CHAPTER 1:

Introduction and Review of literature

Chapter 1

1.1 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, motile, heterotrophic, rod-shaped bacterium about 1–5 µm long and 0.5–1.0 µm wide. *P. aeruginosa* is a facultative aerobe (that grows through aerobic respiration and anaerobic respiration with nitrate as a terminal electron acceptor (Diggle and Whiteley 2020). In 1882, Carle Gessad (French pharmacist) was the first one to describe 'On the blue and green coloration of bandages in his study. He also described pyocyanin as a blue/green phenazine compound having antimicrobial as well as toxin properties. Two typed strains PAO1 (obtained from the chronic wound) and PA14 (obtained from human burn wounds) are widely used for studies. Metabolic and genomic adaptability is the key to *P. aeruginosa* colonization and survive in a diverse range of environments. *P. aeruginosa* is one of the muti-drug resistant (MDR) opportunistic pathogens, which causes acute or chronic infections in immunocompromised individuals with cystic fibrosis (CF), Chronic Obstructive Pulmonary Disease (COPD), burns sepsis and Ventilator-associated pneumonia (VAP) including those caused by COVID-19 (Cendra and Torrents 2021; Jurado-Martín et al. 2021; Rossi et al. 2021). Apart from this, *P. aeruginosa* can form biofilms that help to survive harsh conditions or hypoxic environments (Sinha et al. 2021). Additionally, *P. aeruginosa* infections are difficult to treat due to rapid mutation and adaption to develop resistance to antibiotics (Blomquist and Nix 2021). *P. aeruginosa* stands among the top pathogens responsible for Hospital-acquired infections (HAIs) as a propensity to flourish on wet surfaces of medical devices (Jangra et al. 2022). Importantly WHO has listed carbapenems-resistant *P. aeruginosa* in the priority pathogens list. Antimicrobial resistance (AMR) is an emerging problem in clinical strains.

1.2 What is biofilm?

Biofilms are the population of cells encased in the self-produced matrix. Biofilm-matrix is composed of exopolysaccharides (EPS), proteins (type 4 pili, fimbriae, flagella, etc), extracellular DNA (eDNA), and water (Greenberg et al. 2001; Whitchurch et al. 2002). The formation of biofilm, a well-regulated process having (i) bacterial adhesion through reversible mechanism (via flagella) (ii) attachment through irreversible mechanism (type 4 pili) (iii) microcolony formation (iv) mushroom-shaped colony formation and biofilm maturation (v) dispersal of biofilm. Highly synchronized process, the phenotypic transition from planktonic to a sessile mode in biofilm depends on various genetic and environmental factors that differ

between species (Toole et al. 2000; Sauer et al. 2002; Southey-Pillig et al. 2005; Monds and O'Toole 2009; Lopez et al. 2010). Numerous characteristics differ between biofilm cells and planktonic cells. While cells within the biofilm are exposed to a gradient of nutrients and waste products in contrast to planktonic cells which are exposed to a homogenous environment (Stewart and Franklin 2008). Due to biofilm subpopulations being physiologically variable, it is difficult to examine them as many experimental techniques, namely susceptibility testing and transcriptome analysis of whole biofilm instead of evaluating specific biofilm subpopulations. Also planktonic and biofilm cells do not share the same transcriptomics and proteomes, (Greenberg et al. 2001; Sauer et al. 2002; Resch et al. 2005; Southey-Pillig et al. 2005; Waite et al. 2005; Dötsch et al. 2012; Park et al. 2014). Further, biofilm cells are resistant to antibiotics than planktonic cells that are genetically identical (J. William et al. 1987; Stewart and Costerton 2001). Biofilms cause 80% of chronic microbial human infections, resulting in increasing hospitalisation rates, health-care costs, and mortality and morbidity rates. (Römling and Balsalobre 2012). Biofilm-related infections include those of the upper and lower respiratory tracts, chronic otitis media, native valve endocarditis, chronic wounds, ocular infections, periodontitis, diabetic foot ulcers, and urinary tract infections (UTIs) (Mysorekar and Hultgren 2006; Percival et al. 2012; Belibasakis et al. 2014; Bao et al. 2015; Bispo et al. 2015; Wu et al. 2015). Biofilms can grow on an abiotic surface of medical devices such as artificial cardiac valves, orthopedic prostheses, dentures, coronary stents, intravascular and urinary catheters, neurosurgical, and ventricular-assist, and ocular devices (Percival et al. 2015). In addition, biofilm is more antibiotic-resistant than planktonic cells which makes biofilm-related infections difficult to treat (Costerton et al. 1999). Understanding the biofilm formation mechanism and biofilm-associated antibiotic tolerance can give a better understanding of developing treatment strategies.

1.3 *P. aeruginosa* biofilm and pathogenesis

P. aeruginosa is known for biofilm formation, making it a model organism for studying biofilm formation (Ghafoor et al. 2011; Crespo et al. 2018). *P. aeruginosa* biofilm-matrix consists of polysaccharides (Pel, Alginate, and Psl), proteins (type 4 pili, flagella, CdrA), eDNA, lipids, rhamnolipids (Whitchurch et al. 2002; Allesen-Holm et al. 2006; Ma et al. 2006; Pamp and Tolker-Nielsen 2007). Further, the composition of *P. aeruginosa* biofilms is dependent on (i) strain, (ii) growth conditions, and (iii) age of the biofilm (Pamp and Tolker-Nielsen 2007). **Figure 1.1** depicts the *P. aeruginosa* biofilm formation and biofilm matrix. The secondary messenger cyclic di-guanosine-5′-monophosphate (c-di-GMP) is an important regulator for *P.*

aeruginosa biofilm lifecycle which is controlled by synthesizing [diguanylate cyclases (DGCs)] and degrading [phosphodiesterase's (PDEs)] activities. When c-di-GMP levels in cells are high, adhesins and other extracellular matrix components are produced, which results in the formation of biofilms. Conversely, when c-di-GMP levels are low, biofilms are dispersed, which causes bacteria to grow in a planktonic manner. Apart from this, other systems regulate biofilm such as GacA/GacS two-component (TC) system, Quorum sensing (QS), quinolone-based system (PQS), etc. A resilient biofilm formation by *P. aeruginosa* is a key weapon to compete, survive and dominate in harsh conditions. It also colonizes diverse surfaces such as medical material (implants, catheters, contact lenses).

Biofilm formation starts with the surface attachment of bacteria through type 4 pili (T4P) which is regulated by intracellular c-di-GMP levels for biofilm formation (Leighton et al. 2015; Webster et al. 2021). Furthermore, it promotes cell aggregation and twitching motility. Recent studies have shown the role of T4P in host colonization and infection through adherence to host tissue and surface (catheter). Apart from this, T4P pili can sense the various host signal

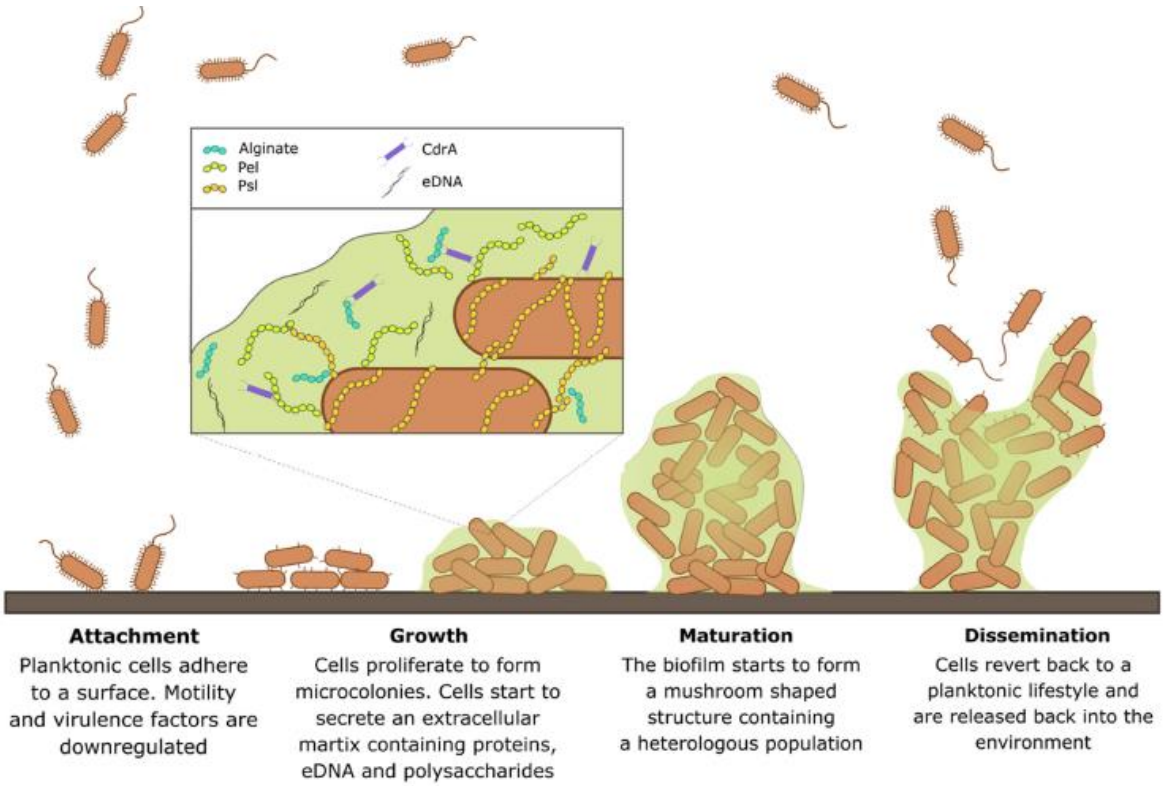

Figure 1.1: Schematic representation of P. aeruginosa biofilm formation. (1) attachment (2) Growth (3) Maturation (4) Dissemination. Permission granted from: (Maunders and Welch 2017)

such as serum albumin (vicinity of epithelium cells), and oligopeptides (present in urine) which further help the colonization of epithelial cells, CF lungs, and implant devices (Nolan et al. 2020). They also showed that T4P and Cyclic Adenosine Monophosphate (cAMP) levels were increased via ChpC one of the Chp chemosensory systems in presence of mucin, Bovine serum albumin (BSA), and oligopeptides. T4P responses to these molecules may affect biofilm formation. T4P can also respond to substrate stiffness through loss/ gain of PilA monomers during T4P extension or retraction (Koch et al. 2022). The same study also showed the surface stiffness measured by T4P activates Vfr genes relies on the Pil-Chp (2 component system). A study showed that isolates having twitching motility have strong biofilm than isolates showing swimming and swarming motility (Horna et al. 2019). The mature biofilm of *P. aeruginosa* also consists of exopolysaccharide, eDNA, pyocyanin, and rhamnolipid.

Exopolysaccharide pel has been shown to play role in establishing and maintaince of cell-cell interactions within biofilm (Colvin et al. 2011; Yang et al. 2011). While, pel exopolysaccharide is cross-linked with eDNA to increase biofilm stability (Jennings et al. 2015). Another exopolysaccharide Psl has an early role in bacterial surface adhesion and is a crucial structural element of the biofilm's extracellular matrix (Byrd et al. 2010; Zhao et al. 2013). Studies on *P. aeruginosa* typed strain (PAO1 and PA14) having Pel and Psl form robust biofilm than the deficient biofilm, in CAUTIs of the murine model (Cole et al. 2014). Another major component of biofilm is eDNA which provides initial bacterium-surface attachment (in terms of charge and hydrophobicity interactions within bacteria and the abiotic surface) and influences the 3-D structure of biofilm and stability by affecting cell-cell interaction and polymer (Whitchurch et al. 2002; Allesen-Holm et al. 2006; Yang et al. 2007; Das et al. 2010). Recent years have focused on the eDNA release mechanism which occurs in multiple ways in *P. aeruginosa* biofilm such as cell autolysis through pyocyanin, prophage induction, and quorum sensing mechanism (Sarkar 2020). The more detailed mechanism of eDNA release is shown in **Figure 1.3**. The above mention diverse mechanism of eDNA release could be one of the reasons for eDNA present in clinical isolates of *P. aeruginosa.* The urea-mediated eDNA release in *P. aeruginosa* was reported by Cole et al 2014, also biofilm formation independent of exopolysaccharide was studied through eDNA release (Cole et al. 2014). Another well-studied extracellular matrix protein CdrA is well studied and known for biofilm stability via cell-cell aggregation and interaction with pel/psl exopolysaccharide (Reichhardt et al. 2020). Apart from this ecotin (PA2755) protein levels were high in the biofilm matrix throughout the biofilm

formation and linked with Psl exopolysaccharide (Tseng et al. 2018). There is scanty information on matrix proteins CdrA and ecotin levels in clinical strains.

In human, bacterial biofilms are responsible for over 80% of persistent and recurring microbial infections (Divakar et al. 2019). Microbes have many benefits from being in a biofilm, such as protection from the host immune system and antibiotics/antimicrobials, tolerance to desiccation, absorption and storage of nutrients, water retention, high extracellular enzymatic activity, adhesion to the infection site, and cell aggregation which initiates virulence gene expression through quorum sensing (QS) (Karatan and Watnick 2009; Rumbaugh et al. 2009; Flemming and Wingender 2010). The ability of microbial biofilms to withstand a high dosage

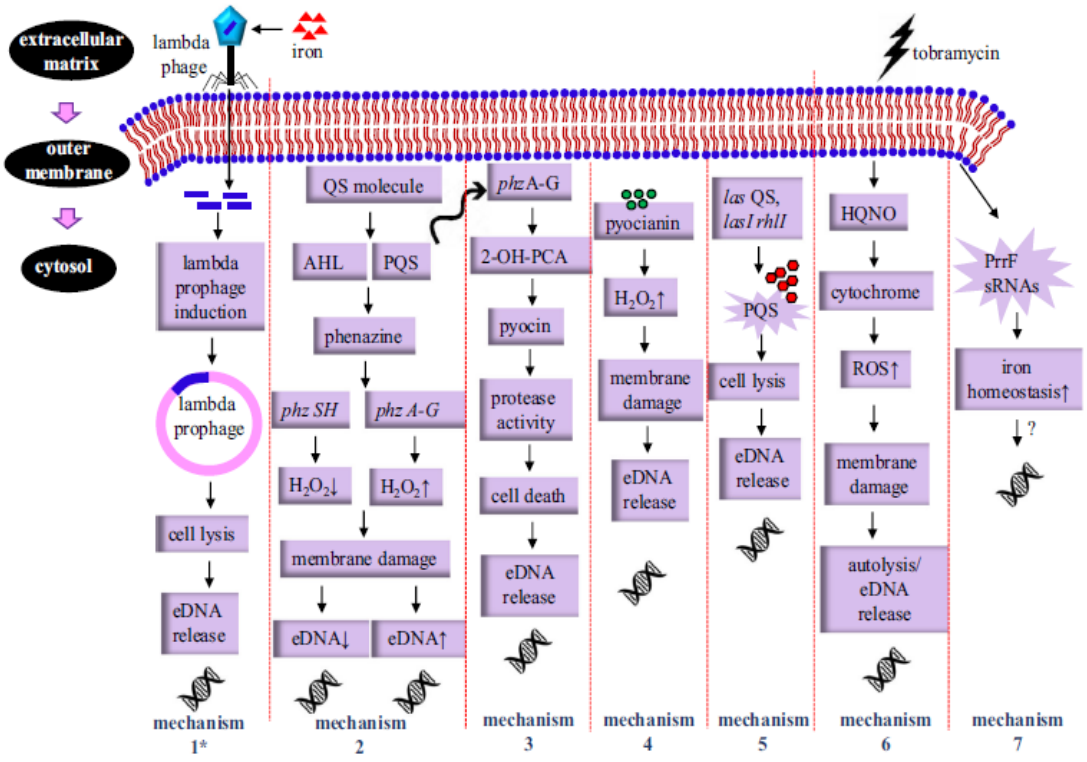

Figure 1.2: *P. aeruginosa* cell death and eDNA release mechanism in extracellular environment. (1) Lambda prophage induction (2) Quorum sensing molecules such as AHL and PQS trigger *phz* expression which leads to an increase in production of H_2O_2 and membrane damage followed by cell lysis. (3) PQS also increases *phz* expression, which in turn elevates the pyocin level and results in cell lysis (4) Pyocyanin causes H_2O_2 production which subsequently damages the membrane (5) On tobramycin exposure, PQS activation is controlled by quorum sensing genes, which causes cell lysis (6) HQNO (cytochrome inhibitor) influence ROS production, which may cause cell autolysis (7) PrrF small RNAs might regulate iron homeostasis under tobramycin treatment and facilitates cell death via QS. Permission granted from (Sarkar 2020)

of antibiotic treatment is one of the distinguishing trait (Costerton et al. 1999). *P. aeruginosa* causes infections such as cystic fibrosis (CF), pneumonia, bacteremia, and urinary tract infections (UTIs) (Shigemura et al. 2006; Gomila et al. 2018). *P. aeruginosa* is also known to form biofilm on the surface of indwelling catheters causing catheter-associated urinary tract infections (CAUTIs), where it is the third most frequent pathogen associated with hospital-acquired CAUTIs (Jarvis and Martone 1992a; Djordjevic et al. 2013). High death rates in hospitalized patients are linked to *P. aeruginosa*-caused UTIs (Lamas Ferreiro et al. 2017). Further, the majority of recent studies have focused on antibiotic resistance and biofilm formation. There are few reports related to biofilm formation by clinical isolates causing UTIs. A better understanding of biofilm formation on catheters (silicone-coated latex and silicone catheters), adhesion of bacteria to the surface, eDNA, and exopolysaccharide (Pel, Psl, and Alginate) would provide strategies to control chronic infections such as CAUTIs. A deeper understanding of biofilm formation and its composition can gain insight into developing the efficient management, prevention, and most importantly eradication of biofilm-associated infections.

1.4 Antibiotic resistance mechanism in *P. aeruginosa* biofilm

For years, *P. aeruginosa* has been used as a model organism to study biofilm formation as well as to study antibiotic tolerance, and resistance in biofilms. The primary clinical repercussion of biofilm tolerance is that antibiotics must be administered in high concentrations to treat (up to 1000 times higher for some antibiotics) (Macià et al. 2014). Schematic presentation of bacterial tolerance and resistance in bacterial biofilm is shown **Figure 1.3**. These include nutrient gradient within the biofilm and less nutrient is found in the core region, matrix exopolysaccharide, eDNA, stress responses (stringent response, oxidative stress, etc.), discrete genetic determinants (*ndvB*, *brlR*, etc) that are expressed only in biofilm stage and might reduce the biofilm susceptibility, multidrug efflux pump, intracellular communication (horizontal quorum sensing, gene transfer, etc.) and persister cells (PCs). Many reviews have recently described multiple molecular mechanisms of *P. aeruginosa* biofilm's resistance and tolerance toward many antibiotics (Hall and Mah 2017a; Moradali et al. 2017; Soares et al. 2020). Majority of the studies to date have focused on planktonic resistance to numerous antibiotics. In addition, these significant studies are done with respect to typed strains PAO1 and PA14. In recent years interest has been gained in persister cells (PCs) as one of the factors for the recurrence of biofilm-mediated infections such as CF, UTIs, etc. Persister cells are defined as

dormant variants and can revert to susceptible cells on the removal of antibiotic pressure (Lewis 2007). **They are also linked with evolution of antibiotic resistance which is even more concern (Levin-Reisman et al. 2017; Lewis and Shan 2017; Barrett et al. 2019). Since they withstand antibiotic treatment, PCs offer a genetic pool for resistance mutations and aid in the evolution of antibiotic-resistant mutants such as multidrug-resistant strains (Barrett et al. 2019; Liu et al. 2020).** Antibiotic treatment given to biofilm/planktonic cells may result in the killing of susceptible cells and expose the PCs. Consequently, antibiotic treatment might eliminate susceptible cells and at the same time upregulate the metabolic pathway for persistence (Balaban et al. 2004). Further, higher treatments of antibiotics (concentration and time) might favour the susceptible one to persister phenotype in the biofilm. Studies on *P. aeruginosa*

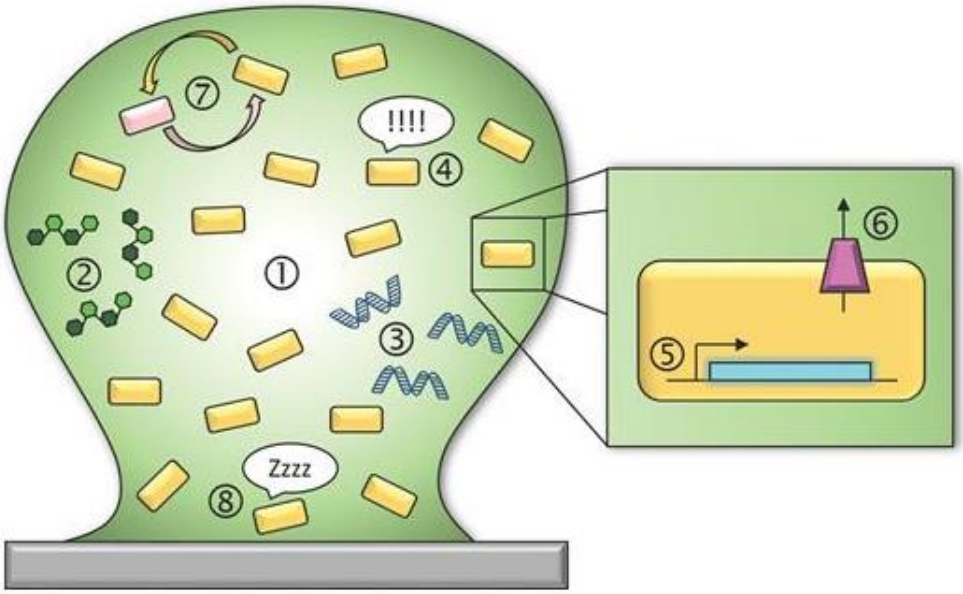

Figure 1.3: Schematic representation of bacterial biofilm resistance. Biofilm is attached to the biotic/abiotic surface (grey color). The bacterial cells (yellow color) are enchased in the biofilm matrix (green color). The multiple biofilm resistance mechanisms are sequentially numbered as follows (i) nutrient gradient (here shown as color intensity gradient) within the biofilm and less nutrient in the core region (ii) matrix exopolysaccharide (iii) eDNA (iv) stress responses (stringent response, oxidative stress, etc.) (v) discrete genetic determinants (*ndvB, brlR,* etc) that expressed only in biofilm stage and might reduce the biofilm susceptibility (vi) multidrug efflux pump (vii) intracellular communication (horizontal quorum sensing, gene transfer, etc.) (viii) persister cells. Permission granted from: (Hall and Mah 2017a)

persistence revealed that many regulatory pathways are involved such as SOS response/ stringent response, and Toxin-Antitoxin (TA) modules (Murakami et al. 2005; Viducic et al. 2006; De Groote et al. 2009).

Recent studies suggest that the diffusion barrier in biofilm is not one of the reasons for the resistant towards antibiotics, but bacteria within the biofilm display specific mechanisms to resist antibiotics (Dingemans et al. 2018). There are two proteins required for biofilm tolerance (i) hybrid SagS one of the two-component systems (TCS) (ii) Biofilm Resistance Locus Regulator (BrlR) is the c-di-GMP-responsive transcriptional regulator (Dingemans, Al-Feghali, Sondermann, et al. 2019; Park et al. 2021). This BrlR transcriptional regulator in turn activates efflux pumps (*mexAB-oprM*, *mexEF-oprM,* and ABC transporter) (Wang et al. 2018), and hence the biofilm tolerance increases.

1.4.1 Persister cell formation in *P. aeruginosa*

1.4.1.1 The Hallmark of persistence "Biphasic kill curve"

Antibiotic treatment of a bacterial population often is observed as a biphasic killing pattern (Mulcahy et al. 2010). Antibiotic treatment leads to the killing of sensitive populations and a small fraction of the population still occurs called persister cells (PCs) in the biphasic way (**Figure 1.4**). At first, majority of the population consists of sensitive cells which get rapidly killed. Remaining surviving population is enriched with PCs and the killing rate slows down. As a result, the killing curve reaches a certain plateau, where the rate at which persisters are killed either directly or by awakening and killing the ensuing normal cells. This phenomenon demonstrates the variability of phenotypic traits in bacterial cultures and the fact that not all bacteria in a clonal population are eliminated at the same rate. Consequently, time-resolved killing assays are the gold standard method for quantitative analysis of persister formation instead of point measurement (Bergh et al. 2017).

Spoering and Lewis group have shown a more number survival fraction on beta-lactam (carbenicillin) antibiotic treatment than on fluoroquinolone (ofloxacin) and aminoglycoside (tobramycin) antibiotic treatment in *P. aeruginosa* planktonic stage (Spoering and Lewis 2001). Furthermore, *P. aeruginosa* clinical isolates showed variation in response to ciprofloxacin treatment, which eradicates non-growing cells (Brooun et al. 2000; Baek et al. 2020). In the biofilm stage, beta-lactam is infective in eradicating the biofilm and only meropenem showed activity against the biofilm under *in vitro* conditions (Haagensen et al. 2017; Gómez-junyent et al. 2019). Further, aminoglycoside treatment resulted in reduction of the biofilm biomass

though still live cells remained in the interior part of biofilm (Rojo-Molinero et al. 2016). Additionally, upon ciprofloxacin treatment there was a deep decline of PAO1 biofilm and a biphasic kill curve was observed having persister cells (Soares et al. 2019). Few studies have reported PC formation in *P. aeruginosa* planktonic as well as biofilm stage at the supra-MIC level of antibiotics exposure. There is need to study PC formation under various antibiotic stress under planktonic as well as biofilm stages.

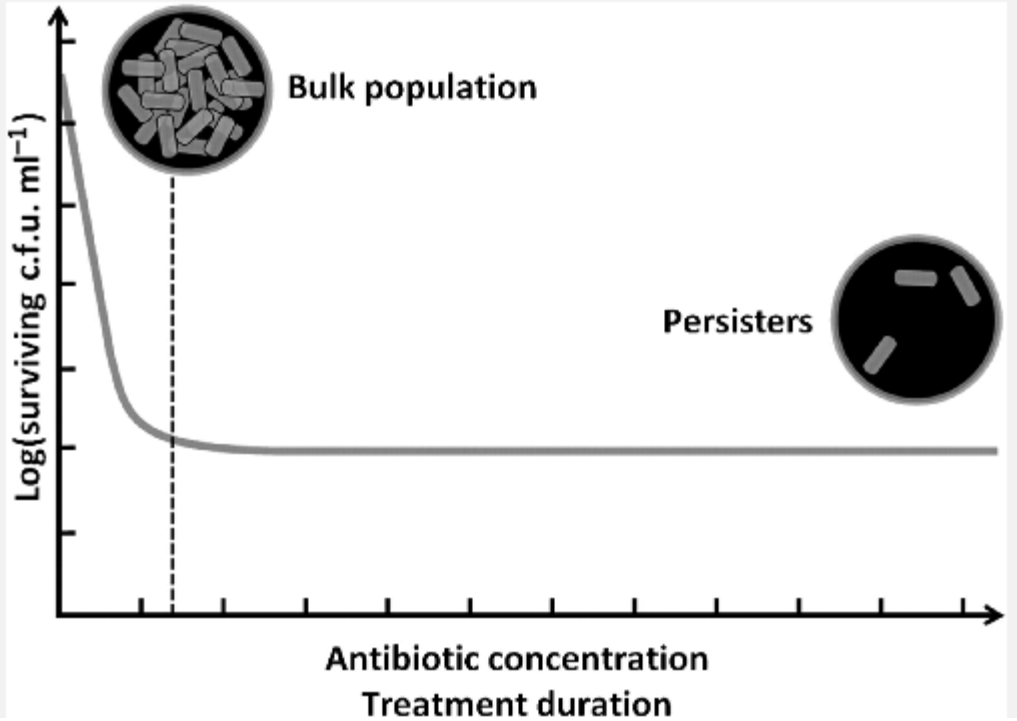

Figure 1.4: Schematic representation of biphasic killing pattern. Viable c.f.u. or Log10c.f. u/ml are plotted against the increasing concentration of antibiotics/ duration of treatment time which induces the rapid killing of the bulk population. A killing plateau is observed after the number of viable cells drops below certain threshold (shown by the dashed line) known as persister cells. Permission granted from: (Fauvart et al. 2011)

1.4.1.2 Microscopy and Flow cytometry analysis:

Many studies have highlighted PC formation study using resuscitation of PC after adding in fresh media using microscopically/ Flow cytometry (FC). Redox Sensor Green (RSG), which is a metabolic stain, is a fluorogenic redox indicator that produces green fluorescence when reduced by bacterial reductases (Orman and Brynildsen 2013). It is non-toxic and does not inhibit the cellular processes. Several studies on PCs formation have been highlighted using 2 fluorophores (i) RSG, metabolic indicator, and (ii) Fluorescent protein (fps) tagged to study cell division, protein expression, or promoter activity which has been studied in *E. coli* (Amato

et al. 2013). There is limited information available with respect to *P. aeruginosa* PC formation using RSG dye under different antibiotic conditions. Using this dye redox activity of PC formation in planktonic and biofilm stage can be known. Studies on *P. aeruginosa* PC formation using Transmission Electron Microscope (TEM) showed morphological changes in response to different antibiotic treatments. On amikacin treatment PCs were elongated and condensed DNA molecules were observed, the cell wall was not affected. Ciprofloxacin-treated PCs had outer membrane vesicles, while on cefepime-PCs had thicker cell walls (Baek et al. 2020).

However, in biofilm stage PC formation in *P. aeruginosa* has been reported using Syto 9 (stains live cells) and PI (stains dead cells) dyes. There is no information on redox activities using RSG dye in the biofilm state. It has been reported that beta-lactam (azithromycin) treatment resulted in the filamentous formation of bacterial cells. (Rojo-Molinero et al. 2016). Whereas on aminoglycosides treatment rod-shaped cells were observed and a decline in biofilm biomass was observed. Also, on ciprofloxacin treatment deep decline in biofilm biomass and rod-shaped cells was observed (Soares et al. 2019).

1.4.1.3 Role of stringent response and Toxin-Antitoxin (TA) system in P. aeruginosa.

Elements required for PC formation in *P. aeruginosa* include ppGpp (guanosine Penta phosphate or guanosine tetraphosphate) alarmone, SpoT, RelA, DksA, and TA system, and regulatory functions of these elements is well defined in typed strain PAO1/PA14 (**Figure 1.6**) (Moradali et al. 2017). In response to external stimuli, cellular levels of (p)ppGpp are regulated by enzymes that synthesize it (RelA) and break down it down (SpoT) (Bremer and Dennis 2008). Under stress conditions (nutritional or antibiotic or environmental stress) bacterial cells encounter amino-acid limitations, and ppGpp molecules are upregulated by ribosome-associated RelA. Together with the transcriptional regulator DksA (global regulator of metabolism), ppGpp interacts with RNA polymerase and inhibits the transcription of rRNA promoters. This inhibiting effect occurs concurrently with stress response gene transcription as well as activation and overexpression of amino acid production pathways (Potrykus and Cashel 2008; Dalebroux and Swanson 2012; Amato et al. 2013; Amato et al. 2014). Apart from this, PC formation is dependent on the activities of TA modules in response to antibiotic treatments, buts its activity is modulated by Lon protease. TA systems (HigBA, RelBE, ParDE, etc.) in *P. aeruginosa* are genomically/plasmid-encoded. Here toxin is the stable protein and the antitoxin might be a protein/small RNA molecule that is most unstable in adervse conditions. Toxin is inhibited by small RNA antitoxin in two different ways: (i) pairs with toxin mRNA to inhibits

translational of toxin or (ii) directly binds to the toxin. While protein toxin degrades toxin mRNA/blocks directly the protein-protein interaction of cognate toxin. When cells encounter stress such as antibiotic stress antitoxin gets impaired while toxin proteins get activated. Now, free toxin disrupt crucial cellular functiond such as synthesis of tRNA, DNA replication, and ATP which leads to cell inhibition/ cell dormancy (Christensen-Dalsgaard et al. 2010; Wen et al. 2014). To date, the well-studied TA modules in *P. aeruginosa* are ParD/ParE (Muthuramalingam et al. 2019), HicA/HicB (G. Li et al. 2016), RelE/RelB (Coskun et al. 2018), and HigB/HigA (Wood and Wood 2016) TA systems. The above-forementioned mechanism is well studied in *E. coli* and *P. aeruginosa* typed strain PAO1 as described in **Figure 1.5**. Recent studies on *P. aeruginosa* persistence in planktonic stage reported on various antibiotics: cefepime, colistin, ciprofloxacin, and amikacin that upregulated the *relA* gene expression and the PCs formation, the mechanism might differ as per antibiotics used (Baek et al. 2020). Lon protease activity is well studied in *E. coli*, however, in *P. aeruginosa,* antibiotics called aminoglycosides have been shown to increase Lon protease (Marr et al. 2007). Also, its importance has been reported for motility, virulence, and biofilm formation (Marr et al. 2007). Among all TA modules, the type 2 TA pair HigB/HigA system is well characterized in typed strain (PAO1 and PA14) in *P. aeruginosa*. As shown in **Figure 1.6**, the genetic organization of HigBA system, the HigBA pair has a genomic order that is "reversed," with the antitoxin gene high after the toxin gene *higB*. Further, HigB carries an additional *higA* promoter inside *higB* gene, which leads to an overexpression of HigA that binds to a common palindrome sequence (TTAACGTTAA) within HigBA operon (Guo et al. 2019). On antibiotic (gentamicin) stress, Lon protease is induced which in turn degrades HigA and activates the HigB toxin (Guo et al. 2019). The released HigB toxin binds the HigA antitoxin which reduces the DNA binding ability and increases the expression of HigB toxin. The HigB/HigA system influences biofilm formation, swarming motility, and virulence factors (pyochelin and pyocyanin) (Wood and Wood 2016; Zhang et al. 2018). HigB decreases the biofilm formation through decreasing the levels of intracellular c-di-GMP and increasing the expression of the type 3 secretion system (T3SS) (Zhang et al. 2018). Additionally, on ciprofloxacin exposure PC formation in *P. aeruginosa* occurs through upregulation of the HigBA system. In the planktonic stage on the sub-inhibitory concentration of ciprofloxacin, there are 1000 times more PC cells due to overexpression of *higB* gene (M. Li et al. 2016). Within PAO1 biofilm there is an early trigger (1 h) of stringent response (*relA* and *spoT*) at the supra-MIC level as well as HigBA system (Soares et al. 2019). Song et al 2020 reported the downstream regulation of HigA (pel operon,)

is more than HigB. They also found HigA regulated genes were involved in the diverse process including carbon metabolism, biofilm formation and ion uptake (Song et al. 2020).

The majority of the studies on planktonic/biofilm persistence are done with *P. aeruginosa* typed strain (PA01/PA14) and limited studies are carried out regarding the role TA modules and Lon protease at supra-MIC levels with respect to different antibiotics. There is a need to study PCs formation with respect to clinical isolates under the supra-MIC level with different antibiotic conditions in the planktonic and biofilm stage.

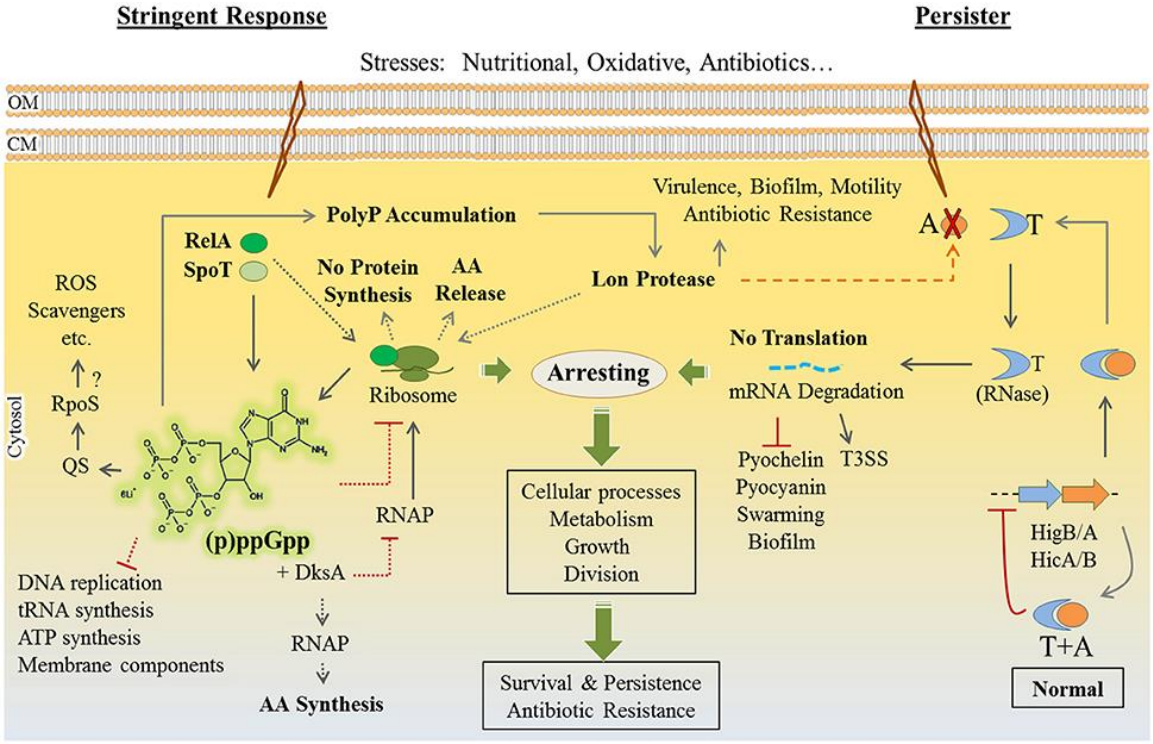

Figure 1.5: Stringent response and persister cell formation in *P. aeruginosa*. When cells encounter stress (such as amino acid/fatty acid starvation, antibiotic stress, oxidative stress, etc.) stringent response gets activated. The (p)ppGpp alarmone is upregulated by RelA/SpoT enzymes. In general, increase in (p)ppGpp level, Lon protease complex disrupts normal cell biological functions by arresting the metabolism, cell division, and cell growth for bacterial survival (the dashed line is well studied in the E. coli model, but not or partially studied in P. aeruginosa). (p)ppGpp signaling in *E. coli* is associated with TA system through the induction of Lon protease which leads to PC formation (dashed orange line). Under stress condition, toxin becomes free and normally hinder the cellular process. In *P. aeruginosa*, HigBA, HicBA, here, endoribonuclease (RNase) activity is carried out on mRNA molecules by toxin components. In the case of *P. aeruginosa*, the (p)ppGpp alarmone is associated with the production of ROS scavengers through quorum sensing (QS) or RpoS regulator. The Lon protease activity is needed for motility, virulence, biofilm formation, and antibiotic resistance. In addition, the

TA system upregulates T3SS while expression virulence factors and biofilm formation are downregulated. The (p)ppGpp signaling, TA systems, and Lon protease are present in *P. aeruginosa*, however, it is unclear how they relate to development of PC. AA, amino acid; CM, cytoplasmicmembrane; CM; cytoplasmic membrane, RNAP, RNA polymerase. Permission granted from (Moradali et al. 2017)

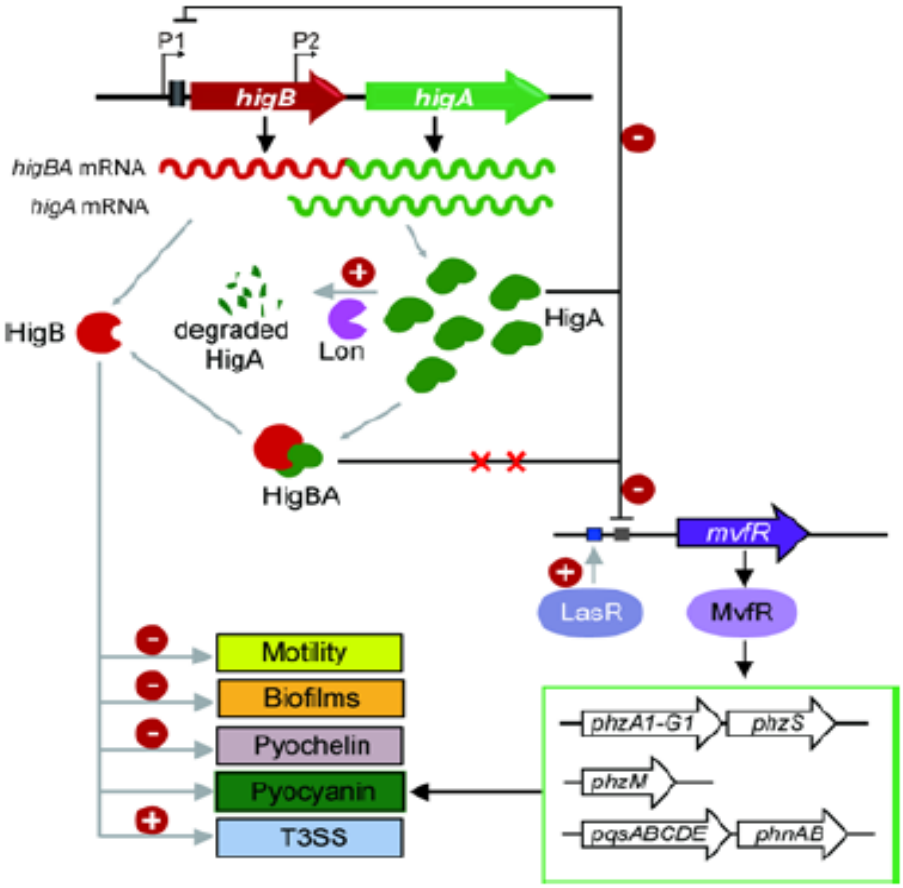

Figure 1.6: Schematic representation of *P. aeruginosa* HigBA regulation. In exponential phase, the HigA and HigB are produced at similar level, however in late stationary phase, HigA is produced at higher level. The excess HigA binds to *mvfR* promoter at the HigA palindromic sequence, inhibiting mvfR and the genes it regulates. When Lon is activated, HigA is degraded by Lon, which causes mvfR and higB to be derepressed. Recent studies have reported that HigB negatively regulates motility, pyochelin production and biofilm formation and positively regulates T3SS. While binding of HigA to HigB counteract the HigB activity, and also decrease the DNA binding of HigA to the promoter of *higB* or *mvfR*. + indicates induction and indicates repression. Permission granted from: Guo et al 2019

1.4.2 Biofilm resistance in *P. aeruginosa* biofilm:

1.4.2.1 Surface attachment and growth sensor hybrid (SagS)

Multicellular communities that are adhered to a certain surface (biotic/abiotic) and encased within a self-produced matrix are known as a biofilm. With regard to physiological changes in cells from planktonic to biofilm transition is induced by complex regulatory networks that play a role for differential synthesis of cell appendages (T4P and flagella) and/or biofilm matrix that affects the development of biofilm (O'Toole and Kolter 1998; Klausen et al. 2003; Jennings et al. 2015) however also provides the dual purpose of allowing signalling relays to detect and incorporate environmental cues. Two-component regulatory systems (TCS) allows the transduction of these environmental signals into the bacterial cells (Hoch 2000; Mitrophanov and Groisman 2008). These systems have receptor histidine kinase that perceives the signals/cues and phosphorylates cognate response regulator, which affects the cellular physiological processes by altering the subset of gene expression. (Gao et al. 2007). There are number of overlapping signaling pathways responsible for planktonic to biofilm switch. In *P. aeruginosa*, the overlapping signals are as follows Gac/Rsm TCS pathway (that precisely adjusts c-di-GMP level changes), surface attachment and motility (Ventre et al. 2006; Brencic et al. 2009; Valentini et al. 2016; Valentini and Filloux 2016). In recent years, one of the well-known crucial players in biofilm regulation is hybrid sensor regulator SagS which has been associated with regulation of c-di-GMP regulation and planktonic-specific Gac/Rsm-dependent signaling (Hsu et al. 2008; Petrova and Sauer 2011). As shown in **Figure 1.7**, SagS has a role in the transition from motile to sessile and susceptible to a resistant state in *P. aeruginosa.* SagS interact with and/or phosphorylates the GacA-dependent histidine phosphotransfer protein HptB (Hsu et al. 2008). In planktonic mode, SagS modifies the level of sRNA (one of the essential elements of Gac/Rsm pathway) in an HptB-dependent manner (Bordi et al. 2010; Petrova and Sauer 2011). Under HpT dephosphorylation conditions, the anti-anti-sigma factor HsbA is phosphorylated by HsbR kinase and phosphorylated HsbA then interacts with the HsbD diguanylate cyclase (Valentini et al. 2016). As a result, the interaction of HsbD increases the c-di-GMP levels and the amount of small rRNA RsmY (Valentini et al. 2016). While in biofilm conditions, SagS alone promotes biofilm formation and triggers biofilm-associated antimicrobial resistance (Petrova and Sauer 2011; Gupta et al. 2013). More precisely, SagS stimulate biofilm development through a phosphotransfer signaling mechanism to the BfiSR two-component regulatory system, further regulating the sRNA and RsmZ levels (Petrova and Sauer 2009; Petrova and Sauer 2010). Additionally, SagS modulate the transition

from antimicrobial susceptible to a highly tolerant state by regulating levels of c-di-GMP and activating Biofilm resistance regulator (BrlR). BrlR is a c-di-GMP responsive and independent of phosphotransfer-independent transcriptional regulator of biofilm resistance. Further, BrlR regulates the expression of ABC transporter and multidrug efflux pumps (Gupta et al. 2013; Gupta et al. 2014; Poudyal and Sauer 2018a).

The transmembrane protein called SagS contains an N-terminal HmsP domain (a histidine kinase), and a C-terminal response regulator receiver (Rec) domain (Petrova et al. 2017). It is anticipated that the SagS periplasmic HmsP domain will detect particular cues or signals and function as a regulating point in biofilm development and biofilm tolerance (Dingemans et al. 2018; Dingemans, Al-Feghali, Sondermann, et al. 2019). It is still unclear how SagS is triggered; modulation of c-di-GMP levels and two separate phenotypic switches (planktonic to biofilm mode). Also, the environmental cue(s) that may activate the SagS to regulate the phenotypic transition is not yet fully understood. Using a mouse model of chronic pneumonia, SagS has previously been shown to promote pathogenicity and the switch from acute to chronic virulence (Dingemans, Al-Feghali, Lau, et al. 2019). Recent studies have shown increased attachment as well as biofilm development by addition of glucose-6-phosphate is SagS dependent. Additionally, glucose-6-phosphate also increases c-di-GMP levels within biofilms and changes in the c-di-GMP levels are associated with diguanylate cyclase NicD and its associated with SagS (Park et al. 2021).

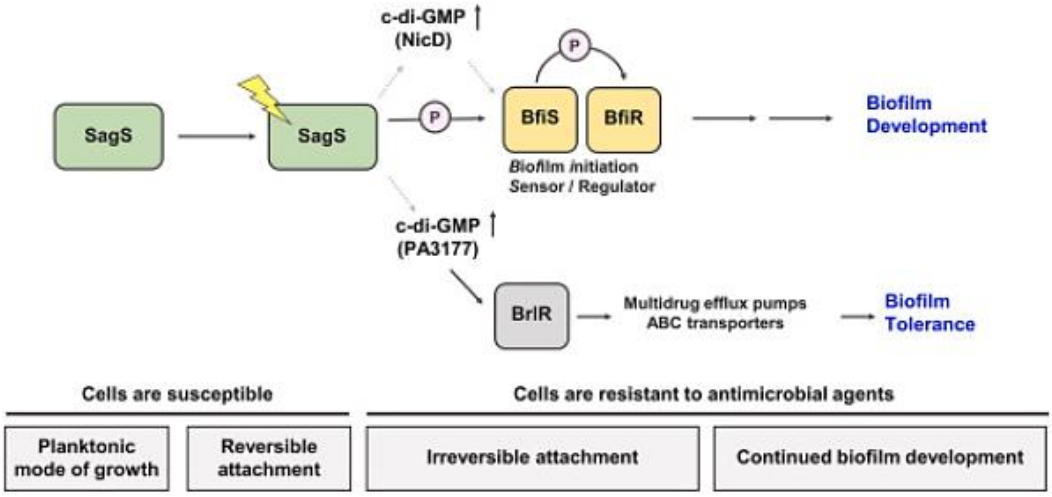

Figure 1.7: Role of SagS in *P. aeruginosa's* transition from motile to sessile and susceptible to resistance. Surface-associated cells begin to develop antimicrobial resistance during irreversible attachment stage, which is characterised by the direct interaction of SagS with and phosphorylation of the TCS BfiSR (Petrova and Sauer 2010; Petrova and Sauer 2011; Gupta et al. 2013). SagS modulates the level of c-di-GMP (Gupta et al. 2014), produced by the diguanylate cyclase PA3177 , and subsequently activating the BrlR in a phosphotransfer-independent way (Gupta et al. 2013; Chambers et al. 2014; Poudyal and Sauer 2018b). In turn, BrlR controls the expression of ABC transporter and multidrug efflux pumps (Liao et al. 2013; Poudyal and Sauer 2018a). Unidentified mechanism is indicated by dashed-line grey arrow(s). Source: (Park and Sauer 2021)

1.4.2.2 Biofilm resistance locus regulator (BrlR)

In *P. aeruginosa*, similar to MerR transcriptional activator the biofilm resistance locus regulator (BrlR) promotes biofilm specific-antibiotic tolerance (Liao and Sauer 2012). BrlR has sequence similarity to the N-terminal DNA binding domain of MerR proteins, however, th

They differ from MerR transcriptional activators in the term that expression of brlR is biofilm specific. BrlR affects drug tolerance in a independent manner of biofilm growth, colony morphology, periplasmic glucans and Psl production (Liao and Sauer 2012). BrlR activates the expression of multidrug efflux pump operons (includes *mexAB-oprM,* and *mexEF-oprN)*, numerous ABC systems including PA1874-77, and inhibits *oprH-phoPQ* operon expression (Liao and Sauer 2012; Chambers and Sauer 2013; Liao et al. 2013; Chambers et al. 2014; Poudyal and Sauer 2018a), inhibiting these efflux pump render the biofilm susceptibility to various antibiotics (colistin). Furthermore, BrlR expression is SagS dependent manner (Gupta et al. 2013). Studies have shown that ΔSagS had a decrease in BrlR production levels which

leads to impairment in the binding of *brlR* promoters and promoters of efflux pump operons which includes *mexAB-oprM* and *mexEF-oprN* (Gupta et al. 2013). In ΔSagS biofilms, restoration of c-di-GMP pool to wild type restored the level of *brlR*, and resulted in biofilm tolerance to antimicrobial agents in the cells of Δ*sagS* biofilms (Gupta et al. 2014). The same study demonstrated that decreasing the cellular cyclic-di-GMP levels (up to ≤40 pmol/mg) of biofilms cells increased biofilm susceptibility and a reduction in *brlR* expression (Gupta et al. 2014). Conversely, increasing the cellular c-di-GMP levels in the planktonic cells leads to increase resistance with an increase in the gene expression *brlR*, *mexA,* and *mexE*. In addition to *brlR* increasing the expression of the efflux pump of ABC transporter, *mexAB-oprM, and mexEF-oprN*, at the same time *brlR* also modulates the gene expression of LPS, the composition of membrane protein, metabolism, and energy generation. Also, *brlR* downregulates *oprH-phoPQ* operon expression (Liao and Sauer 2012). The fact that BrlR is involved in the control of biofilm resistance to multiple antibiotics may be explained by a large number of possible targets (Poudyal and Sauer 2018a). It is demonstrated that BrlR is both a c-di-GMP and pyocyanin-responsive regulator (Wang et al. 2018). When both of them bind to BrlR it gets activated and then it further upregulates the transcription of efflux pumps and ABC transporters as shown in **Figure 1.8**.

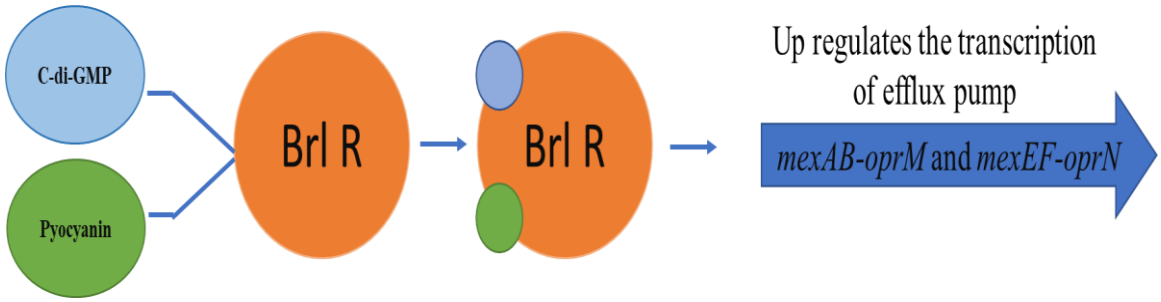

Figure 1.8: BrlR activation and its regulation

1.4.2.3 Efflux pump

P. aeruginosa genome sequences have a larger number of primary and secondary active transporters. Transcriptional activators and/or repressors regulates the overexpression of multidrug efflux transporter's in response to toxic compounds. (Sun et al., 2014). *P. aeruginosa,*

a gamma-proteobacterium, has at least 12 operons that encode Resistance Nodulation factor (RND) pumps (Kumar and Schweizer 2005; Fernández and Hancock 2012). **Figure 1.9** shows the structure of the RND system. The preliminary studies on *P. aeruginosa* efflux pump: *mexAB-oprM*, *mexEF-oprN*, *mexXY efflux*, and *mexCD-oprJ* found that they did not play a substaintial role in biofilm resistance, however, it was later observed that these efflux pump expressions are heterologous in biofilm, where nearest to substrate showed highest expression levels (De Kievit et al. 2001).

So far, the regulation of the efflux pump in the biofilm is still poorly understood. Although, Karin Sauer and co-workers' studies on the *P. aeruginosa* transcriptional regulator BrlR, which is exclusive to biofilms, suggest a potential molecular connection between the expression of the efflux pump and the biofilm phenotype. Additionally, Sauer and colleagues (Liao et al. 2013) showed that BrlR is necessary for the MexAB-OprM and MexEF-OprN efflux pumps to express their highest levels in *P. aeruginosa* biofilms. Same study also showed that BrlR directly regulates these pumps by binding to the promotor regions of these efflux pumps. Expression of *mexAB-oprM* is enhanced by C4-HSL (L-homoserine lactone) whereas mexT, a positive regulator of the *mexEF-oprN* operon, supresses it. Further, two promoter controls the expression *mexAB-oprM* efflux system, the distal promoter MexR which is the negative regulator, and the distal promoter NalD (Saito et al. 1999; Ramakrishnan Srikumar and Poole Keith 2000; Morita et al. 2006). By regulating the level of ArmR, an anti-MexR protein, production, a third repressor called NalC indirectly affects the expression of the *mexAB- oprM* operon.

Mutations in any of the repressors of MexR, NalD, and NalC could enhance the expression of the *mexAB-oprM* operon (Saito et al. 1999; Ramakrishnan Srikumar and Poole Keith 2000; Cao et al. 2004; Llanes et al. 2004; Morita et al. 2006). In *P. aeruginosa mexAB-oprM* operon is regulated intricately due to the presence of several regulatory components. Another efflux pump MexEF-OprN which is active under BrlR production is also regulated by the transcriptional activator MexT (Köhler et al. 1999; Liao et al. 2013). In contrast to the majority of other RND-efflux systems, which are negatively regulated by a repressor protein , MexT positively regulates *mexEF-oprN* production (Poole 2005). The expression of *mexEF-oprN* is, however, frequently suppressed in wild-type *P. aeruginosa* strains due to an inactive *mexT* gene (Maseda et al. 2000). Studies show strains with *nfxC* mutation have overexpression of the MexEF-OprN efflux pump which transports quinolones and chloramphenicol (Köhler et al. 1997).

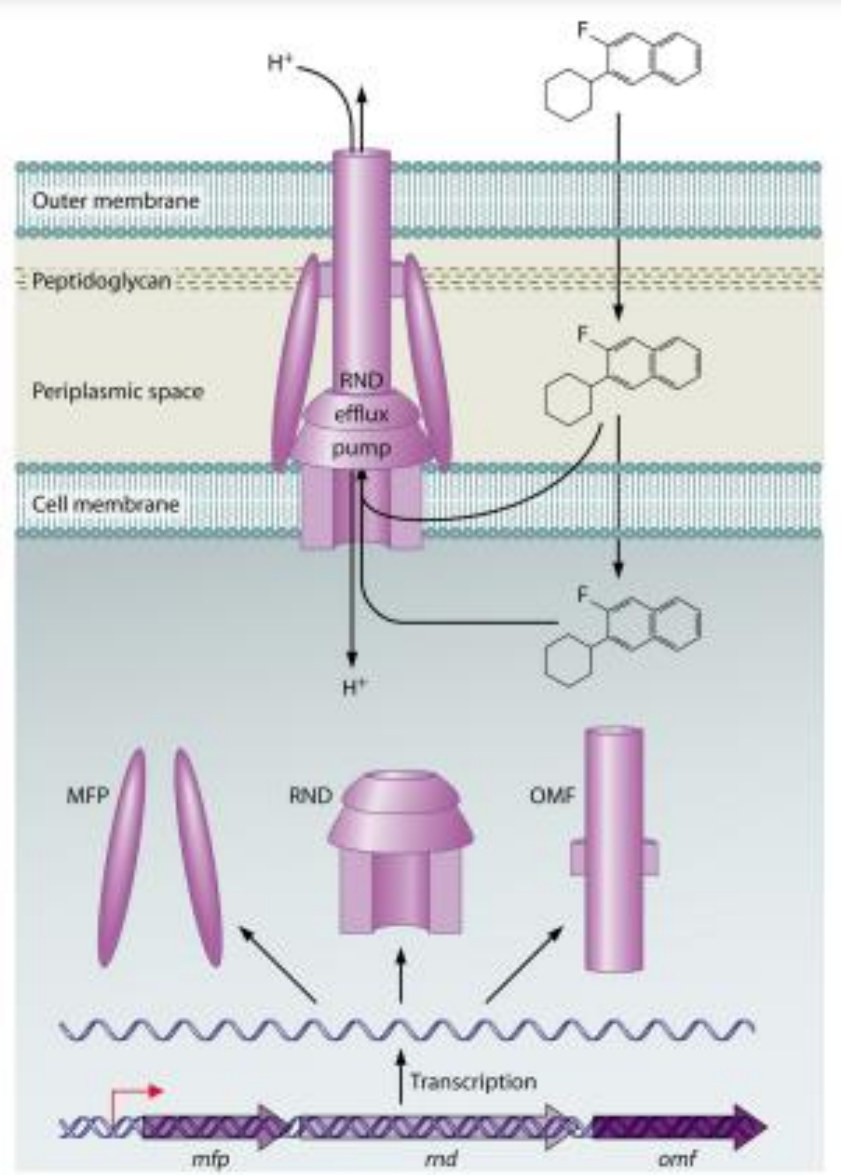

Figure 1.9 Structure and function of *P. aeruginosa* of RND efflux pump. The tripartite system makeup RND pumps having RND cytoplasmic membrane transporter (RND), the periplasmic membrane fusion protein (MFP) and an n outer membrane factor (OMF). The complex creates a channel that span the entire membrane, enabling the passage of drugs from cell cytoplasm through the cytoplasmic membrane, the periplasmic space, the peptidoglycan and the outer membrane with the help of protons. Permission granted from: (Lister et al. 2009)

However, there is scanty information regarding the expression of these efflux pumps in presence of mutation regulators in the biofilm stage. The majority of the studies come from the planktonic stage of *P. aeruginosa*. The genomic analysis and expression analysis within the biofilm stage would give a better in-depth understanding of biofilm resistance.

Understanding the processes that underlie antibiotic tolerance and resistance, particularly to biofilms would assist in the development of therapies that would interfere with these mechanisms and make biofilms more susceptible to antimicrobial therapy.