In silico structure analysis revealed that, PA7 like PelA do not have tat recognition motif in N terminal region and have structural changes in important hydrolase and deacetylase domain, PA7 like PelB have distorted PelA binding TPR region and altered ß-barrel structure, PA7 like PelC have structural changes that may interfere with its characteristic deodecamer formation around PelB and PA7 like PelF have conformational changes catalytic sites that may affect UDP-Glucose binding to PelF. Expression analysis also showed that, lower amount of *pel*B transcripts is present in WBF 30b biofilm cells in comparison with SBF 27b. These distinct features separate PA7 like Pel operon proteins from reference PAO1 like Pel operon proteins. Phenotypic assays indicates that, these sequence and structure variations may have interfered with Pel production pathway and result in impaired Pel production in weak biofilm former isolate 30b that contain PA7 like Pel operon proteins. In conclusion, our analyses suggests that, strains of *P. aeruginosa* possess significant genomic divergence in biofilm related genes that affect their biofilm phenotypes.

Chapter 6

REFERENCE



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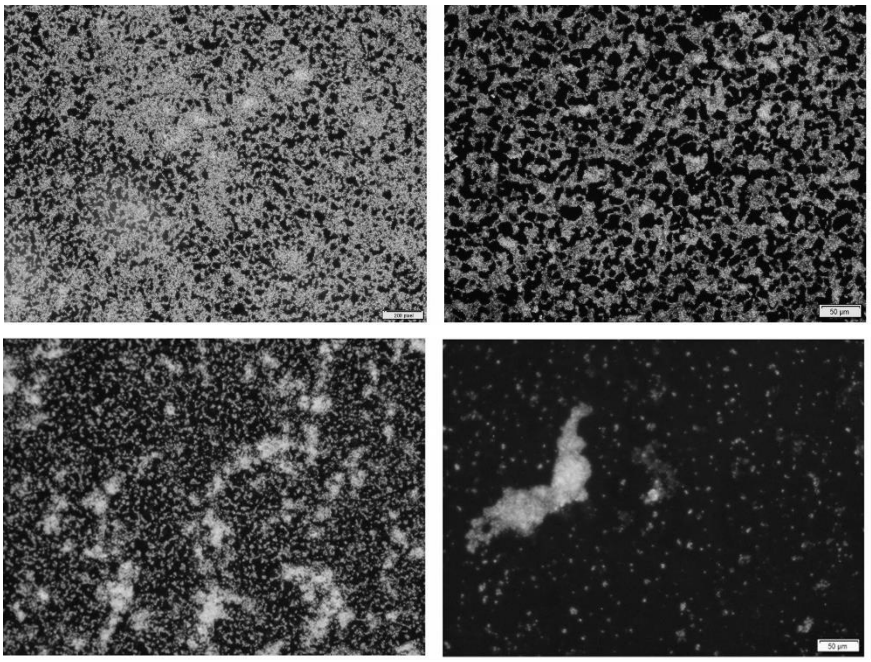
APPENDICES



APPENDIX-I

Comparison of Biofilm related gene products of 3 *Pseudomonas aeruginosa* strains

(in comparison with reference strain *Pseudomonas aeruginosa* PA01)

Name of the Genes/ Gene Products	Identity DMC-27b	Identity DMC-30b	Identity DMC-20c
pilI; twitching motility protein PilI	100	100	100
pilJ; twitching motility protein PilJ	99	100	99
pilH; twitching motility protein PilH	100	100	100
pilG; pilus biosynthesis/twitching motility protein PilG	100	100	100
chpA; chemotactic signal transduction system protein	98	99	99
chpC; chemotaxis protein	98	98	98
cyaB; protein CyaB	99	100	100
cpdA; cAMP phosphodiesterase	98	99	99
vfr; cAMP-regulatory protein	100	100	100
fleQ; transcriptional regulator FleQ	100	100	100
exsA; exoenzyme S transcriptional regulator ExsA	100	100	100
phnB; anthranilate synthase component II	99	99	99
pqsA; anthranilate--CoA ligase	99	100	100
pqsB; hypothetical protein	98	100	99
pqsC; hypothetical protein	99	99	100
pqsD; 3-oxoacyl-ACP synthase	99	99	99
pqsE; thioesterase PqsE	100	100	100
pqsH;	100	100	100
mvfR; transcriptional regulator MvfR	99	99	99
rhlI; acyl-homoserine-lactone synthase	99	99	99
rhlR; transcriptional regulator RhlR	100	100	100
rhlA; rhamnosyltransferase subunit A	99	99	98
rhlB; rhamnosyltransferase subunit B	100	100	99
rhlC; rhamnosyltransferase	100	99	99
lecA; PA-I galactophilic lectin	99	100	99
fucose-binding lectin PA-IIL, LecB	87	100	100
sensor histidine kinase SagS	99	100	100
hybrid sensor kinase, PA1611	99	99	99
ercS'; sensor histidine kinase	99	99	99
histidine phosphotransfer protein HptB	99	99	99
biofilm regulator HsbR	99	100	100
anti anti-sigma factor HsbA	100	100	100
gacA; response regulator GacA	100	100	100
gacS; sensor/response regulator hybrid protein	99	100	100

retS; sensor histidine kinase MifS	99	99	99
rsmY; regulatory RNA RsmY	100	100	100
rsmZ; regulatory RNA RsmZ	100	100	100
rsmA; carbon storage regulator	100	100	100
ppkA; serine/threonine protein kinase PpkA	99	99	98
wspD; hypothetical protein	100	100	100
wspA; chemotaxis transducer	98	99	99
wspE; chemotaxis sensor/effector fusion protein	99	99	100
probable methylesterase, WspF	100	100	99
wspR; two-component response regulator	99	100	100
sadC, protein SadC	99	100	100
siaD, protein SiaD	99	99	99
diguanylate cyclase,TpbB	99	99	99
roeA, Protein RoeA	98	100	98
mucR; signaling protein	99	99	100
bifA; protein BifA	100	100	100
transcriptional regulator, FleQ	100	100	100
pslB; biofilm formation protein PslB	99	99	99
pslC; biofilm formation protein PslC	99	99	99
pslD; biofilm formation protein PslD	100	100	100
pslE; biofilm formation protein PslE	99	99	99
pslF; biofilm formation protein PslF	99	99	99
pslG; biofilm formation protein PslG	100	100	100
pslH; biofilm formation protein PslH	99	99	99
pslI; biofilm formation protein PslI	100	99	99
pslJ; biofilm formation protein PslJ	100	100	100
pslK; biofilm formation protein PslL	98	99	99
pslL; hypothetical protein	99	100	99
pslM; biofilm formation protein PslM	99	99	99
pslN; biofilm formation protein PslN	99	100	99
pelA; hypothetical protein	99	93	99
pelB; pellicle/biofilm biosynthesis protein PelB	98	87	99
pelC; pellicle/biofilm biosynthesis protein PelC	100	95	100
pelD; pellicle/biofilm biosynthesis protein PelD	98	90	98
pelE; pellicle/biofilm biosynthesis protein PelE	97	90	99
pelF; pellicle/biofilm biosynthesis glycosyltransferase PelF	99	90	99
pelG; pellicle/biofilm biosynthesis transporter PelG	100	96	100
alginate biosynthesis protein, Alg44	100	100	100
trpE; anthranilate synthase component I	99	99	99
anthranilate synthetase component II, trpG	98	98	98
flgM; protein FlgM	100	99	100
fliA; flagellar biosynthesis sigma factor FliA	100	100	100
lasI; acyl-homoserine-lactone synthase	100	100	100
lasR; transcriptional regulator LasR	98	100	100

pppA; serine/threonine phosphatase PppA	99	99	99
stp1; phosphatase Stp1	100	100	100
clpV1; secretion protein ClpV1	99	99	99
hcpA; secreted protein Hcp	100	100	100
hcp1; protein secretion apparatus assembly protein	100	100	100
icmF1; type VI secretion protein IcmF	99	100	99
phnA; anthranilate synthase component I	98	98	98
pslA; biofilm formation protein PslA	100	99	100
algA; isomerase	100	99	100
fha1; Fha domain-containing protein	99	98	99
ladS; lost adherence sensor LadS	99	98	99

APPENDIX-II

Protein sequences

PAO1 like LecB

MATQGVFTLPANTRFGVTAFANSSGTQTVNVLVNNETAATFSGQSTNNAVIGTQVLNS
GSSGKVQVQVSVNGRPSDLVSAQVILTNELNFALVGSEDGTDNDYNDAVVVINWPLG*

PA14 like LecB

MATQGVFTLPANTQFGVTAFANSAGTQTVNVQVNNETVATFTGQSTNNGIIGSKVLNS
GGGGKVQILVSVNGRSSDLVSAQVILANELNFALVGSEDSTDNDYNDAVVVINWPLG*

PAO1 like PelA

MRFSKKGIAVLRLPSRRNILRPIECPLAWLAGLALALCAGTAAGAAGGPSSVAFWYAER
PPLAELSQFDWVVLEAAHLKPADVGYLKEQGSTPFAYLSVGEFDGDAAAIADSGLARG
KSAVRNQAWNSQVMDLAAPSWRAHLLKRAAELRKQGYAGLFLDTLDSFQLQAEERRE
GQRRALASFLAQLHRQEPGLKLFFNRGFEVLPELPGVASAVAVESIHAGWDAAAGQYR
EVPQDDRDWLKGHLDALRAQGMPIVAIDYLPPERRDEARALAARLRSEGYVPFVSTPA
LDYLGVSDVEVQPRRIALLYDPREGDLTLSPGHVYLGGLLEYLGYRVDYLPTDQPLPER
PLSGLYAGVVTWMTSGPPLASDAFDNWIAARLDEKVPVAFLAGLPTENDGLLQRLGIR
RLSQKLKVKPSTETHDQALLGSFEAPLVIRIRDLPALTVLDPARVTPALKLKGDGKEYVP
VATADWGGFALAPYVLEEGSEHRRWILDPFAFLRKALRLVPLPSPDATTENGRRIATVHI
DGDGFVSRAEVPGSPYAGQQVLEDFIKPYPFLTSVSVIEGEVGPKGMYPHLARELEPIAR
RIFADDKVEVASHTFSHPFFWQPQLAEQGENFEAQYGYKMAIPGYDKVDFVREVIGAR
DYIEQRLTTPRKPVKMIFWSGDALPDAATIKLAYDAGLMNVNGGNTALTRAFPSLTGLY
PLIRPTRGGVQYYAPIINENVYTNLWQGPYYGFRGVIDTFALTDSPRRLRGLHLYYHFYS
GTKQASIRTMHQIYAAMQAEHPLSLWMSDYIPRLEGLHRASLAKRADGSWQLRGFAAL
RTVRLDPALGWPDLGRSTGVAGVRDLPQGRYVHLSAANARLVLRDSRDPRPALEEANL
PLKHWRYRDDGRVEFAFAGHLPLRLVVRAAGDCRLSAAGKAFPGKAGNGLWTFELPM
EQVRDGQLVCR

PAO7 like PelA

MRASKKGITVSSSRKIVRPFVSFLARFAGFVLALSAGAAAGAPSSVAFWYAERPPLAELS
QFDWVVLEAAHLQPADVRYLREQGSTPFAYLSVGEFDGDAAAIADSGLVQGKSAVRNE
AWNSQVMDLAAPSWRAHLLRRAAELRKQGYAGLFLDTLDSFQLQAEERREGQRQALA
SFLAQLHRQEPGLKLFFNRGFEVLPELPGVASAVAVESIHAGWDAAAGQYREVPQEDR
DWLRGHLDKLRARGTPIIAIDYLPPERRDEARKLAARLRGEGYVPFVSTPALDYLGVSNI
EVQPRRIALLYDPREGDLTLSPGHVYLGGLLEYLGYRVDYLPTDQPLPERPLAGLYAGV
VTWMTSGPPVASDAFDNWIGARLDEKVPVAFLAGLPTENDGLLQRLGIRRLSQKLRVK
PSTETHDQALLGSFEAPLVIRIRDLPALTVLDPARVVPALRLKGDGKEYVPVATADWGG
FALAPYVLEEGSEHRRWILDPFAFLHKALRLAPLPSPDATTENGRRIATVHIDGDGFVSR
AEVPGSPYAGQQVLEDFIRPYPFLTSVSVIEGEVGPKGMYPHLARELEPIARRIFADDKVE

VASHTFSHPFFWQPQLAERGENFEAQYGYKMAIPGYDKVDFVREVIGARDYIDQRLTTP
RKPVKMIFWSGDALPDAATIKLAYDAGLMNVNGGNTALTRAFPSLTGLYPLIRPTSGGV
QYYAPIINENVYTNLWQGPYYGFRGVIDTFALTDSPRRLRGLHLYYHFYSGTKQASIRT
MHQIYTAMQAEHPLSLWMSDYIPRLEGLHRASLARRADGSWQLRGFAALRTLRLDPAL
GWPDLGRSEGVAGVRDLPQGRYVHLSAANARLVLRDSRDPRPALEEANLPLERWRYR
DDGRVEFAFAGHLPLRLVVRAAGECRLSVAGTAYPGKAGRGLWTFELPMEQVRDGQL
VCR

PAO1 like PelB

MANSSAADKHPQARLLNPWALLPVALGVALVLWLTFNSEEVFMPSGDGEPDAVSVNY
AELLLQAHPENDALRLTLIDLLVKLGDFEQARHHLARLRGKDRLATPFYEVELDILGAL
ARPEGMDEEQTRRLLERLRKIEHVSLNDAMLERLARHALALDAPDLAARTFAELAGRD
PQGRQRWLDEVARWYLASGEPLPAADIQRQLAEAQTEPAKRLAYLRQAFASLLAGERG
EQAALLLDERLDALPEDESTLAWLAEGVRAAEGSQRYDLAERFIRRWRELRPEDHEAL
AADLRLNMAAGRVERAWEVGQELLALRPEDRTLLADLARLGEWTGNGPRALGFWKQ
LLAGADDPALREHAWRLSLQMFDFDSAIELLAPIGAQRQMTDEELDALVYSHETRGTPE
EGEAWLRGYVQRYPKQRLAWQRLQQILEHTQQLQEETGVVWARMARHFPLSVKERMQ
WAETHWNLFDPRQAWKVLAGVDTRAIREPEFWRLRAALAWALEQDDDARAAYERML
ALDIRLNSSDEDQLIALYRDSNPKQALQVLIGSWQRSRDPRRLASALQLAENLHDWPAL
KSLLAEAEGLPEAQGSPYYWVARARLAEQEGHGDVAERLYREALVRFPGENLVRERLL
WFYIDRGRRDSLAPLLAQWHGLALRDSTLWLPFASASLLLERNDQALAWFRLYLKSNP
NDWLVQAAYADALDASGYQDKALRLRRLLLRRLDREAVRATPDSFAAYLRLLAVAQG
PLLAQGEARRAWNGEPAMLQLWFEQFLDQLAATNQEPLKDDWLAWARGRGLKIGRN
EEIQAALRSQNRAALQRLLERGELDPAQRVEALVRLGHGGEALGEALGALGDGHSRDN
REQLRRQAAEILERTPQGLQLGWNKRDFGGLDFKGPTLRAARHLGDDWYADLELGSGR
YHGDALDSSLLGSERNARLTLRRELADGFAAATLDGSWRDDEDRHGLGVLRNWRLSS
RDELEAGLDWHRETDETGLMRALGMRDSLRLGGRHTLSGRDQLSWSLAHNRFSTRQG
DDLGNGEALSLEWAHTLFFDGPAWQLRGGIDYQRNRLENRVPDDLLAAHGGALALDG
ARSQDLLQDRYGQVYLGSTWRRGFPGALNRSRPQYTWIVDTLAGWQWTEKEFNYGID
LGIGMELLGDDELAFTFGYQSAPQGGGGDAGGTLGVTYSTRFGR*

PAO7 like PelB

MANSSAARKQPRARLLNPWALLFIALAVVLVLWLTFNSEEVFMPSGDGEPDAVSVNYA
ELLLKAHPENDALRLTLIDLLIKLGDFERARHHLAGLRGKDRLAAPFYETELDILTALAK
PEGLDEAQTLALVERLRKIDHASLNNAMLERFARHALALDAPALAAAAFAELAGRDPR
HRQRWLDEAARWFLASAQPGRAADIYRQLAETEADPARRLDYLRQAFASLLAGERGEQ
AALLLAEHLGELPDDTRSLAWLAEGVRAAEGNQRYDLAERFVERWRTLRPGDSDAVA
ADLRLNMASGRIERAWAIGQELLALRPDERTLLADLARLGEWTGNTREALGFWMQLLA
GNDDPALREHAWRLSLQMFDFDSAIELLTPIGAQRQMSDEELDTLVYSHETRGTPEEGE
AWLRTYLERYPKQRLAWQRLQQILEHTQQLQEETEVWARMARHFPLSIKERMQWAET
HWNLFDPRQAWEVLDAADEHAIREAEFWRLRAALAWALERDDDARAAYERMLALGI
RLNSSDEDQLITLYRDSDPKKALRILIGSWQRSRDPRRLASALQLAENLHDWPTLRSLLA
EAEGVPQAQGSPYYWVARARLAEQEGRGEDAERLYRQALARFPGENLVRERLLWFYV

ERGRRDALAPLLTQWRGLARRDSTLWLPFASANLLLERNDQALAWFRLYLKSNPNDW
LAQAAYADALDASGYRDRALRLRRYLLGRIEHGTVRATPDSFAAYLRLLAVAQGPLLA
QEEARRAWNGEPAMLQLWFEQFLDQLAATNQDALKDGWLAWARERGLKIGRHEEIQA
ALRSQSRAALQRLLARGELDPAQRVEALMRLGHGGEALGEALGALGDGQSRDNREQL
RRQAAEILERTPQGLQLGWNSRDFGGLDFKGPTLRAARHLGDDWHAGLELASGRYHG
DALDSSVLGSERNARLTLQRELADGFAAAVFDGSWRDDEDRHGLGLLRNWRLSSRDEL
EAGLDWHRETDETGLMRALGMRDSLRLGGRHNLSGRDQLSWSLAHNRFSTRQGDDLG
NGEALSLEWAHTLFFDGPAWQLRSGIDYQRNRLENRLPDDLLASHGGALVLDGARSQD
LLQDRYGQLYFGSTWRRGFPGALNRSRPQYTWIVDTLAGWQWTEKEFNYGLNLGVGV
ELLGDDELAFTFGYQSAPQGGDGEAGGTLGVTYSTRFGR*

PAO1 like PelC

MQSIRCLALAAVALFMAGCSSFTSESATPLARGAQWGLVPLLNYSQAPQAGERAEQILL
SVLAEEGVRPRLYPAQPQGDLQLVDDRERQQRALDWARQQKLAYVVTGSVEEWQYK
NGLDGEPAVGVSLQVLEPASGRVLWSTSGARAGWSRESLAGAAQKVLRELVGDLRLE*

PAO7 like PelC

MQSIRCLTFVVVALFMAGCSSFTSESATPLARGAQWGLAPLLNYSQAPQAGERAEQILL
SVLAEEGVRPRLYPAQPQDDLQLVDDRERQQRALDWARQQKLAYVVTGSVEEWQYK
NGLDGEPAVGVSLQVLEPASGRVLWSTSGARAGWSRESLSGAAQKVLRELVGDLRLE*

PAO1 like PelD

MSAHKDFTLAPRASGSVSWVETLVISALALGLGWWFSPDDPLQVNATFPWVILAPLLLG
MRYGFVRGLASAALLVAALFAFRARGVEAYAQVPAAFIVGVLLCAMLVGEFRDIWERR
LERLELANEYRQLRLDEFTRAHHILRISHDRLEQRVAGNDQSLRSSLLGLRQLLRELPGD
EAPLDALAETVLALLAQYGSLRIAGLYRVRYDRTPEPQPLATLGEMPALDADDLLVRTC
LERGELVSVRQELLERGEQRAHSALQVCVPLVDTDGRILALLAVEQMPFFVFNERTFSL
LAILAGHIADLLQSDRRALQLADIDAQRFSQYLKRSLLDARDHGLPACLYAFELTDARY
GEEVQRLLEGSQRGLDVQLRLRNDEGRRVLLVLLPLTSAEGSQGYLQRLRILFAERFGQ
ARELESLGVRIRQYELDAGNDRQALGHFLFNECGLNDQQVAI*

PAO7 like PelD

MSAHKDFTLAPRASGSVSWIETVLITALALGLGWWFEPSDPLLVQASFPWVILAPLLLG
MRYGFVRGLASAALLVAALFAFRAQGLDAYAQVPAAFIVGVLLCAMLVGEFRDIWER
RLERLELANEYRQLRLDEFTRAHHILRISHDRLEQRVAGNDQSLRSSLLGLRQSLRELPD
DEEPLAALAETVLALLAQYGSLRIAGLYRVRQDRAADPRPLATLGEMPALDGDDLLVR
TCLERGELVSVRQDLLERGEQRAHSALQACVPLIDTEGRVLALLAVAQMPFFVFNDRTF
SLLAILAGHVADLLQSDPRALRLADVDAQRFSQYLKRSLLDARDHSLPACLYAFELTDE
RCGEEVQRLLESSQRGLDVQLRLRNAEGRRVLLVLLPLTSTEGSQGYLQRLRILFAERFG
QASELESLGVRIHGYELDAGNEHQALGHFLYNECGLNDQQVAI*

PAO1 like PelE

MISKWLFSGAFLFELSSWASVFADLPFGQALALYLFAHGLGSALLCVGVWLLLPRRYKF
PLPWSPLFLFSLAFFVPLIGAVGVAAAVFPALYLPRQRGEQAWQAMGVPELPFRPKEKR
LDMMFSDGGLQDVLRHAPDPNQRLTAIFATRRMPGKEAIPILKLALRDPADDVRLLAYS
MLDQKESRINQRIEAALGRLAGATPARRGALHGTLARWYWELAYLGLAQGSVLEHILE
QAREHTDQALRGAPSADLHLLAGRIALEQGRLEDAGRALQAAEEAGIDSAQLAPFRAEV
AFFQRRYRDIPRLLAGMPDDMLQRPPFAALARYWT*

PAO1 like PelE

MISKWLFSGAFLFELGSWASVFAGLPVAQALPLYLGAHGLGSLLLCGGVWLLLPRRYK
FPLPWSPLFIFSLAFFVPLIGAIGVAAAVFPALYLPRKRDEQAWQAMGVPELPFRPKEKR
LDMMFSDGGLQDVLRHAPDPNQRLTAIFATRRMPGREAIPILKLALRDPADDVRLLAYS
MLDQKESRINQRIESALGNLAGASPARRGALHGTLARWYWELAYLGLAQGSVLEHVLE
QAREHADQALRGSPSADLHLLAGRIALEQGRLDAAAEALQAAEQAGIEAAQLASFRAEI
AFFQRRYGDIPRLLAEMPEDMLQRPPFAALARYWT*

PAO1 like PelF

MTEHTAPTAPVADVCLLLEGTWPYVRGGVSSWVNQLILGLPDLTFSVFFIGGQKDAYG
KRHYPIPDNVLHIEEHFLETAWSSPNPQTRQGSSETEKALRDLHRFFHYPETPDVEEGDA
LLDLLAEGRIGREDFLHSKASWEAITAGYERYCTDPSFVNYFWTLRSMQAPVFMLAEA
ARRMPRARILHSISTGYAGLLGCILQRRWGCRYLLSEHGIYTKERKIDLAQANWIAENPD
EQLSTGLDAEVSYIRRLWIRFFERVGLLTYRAANPIVALYEGNRQRQVLDGAEPRRTRVI
PNGIDLDAWAGALERRPPGIPPVVGLVGRVVPIKDVKTFIRAMRGVVSAMPEAEGWIVG
PEEEDPDYASECRSLVASLGLQDKVKFLGFRRIGEVLPQLGLMVLTSISEAQPLVILEAW
AAGAPVVSSDVGSCRELIEGADAEDRALGRAGEVVAIADPQATSRAILALLRNPQRWQ
AAQAVGLQRVERYYTEALMLGRYRGLYREATEIA*

PAO7 like PelF

MTEHTTPMAPVADICLLLEGTWPYVRGGVSSWVNQLILGLPELTFSVFFIGGQKEAYGK
RHYAIPSNVVHIEEHFLETAWTSSDPRARRADADIAQALRDLHRFFHYPETPTGEEGGAL
LDLLAEGRIEREDFLHGKASWEVITEGYERYCTDPSFVNYFWTLRSMQSPVFMLAEAAR
RMPRARMLHSISTGYAGLLGSILQRLWGCRYLLSEHGIYTKERKIDLAQASWIAENPDE
QLSTGLDAEVSYIRRLWIRFFERVGLLTYRSADPIIALYEGNRQRQVLDGADPLRTRVIPN
GIALDAWAGALERRPPGIPPVVGLVGRVVPIKDVKTFIRAMRGVVSAMPEAEGWIVGPE
EEDPDYASECHSLVASLGLQDKVKFLGFRRIDEILPQLGLMVLTSISEAQPLVILEAWAA
GVPVVSSDVGSCRELIEGAGVEDRALGPAGEVVAIADPQATSRAILALLRNPQRWRAAQ
AVGLQRVERYYTEALMLGRYRGLYREATETA*

PAO1 like PelG

MAGIGFELRKILSRDSYTATLRAYLYAGLISSGPWVLSIVSVMLIGVLSLGVVVPDVLVR
QFLITVTYLMALSLIFTGGLQLFFTRFISDRLFERKHEAILPNLVGVLLLVTLAAGLLSAIL
LATLFDEPFAYRLLVMANFVVLCNLWLVIIFLSGMKAYKRILLVMFIGYALMVACAYLL
RFMQMDGLLLALLIGHASLLFVFLYDILREYPARRMVAFDFLDRRQVFVSLLLTGLCYN

LGIWIDKFIFWFNPSTSDLVIGPLRASILYDLPIFLAYLSIIPGMAVFLVRIETDFAEWYERV
YEAIRGGETLQHIGLLKEQMILAIRQGLLEICKVQGLAVVLLFLLAPQLLGWLGISRYYLP
LFYIDLIGVSIQVVFMALLNVFFYLDKRRIVLELCVLFVIVNGALTFVSLLLGPSFFGYGF
TLSLLVCVLVGLYRLTTALDDLEYETFMLNR*

PAO7 like PelG

MAGIGFELRKILSRDSYTATLRAYLYAGLISSGPWVLSIISVMLIGVLSLGVVVPDVLIRQ
FLITVTYLMALSLIFTGGLQLFFTRFISDRLFERKHEAILPNLVGVLLLVTVAAGLLSAVLL
GTLFDQPFAYRLLVMANFVVLCDLWLVIIFLSGMKAYKRILVVMFIGYALMVACSYLL
RFMQMDGLLLALLIGHASLLFIFLYDILREYPARRMVAFDFLDRRQVFVSLLVTGFCYNL
GIWIDKFIFWFNPATSDVVIGPLRASILYDLPIFLAYLSIIPGMAVFLVRIETDFAEWYERV
YEAIRGGETLQHIGLLKEQMILSIRQGLLEICKVQGLAVVLLFLLAPQLLAWLGISRYYLP
LFYIDLIGVSIQVVFMALLNVFFYLDKRRIVLELCVLFVIVNGALTFVSLLLGPSFFGYGF
TLSLLVCVLVGLYRLTTALDDLEYETFMLNR*

APPENDIX-III

BLASTN search result of Pel operon genes of isolate 30b (PA7 like)

30b PelA

Sequences producing significant alignments Download New Select columns Show 10

select all *10 sequences selected* GenBank Graphics Distance tree of results New MSA Viewer

Description	Query Cover	E value	Per. Ident	Accession
Pseudomonas aeruginosa strain FDAARGOS_570 chromosome, complete…	100%	0.0	100.00%	CP033835.1
Pseudomonas aeruginosa PA7, complete genome	100%	0.0	99.82%	CP000744.1
Pseudomonas aeruginosa strain CR1 chromosome, complete genome	100%	0.0	99.36%	CP020560.1
Pseudomonas aeruginosa strain AR_0356 chromosome, complete genome	100%	0.0	99.36%	CP027169.1
Pseudomonas aeruginosa strain AR441 chromosome, complete genome	100%	0.0	99.33%	CP029093.1
Pseudomonas aeruginosa strain AZPAE15042 chromosome, complete gen…	100%	0.0	99.26%	CP041354.1
Pseudomonas aeruginosa strain PA-3 chromosome, complete genome	96%	0.0	92.83%	CP033084.1
Pseudomonas aeruginosa strain LW chromosome, complete genome	96%	0.0	92.79%	CP022478.1
Pseudomonas aeruginosa PA96 genome	96%	0.0	92.79%	CP007224.1
Pseudomonas aeruginosa strain FDAARGOS_505 chromosome, complete…	95%	0.0	92.93%	CP033832.1

30b PelB

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Description	Query Cover	E value	Per. Ident	Accession
Pseudomonas aeruginosa strain FDAARGOS_570 chromosome, complete…	100%	0.0	100.00%	CP033835.1
Pseudomonas aeruginosa PA7, complete genome	100%	0.0	99.78%	CP000744.1
Pseudomonas aeruginosa strain AZPAE15042 chromosome, complete gen…	100%	0.0	99.64%	CP041354.1
Pseudomonas aeruginosa strain CR1 chromosome, complete genome	100%	0.0	99.58%	CP020560.1
Pseudomonas aeruginosa strain AR441 chromosome, complete genome	100%	0.0	99.53%	CP029093.1
Pseudomonas aeruginosa strain AR_0356 chromosome, complete genome	100%	0.0	99.53%	CP027169.1
Pseudomonas aeruginosa strain PAC1 chromosome, complete genome	100%	0.0	88.99%	CP053706.1
Pseudomonas aeruginosa strain DVT414 chromosome, complete genome	100%	0.0	88.97%	CP050331.1
Pseudomonas aeruginosa strain PAAK095 chromosome, complete genome	100%	0.0	88.97%	CP054473.1
Pseudomonas aeruginosa strain FDAARGOS_505 chromosome, complete…	100%	0.0	88.97%	CP033832.1

30b PelC

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Description	Query Cover	E value	Per. Ident	Accession
Pseudomonas aeruginosa strain FDAARGOS_570 chromosome, complete...	100%	0.0	100.00%	CP033835.1
Pseudomonas aeruginosa strain AZPAE15042 chromosome, complete gen...	100%	0.0	99.81%	CP041354.1
Pseudomonas aeruginosa PA7, complete genome	100%	0.0	99.81%	CP000744.1
Pseudomonas aeruginosa strain AR441 chromosome, complete genome	100%	0.0	99.61%	CP029093.1
Pseudomonas aeruginosa strain CR1 chromosome, complete genome	100%	0.0	99.61%	CP020560.1
Pseudomonas aeruginosa strain AR_0356 chromosome, complete genome	100%	0.0	99.61%	CP027169.1
Pseudomonas aeruginosa strain DVT423 chromosome, complete genome	100%	0.0	94.80%	CP050326.1
Pseudomonas aeruginosa strain DVT429 chromosome, complete genome	100%	0.0	94.80%	CP050323.1
Pseudomonas aeruginosa strain PAAK095 chromosome, complete genome	100%	0.0	94.80%	CP054473.1
Pseudomonas aeruginosa strain PSE6684 chromosome, complete genome	100%	0.0	94.80%	CP053917.1

30b PelD

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Description	Query Cover	E value	Per. Ident	Accession
Pseudomonas aeruginosa strain FDAARGOS_570 chromosome, complete...	100%	0.0	100.00%	CP033835.1
Pseudomonas aeruginosa strain AZPAE15042 chromosome, complete gen...	100%	0.0	99.81%	CP041354.1
Pseudomonas aeruginosa PA7, complete genome	100%	0.0	99.81%	CP000744.1
Pseudomonas aeruginosa strain AR441 chromosome, complete genome	100%	0.0	99.61%	CP029093.1
Pseudomonas aeruginosa strain CR1 chromosome, complete genome	100%	0.0	99.61%	CP020560.1
Pseudomonas aeruginosa strain AR_0356 chromosome, complete genome	100%	0.0	99.61%	CP027169.1
Pseudomonas aeruginosa strain DVT423 chromosome, complete genome	100%	0.0	94.80%	CP050326.1
Pseudomonas aeruginosa strain DVT429 chromosome, complete genome	100%	0.0	94.80%	CP050323.1
Pseudomonas aeruginosa strain PAAK095 chromosome, complete genome	100%	0.0	94.80%	CP054473.1
Pseudomonas aeruginosa strain PSE6684 chromosome, complete genome	100%	0.0	94.80%	CP053917.1

30b PelD

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Description	Query Cover	E value	Per. Ident	Accession
Pseudomonas aeruginosa strain FDAARGOS_570 chromosome, complete...	100%	0.0	100.00%	CP033835.1
Pseudomonas aeruginosa PA7, complete genome	100%	0.0	99.85%	CP000744.1
Pseudomonas aeruginosa strain AR441 chromosome, complete genome	100%	0.0	98.61%	CP029093.1
Pseudomonas aeruginosa strain AR_0356 chromosome, complete genome	100%	0.0	98.61%	CP027169.1
Pseudomonas aeruginosa strain AZPAE15042 chromosome, complete gen...	100%	0.0	98.54%	CP041354.1
Pseudomonas aeruginosa strain CR1 chromosome, complete genome	100%	0.0	98.46%	CP020560.1
Pseudomonas aeruginosa strain WPB100 chromosome	100%	0.0	87.73%	CP031877.1
Pseudomonas aeruginosa strain WPB101 chromosome	100%	0.0	87.73%	CP031876.1
Pseudomonas aeruginosa strain WPB099 chromosome	100%	0.0	87.73%	CP031878.1
Pseudomonas aeruginosa strain AR_0353 chromosome, complete genome	100%	0.0	87.73%	CP027172.1

30b PelE

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Description	Query Cover	E value	Per. Ident	Accession
Pseudomonas aeruginosa strain FDAARGOS_570 chromosome, complete…	100%	0.0	100.00%	CP033835.1
Pseudomonas aeruginosa PA7, complete genome	100%	0.0	99.80%	CP000744.1
Pseudomonas aeruginosa strain CR1 chromosome, complete genome	100%	0.0	99.70%	CP020560.1
Pseudomonas aeruginosa strain AZPAE15042 chromosome, complete gen…	100%	0.0	99.60%	CP041354.1
Pseudomonas aeruginosa strain AR441 chromosome, complete genome	100%	0.0	99.60%	CP029093.1
Pseudomonas aeruginosa strain AR_0356 chromosome, complete genome	100%	0.0	99.60%	CP027169.1
Pseudomonas aeruginosa strain PA59 chromosome, complete genome	100%	0.0	90.45%	CP024630.1
Pseudomonas aeruginosa strain PAER4_119, complete genome	100%	0.0	90.45%	CP013113.1
Pseudomonas aeruginosa YL84, complete genome	100%	0.0	90.45%	CP007147.1
Pseudomonas aeruginosa strain PAAK088 chromosome, complete genome	100%	0.0	90.35%	CP054472.1

30b PelF

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Description	Query Cover	E value	Per. Ident	Accession
Pseudomonas aeruginosa strain FDAARGOS_570 chromosome, complete…	100%	0.0	100.00%	CP033835.1
Pseudomonas aeruginosa PA7, complete genome	100%	0.0	99.93%	CP000744.1
Pseudomonas aeruginosa strain AZPAE15042 chromosome, complete gen…	100%	0.0	98.69%	CP041354.1
Pseudomonas aeruginosa strain CR1 chromosome, complete genome	100%	0.0	98.56%	CP020560.1
Pseudomonas aeruginosa strain AR_0356 chromosome, complete genome	100%	0.0	98.56%	CP027169.1
Pseudomonas aeruginosa strain AR441 chromosome, complete genome	100%	0.0	98.49%	CP029093.1
Pseudomonas aeruginosa strain Ps33 chromosome	100%	0.0	89.78%	CP044533.1
Pseudomonas aeruginosa strain AES1M chromosome, complete genome	100%	0.0	89.72%	CP037925.1
Pseudomonas aeruginosa strain AES1R chromosome, complete genome	100%	0.0	89.72%	CP037926.1
Pseudomonas aeruginosa strain H5708, complete genome	100%	0.0	89.71%	CP008859.2

30b PelG

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Description	Query Cover	E value	Per. Ident	Accession
Pseudomonas aeruginosa strain FDAARGOS_570 chromosome, complete…	100%	0.0	100.00%	CP033835.1
Pseudomonas aeruginosa PA7, complete genome	100%	0.0	99.85%	CP000744.1
Pseudomonas aeruginosa strain AZPAE15042 chromosome, complete gen…	100%	0.0	99.49%	CP041354.1
Pseudomonas aeruginosa strain CR1 chromosome, complete genome	100%	0.0	99.49%	CP020560.1
Pseudomonas aeruginosa strain AR441 chromosome, complete genome	100%	0.0	99.20%	CP029093.1
Pseudomonas aeruginosa strain AR_0356 chromosome, complete genome	100%	0.0	99.20%	CP027169.1
Pseudomonas aeruginosa strain WPB100 chromosome	100%	0.0	93.29%	CP031877.1
Pseudomonas aeruginosa strain WPB101 chromosome	100%	0.0	93.29%	CP031876.1
Pseudomonas aeruginosa strain WPB099 chromosome	100%	0.0	93.29%	CP031878.1
Pseudomonas aeruginosa strain LW chromosome, complete genome	100%	0.0	93.29%	CP022478.1

APPENDIX-IV

Unless otherwise mentioned, all media were sterilized by autoclaving at 121° C for 15 minutes at 15 lbs pressure. Distilled water was used for preparation of all media. The media used in this thesis have been given below:

1. Tryptic Soy Agar (OXOID)

Ingredients	Amount (g/L)
Pancreatic Digest of Casein	17.0
Papaic Digest of Soya Bean	3.0
Sodium Chloride	5.0
Dipotassium Hydrogen Phosphate	2.5
Glucose Monohydrate	2.5
Bacteriological Agar	1.5
pH	7.3

2. Tryptic Soy Broth (OXOID)

Ingredients	Amount (g/L)
Pancreatic Digest of Casein	17.0
Papaic Digest of Soya Bean	3.0
Sodium Chloride	5.0
Dipotassium Hydrogen Phosphate	2.5
Glucose Monohydrate	2.5
pH	7.3

APPENDIX-V

Solutions and Reagents Used

Preparations of the stock solutions used in this work are given below: (all the working solutions used in this work were prepared from the stock solutions).

Normal Saline

Normal saline was prepared by dissolving 0.85 g NaCl in 100ml of distilled water and sterilized by autoclaving, pH was adjusted to 7.8.

0.5 M EDTA

186.1 g of $Na_2EDTA.2H_2O$ and 20.0 g of NaOH pellets were added and dissolved by stirring to 800 ml distilled water on a magnetic stirrer. The pH was adjusted to 8.0 with a few drops of 10 M NaOH and final volume was made up to 1L with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

TAE buffer

242 g of tris-base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA (pH 8.0) was taken and distilled water was added to the mixture to make 1L. 1X concentrated TAE buffer was made by adding 10 ml 50X TAE buffer with 490 ml distilled water and stored at room temperature.

Ethidium bromide solution

10 µl of ethidium bromide was dissolved in 100 ml TAE buffer to make a final concentration of 20 mg/ml and stored at 4°C in the dark.

Phosphate buffer solution:

359 µl of 1M K_2HPO_4 solution and 142µl of 1M KH_2PO_4 solution was dissolved in distilled water to make the final volume of 50 ml and final concentration of 0.01 M. The p^H was adjusted to 7.2 and stored at 4°C

0.1% SDS solution

0.1g of SDS was dissolved in 100 ml of distilled water to prepare final concentration of SDS 0.1% It was the stored at room temperature.

0.1% Crystal Violate solution

0.1g crystal violate solution was dissolved in distilled water to make 0.1% (w/v) concentration and stored at room temperature.

30% Acetic Acid solution

30 ml of 100% acetic acid solution was mixed with 70 ml distilled water to make a final 30% (v/v) solution.

Sodium Hypochlorite solution (0.02% Chlorine)

8 ml standard bleach solution was mixed with 992 ml distilled to make the working sodium hypochlorite solution.