Chapter

Pseudomonas aeruginosa Secreted Biomolecules and Their Diverse Functions in Biofilm Formation and Virulence

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Abstract

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium accountable for causing life-threatening infections in humans. According to the World Health Organization, *P. aeruginosa* classified as a critical pathogen. Specifically, *P. aeruginosa* in its colonized or biofilm state presents a major infection threat to immunocompromised (HIV) patients, Cystic fibrosis, burns, wounds and surgery associated infection. It is also a common pathogen responsible for causing hospital acquired/nosocomial infection and Urinary tract infections. *P. aeruginosa* biofilm is made up of bacterial self-synthesized biomolecules includes extracellular DNA, polysaccharides, proteins, RNA, siderophores and metabolites such as pyocyanin. This chapter will elaborate the manifold functions of *P. aeruginosa* secreted biomolecules in establishing and stabilizing biofilms, triggering virulence and pathogenicity in host, and resisting antibiotics and antibacterial agents.

Keywords: *Pseudomonas aeruginosa*, pyocyanin, extracellular DNA, biofilms, alginate, rhamnolipids, pyoverdine

1. Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative bacilli bacterium which holds a greater clinical significance in relation to its infection causing ability in humans [1]. *P. aeruginosa* is commonly found in environment (soil and water) and can be a source of contamination of drinking water and food spoilage [2, 3]. Prevalence of *P. aeruginosa* and its associated infection is commonly found in cystic fibrosis patient and chronic obstructive pulmonary disease (COPD) lungs, urinary tract, immunocompromised (HIV) patients, skin and soft-tissue, diabetic leg wounds, burns and surgical site infections [1, 4]. It is also a common pathogen responsible for causing healthcare associated (nosocomial) infection and microbial keratitis (eye infection due to contamination of contact lenses) [4]. World Health Organization (WHO) has listed *P. aeruginosa* as a most critical pathogen, due to the threat of causing blood stream infection (septicemia) and its antibiotic resistance ability [5]. *P. aeruginosa* or in general many other bacterial pathogens (e.g. *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus epidermidis*,

Streptococcus pyogenes, *Proteus mirabilis*, etc.) has an inheritance ability to colonize and form biofilms on biotic (e.g. mucosa, tissue) or abiotic surface (e.g. medical implants, surgical instruments, hospital beds, wash basins, sinks, bath tub, etc.). Bacterial colonization on these surfaces directly leads to the contamination of surfaces, food and water and consequently precedes to infections in host. Bacterial biofilms are liable for approximately 80% of hospital and community-associated infections [6]. The most serious concern is antibiotic/antibacterial agents' resistance by the infecting bacteria that threatens the very core of modern medicine and impose a greater burden on global public health and economy.

2. Bacterial infection and antibiotic resistance are a global concern

National Institutes of Health (NIH), USA statistics data reports 550,000 death a year and about $94 billion total cost annually associated with biofilm infections in USA alone [7]. In Australia, billions of dollars expended annually dealing with antibiotic-resistant infections [8]. Statistics on antibiotic-resistance bacteria causing healthcare associated infections (HAI's) and death in European countries is alarming. Around, 8.9 million HAI recorded each year in combined hospitals and long-term care facilities and one in three bacteria associated with HAI's are antibiotic resistant [9]. In European population death associated with antibiotic resistance bacteria is estimated to be around 33,000 annually, this statistic is comparable to death associated with combined influenza, tuberculosis, HIV/AIDS [10]. Antibiotic resistance associated infections also triggers massive loss in productivity and healthcare incidentals estimated to be approximately 1.5 billion Euro's each year [11]. Bacterial resistance profile to different antibacterial agents is depends of several factors including geographical location of the strain (genetic mutations influenced by temperature, nutrient, oxygen), antibiotic prescribing practice to patients around the globe, poor hygiene and sanitation practice by common public and health care workers in hospitals. For instance, report released by Australian Commission on Safety and Quality in Health Care (Antimicrobial use and Resistance in Australia, AURA 2019) suggest more than 26.5 million antibiotic prescriptions were give out to patients [12]. The same report also highlighted that 23.5% of hospital prescribed antimicrobials in Australia are inappropriate and also community associated increase in antibiotic resistance bacteria (MRSA) are higher among old age people living in aged care facilities and in remote regions of the country [12]. Misuse or unethical use of antibiotics in agriculture, meat and poultry industry and fish farming, is a primary concern. Study published by Chinese Academy of Sciences reports use of 162,000 tons of antibiotics in the year 2013 alone in which more than half (52%) was used for animal husbandry and 48% by humans in addition, massive amount (50,000 tons) of antibiotics drained in the environment (water and soil) [13, 14]. USA also reported 10,000 tons of antibiotics annually used for livestock [15]. India, China, USA, Russia, Brazil, and South Africa are the world leaders in consuming antibiotics [16]. South China Morning Post (SCMP) Newspaper published an article in 2017 stating "Antibiotic overuse is a ticking time bomb for Asia" and health care workers act instantly to restrain misuse of antibiotics to stop public health calamity [17]. World Health Assembly, WHO, United Nations (UN), and countries respective government, local health organization and institutes are adopting a global action plan to crumb antibiotic resistance by educating common public, health care workers on effective sanitation, hygiene and infection prevention measures; and spreading awareness on responsible use

of antibiotics in human and animal health, investing more funding in research and development in developing novel antibacterial agents, diagnostic tools, vaccines, improving hospital facilities especially in low-and-middle income countries [18].

3. *P. aeruginosa* Antibiotic resistance profile around the globe

P. aeruginosa inherent and adaptive antibiotic resistance character thus consequently making many existing antibiotics and anti-pseudomonal agents unusable against this bacterium and present a significant challenge for medical practitioners to treat infections. In this section, we exhibited few cases based on *P. aeruginosa* antibiotic resistance profile from different parts of the world by referring to previously published literature.

A comprehensive review by Wozniak et al., (2017), that covered the Australian data from the year 1990 till 2017, on antibiotic resistance Gram-negative bacteria [19]. Their study highlighted that *P. aeruginosa* isolates from different infection site showed resistance to many commonly used antibiotics. Among the *P. aeruginosa* isolates that were collected from surgical site between years 2002–2013, approximately 0.5%, 7.7% and 0.5% of the isolates showed resistance to fluoroquinolone, third generation cephalosporin and gentamicin respectively [20]. Survey on antibiotic resistance profile of *P. aeruginosa* isolates from patient's sputum between years 2007–2010 showed resistance to aminoglycosides (43%), beta-lactam (21%) and fluoroquinolone (30%) class of antibiotics [21]. Epidemiology studies on *P. aeruginosa* isolates from blood (years: 2001–2009) showed resistance to fluoroquinolone and meropenem about 12.7% and 14.3% respectively [22]. National Healthcare Safety Network (NHSN), USA survey on antimicrobial resistance patterns for the year 2009–2010, reported about 20% of pathogens (from 69,475 HAI's incidence) are antibiotic resistance in which 2% is carbapenem-resistant *P. aeruginosa* [23]. Microbial analysis on patients affected with Nosocomial and ventilator-associated pneumonia (VAP) in a period 2011–2012 in Georgia, USA reported *P. aeruginosa* as most prevalent Gram negative (40%) and highest prevalence of multi drug resistance [24]. Similar multi-drug resistance profile of *P. aeruginosa* was recorded in Asian countries. For example, antibiogram of total 2444 Pseudomonas species isolated from different clinical specimens (blood, pus, tracheal aspirate, urine and sputum from wards, intensive care units (ICUs) and follow up patients) of trauma patients from tertiary care hospitals in India over a period 2012–2016 revealed dominance of *P. aeruginosa* (95%) [25]. Among 69%, 68%, 67% 66%, 63% and 51% were levofloxacin, gentamicin, ciprofloxacin, ceftazidime, meropenem and tobramycin resistance, respectively [25]. Antibiotic profile of 121 *P aeruginosa* strains isolated from hospitals of Makkah and Jeddah, Saudi Arabia showed high resistance to antibiotics: meropenem (~30.6%), ticarcillin (22.3%), and imipenem (19%) [26]. A study reported that in mainland china hospitals prevalence of *P. aeruginosa* related ventilator-associated pneumonia (VAP) and hospital-acquired pneumonia were 19.4 and 17.8% respectively [27]. National Healthcare Safety Network (NHSN) USA, reports prevalence of *P. aeruginosa* is common among possible VAP [28]. These isolates exhibited high level of resistance to antibiotics: Gentamicin (up to 51.1%), cefoperazone (50%), and about 22.5% for amikacin [28]. *P. aeruginosa* resistance to ciprofloxacin has also risen a global concern, especially in Asian countries for example, Bangladesh reported 75.5% resistance to ciprofloxacin whereas, India, Iran, Turkey, and Saudi Arabia reported 49%, 58%, 48.9% and 50.9% respectively [29–33].

4. Role of *P. aeruginosa* secreted biomolecules in biofilm formation and virulence

Biofilm formation is the most preferred stage of many bacterial pathogens. Biofilm formation is a multi-step process to start with i) initial attachment of bacteria to the surface (adhesion) and to each other (aggregation), ii) growth regulations and microcolony formation and production of extracellular polymeric substances (EPS) and other exogenous molecules, iii) maturation of biofilms includes structural stability and iv) dispersal of bacterial cells from the mature biofilm into the environment and reestablishment at a new site [34].

Bacteria in its biofilm state are known to withstand antibacterial agents by many ten's and 100's-fold in comparison to its sessile/planktonic state [35]. Biofilm main composition includes up to 90% bacterially self-secreted biopolymers also known as extracellular polymeric substances (EPS) and other exogenous molecules and 10% bacterial cells [36]. These molecules in combined has been termed as house of bacteria and it shelter bacterial cells from numerous challenges includes antibiotics, antiseptics, detergents, shear mechanical stress, etc. [36]. Exogenous molecules synthesized by *P. aeruginosa* is primarily structured by a complex Quorum Sensing (QS) mechanism [37, 38]. In simple terms, QS is an intracellular communication phenomenon in which bacterial species able to detect and respond to its own cell population and ecological cues by regulating genes that facilitates them in survival and colonization in both biotic and abiotic environment. In *P. aeruginosa* QS is hierarchical and its driven through four known signaling system. At the top or first stage is driven by *las* system that activates the biosynthesis of autoinducing molecules N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL). Binding of LasR-HSL molecules triggers the transcription of second QS system: *rhlR*, *rhlI*, *lasI. LasR system further regulates the third and fourth:* 2-heptyl-3-hydroxy-4-quinolone *(HHQ) and* pseudomonas quinolone signal (PQS) [39]. These four QS circuits are interconnected and depends on each other regarding *P. aeruginosa* biosynthesis of various secreted and surface attached molecules. These includes extracellular biopolymers (Extracellular DNA, polysaccharides, proteins/enzymes), biosurfactant (rhamnolipids), metabolites (phenazine/pyocyanin), iron chelator (siderophore: pyoverdine, pyochelin), and bacterium cell surface anchored flagella and pili for swarming and twitching motilities [37–40]. These biomolecules and cell appendages independently or in coordination with each other plays dominant role in *P. aeruginosa* growth, fitness, biofilm formation, virulence, pathogenicity in host during infection, antibacterial resistance, and persistence. In this chapter we emphasize only on the diverse role of *P. aeruginosa* secreted extracellular biomolecules. **Figure 1** summarizes the diverse function of *P. aeruginosa* secreted extracellular biomolecules.

4.1 Extracellular DNA production, role in *P. aeruginosa* biofilm formation and stability

The role of extracellular (eDNA) in *P. aeruginosa* biofilm was first highlighted by Whitchurch et al. (2002) [41]. Their study revealed that eDNA is predominant in *P. aeruginosa* matrix component and its essential for *P. aeruginosa* biofilm formation [41]. Followed which numerous discoveries were done highlighting several roles of eDNA in *P. aeruginosa* and in other bacterial pathogens as well as in fungi [42–46]. Structural analysis study revealed that eDNA is similar to bacterial chromosomal DNA in its primary structure and it is not surprising because when chromosomal DNA release from bacterial cells (either via membrane vesicles or cell lysis) into

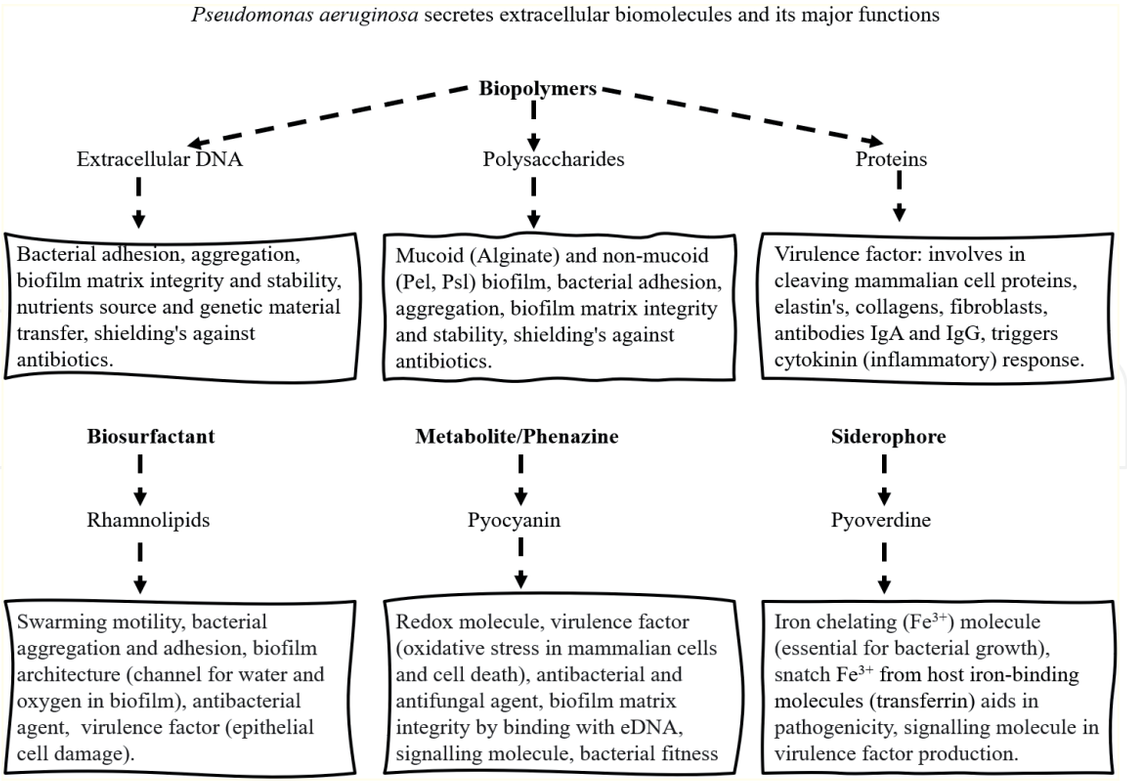

Figure 1.
Highlighting the major role of biomolecules secreted by P. aeruginosa*. These biomolecules are essential for establishment of biofilm, bacterial growth, fitness, and survival, induce virulence/pathogenicity and triggering immune response in host during infection, evading antibiotics, and other antibacterial agents.*

its immediate environment is termed as eDNA [47]. eDNA in *P. aeruginosa* cell population is released primarily through QS mechanism [48]. QS system (las and rhl -acyl homoserine lactone and pqs-*Pseudomonas* quinolone signaling), as well as flagella and type IV pili (*fliMpilA)* facilities prophage induction in *P. aeruginosa* cell population and consequently trigger cell lysis and eDNA release [48]. Virulence factor pyocyanin/phenazine biosynthesis also shown to trigger cell lysis (via oxidative stress mediated by hydrogen peroxide) and eDNA release in *P. aeruginosa* [49]. Outer membrane vesicles in *P. aeruginosa* cell also demonstrated to actively release eDNA [50].

Studies have confirmed that eDNA plays a key role in different stages of biofilm formation including initial bacterial to surface attachment (adhesion), bacteria-to-bacteria interaction (aggregation), colonization and biofilm formation by connecting cells to cells like nanowires [41–45]. Presence of eDNA on *P. aeruginosa* cell surface have shown to dictates physical surface properties of bacterial cell such as increase in cell surface hydrophobicity and consequently enables physico-chemical interactions forces such as Van der Waals interactions, Acid–Base interactions, hydrophobic interactions that aids in bacterial interactions and biofilm formation [51, 52]. eDNA have proven to induce electrostatic interactions with divalent cations like calcium (Ca^{2+}) and triggers bacterial aggregation [53]. eDNA has been established being an essential factor in structural integrity of *P. aeruginosa* biofilms and many studies have shown that cleaving of DNA using DNase I (enzyme that cleaves DNA through hydrolysis of phosphate di-ester bonds that links nucleotides in DNA) disrupts *P. aeruginosa* adhesion and biofilm formation [41, 44, 45, 54]. Other general roles of eDNA includes nutrient (e.g. good source of carbon, nitrogen, phosphorus) for starving bacteria and facilitate growth, horizontal gene transfer among bacteria cell (antibiotic resistance genes, virulence factor genes, etc), protects biofilms from shear stress by increasing biofilm viscosity. eDNA

directly bindings to cationic antibiotics thus inhibits antimicrobial agents' interaction with bacteria within biofilm, removal of eDNA from biofilms have shown increase of bacterial susceptibility to antimicrobial agents [55]. In *P. aeruginosa* biofilm, eDNA release has shown to lower the pH of the local environment and subsequently these acidification initiates antibiotic resistance phenotype genes (PhoPQ and PmrAB) that fosters alteration of lipid A and the manufacture of spermidine on the *P. aeruginosa* outer membrane and consequently decrease entry/intake of aminoglycoside antibiotics [56].

4.2 Multitude task of polysaccharides secreted by *P. aeruginosa*

Many studies have concluded that polysaccharides as a chief component of many bacterial EPS/biofilm matrix. *P. aeruginosa* biosynthesis alginate, psl, and pel as their three predominant extracellular polysaccharides. Alginate producing isolates of *P. aeruginosa* have been acknowledged as a mucoid phenotype regulates through mutation in the alginate biosynthesis of *algA-algD* operon and *mucA* [57]. *AlgD* is the key gene that promotes alginate production followed by combined action of *mucA* and *algU* genes [57]. The physical characterizes of alginate positive *P. aeruginosa* colonizes are highly viscous and gelatinous structure on the edge of the cells [58]. This feature is due to its heavy molecular weight structure of alginate which mainly composed of O-acetylated D-mannuronic acid and its C5′ epimer L-guluronic acid [59]. Alginate productions make *P. aeruginosa* virulent strain and a foremost cause for respiratory infections and mortality in CF patients [60]. Alginate production enhances bacterial adhesion due to its sticky nature and its plays key role in shielding *P. aeruginosa* from host immune defense system by scavenging reactive oxygen species (ROS) and evading neutrophils and macrophages mediated phagocytosis [61, 62]. A study by McCaslin in rat alveolar macrophages, showed that alginate in combination with lipopolysaccharide produced by *P. aeruginosa* plays a synergy role in sparking airway inflammation by impeding alveolar function in removal of apoptotic cells and debris [63]. The anionic (negative charge) feature of alginate undergoes electrostatic interactions with cationic aminoglycosides and thus constrains their dissemination into biofilms [64]. Alginate also induce structural and conformational alteration and aggregation in the antimicrobial peptides by binding with it thereby, hinders its antimicrobial activity against pseudomonas [65].

In absence of alginate biosynthesize, *Psl* or *Pel* genes in *P. aeruginosa* isolates up-regulates and activates over production of psl and pel polysaccharide [58]. These polysaccharides by itself or in combination with each other exhibit non-mucoid bacterial colonies/biofilm and these colonies are termed as rugose small colony variant (RSCV) [58]. Psl biosynthesis in *P. aeruginosa* is induced through a QS (*las*) mediated set of *Psl* genes (*PslA-PslL*) and each or group of *Psl* genes and its corresponding protein/enzyme plays a unique role in synthesizing and integrating Psl polysaccharide [58]. For instance, PslB enzyme is responsible for sugar-nucleotide precursor production, whereas, PslA/PslE/PslJ/PslK/PslL and PslF, PslH, and PslI set of enzymes deals with polymerization of polysaccharide, and integration of the activated sugar subunits into the polysaccharide repeating structure [58]. Psl is a neutrally charged polysaccharide comprised of repeating sugar groups: D-mannose, L-rhamnose, and D-glucose [66, 67]. This polysaccharide plays a crucial role in bacterial cell-to-cell communication by enhancing intracellular c-di-GMP (secondary messenger molecule) and essential for initial *P. aeruginosa* attachment to a surface as tested on various clinical, environmental and common laboratory strains, biofilm biomass and antibiotic tolerance (tested on gentamicin) [68, 69].

Pel is a positively charged polysaccharide comprised of amino sugar groups and is biosynthesized is regulated via QS (*rhl* sytem) through activating *pel* operons (*pelA-pelG)* [70, 71]. Pel composed of acetylated 1–4 glycosidic linkages of N-acetylgalactosamine and N-acetylglucosamine [71]. PelA protein is responsible for the deacetylase of the sugar amino group, whereas PelD, PelE, PelF, and PelG enzymes, these set of enzymes accountable for Pel polymerization and passage across the *P. aeruginosa* cytoplasmic membrane [58, 71]. Study also speculated that pel is adapted version of LPS [71]. Pel polysaccharide biosynthesize is a strain dependent, and studies shown that in absence of psl polysaccharides pel genes up regulated to form primary structural framework in non-mucoid *P. aeruginosa* biofilms. This indicates that pel plays important role on later stage of biofilm and not during initial adhesion, aggregation, and colonization [58]. Pel being a cationic biopolymer binds to negatively charged eDNA in *P. aeruginosa* biofilm matrix via ionic bonding/electrostatic interactions henceforth, stabilize biofilm matrix frame [72].

4.3 *P. aeruginosa* exotoxins proteins role in pathogenicity

The biosynthesize and secretion of exogenous proteins/enzymes by *P. aeruginosa* is mediated by QS (*las-rhl*) system [73]. The common proteins virulence factor *P. aeruginosa* secrets includes elastase/LAS A and B, exotoxin A, U, S, T, Y phospholipase C, alkaline protease, type IV protease, phospholipase H and lipolytic enzymes [74]. The primary function of these proteins is to play as a virulence factor and induce bacterial pathogenicity in host. To induce pathogenicity, evade host immune defense and damage epithelial cells, *P. aeruginosa* secrets these proteins predominantly via type II and type III secretion system (out of five protein secretion system) [75, 76]. Type II system constituent of protein secretons that facilitate release of exotoxin A, elastase/LasA and LasB proteases, type IV protease, and phospholipase H, as well as lipolytic enzymes into the host cells. Whereas exotoxins U, S, T, and Y are released into host cells via type III secretion system (T3SS) [76]. T3SS forms needle like membrane structure that are anchored to the bacterial cell surface and facilitates delivery of bacterial protein virulence factors into the host epithelial cells [76].

Some actions of *P. aeruginosa* virulence proteins are discussed below. For example, *P. aeruginosa* toxin A protein have shown to impair protein elongation factor in mammalian cells thereby interferes with host essential protein synthesis [77]. The T3SS proteins (Exo U, S, T, Y) have diverse functions such as hinder DNA synthesis and modulates cell morphology in host, escaping host phagocytosis by impairing host cell actin cytoskeleton polymerization and endothelial barriers, phospholipase activity (cleaving host cell lipid layer and increase cell membrane permeability), modulates host inflammatory response and consequently extending bacteria and its virulence factors into host blood stream, different organs to cause bacteraemia and septicaemia and organ failure [78–80]. Metalloproteases are another group of enzymes such as elastase whose main function is to cleave human elastin and leukocyte elastase and neutrophil elastase and consequently alters host tissue elastic property and stimulate tissue damage. Elastases also proven to degrade human collagen II and IV, impair fibroblast growth and destroy wound healing proteins which are essential for mammalian cell and tissue development and wound repair [81–84]. Other crucial role of *P. aeruginosa* elastases includes cleaves host immunoglobulins (IgA and IgG) that aids bacterium to evade host immune response [85, 86]. Clinical studies in burn and wound patients infected with *P. aeruginosa*, showed protease biosynthesis by this bacterium trigger host

cytokinin (interleukins IL6 and IL8) production and induce severe inflammation, septicaemia and elevates mortality level in patients [87–89].

4.4 Rhamnolipids *P. aeruginosa* biosurfactant

Rhamnolipids is a glycolipid biosurfactant produced by *P. aeruginosa* mediated through *rhl* QS system involving operons *rhlA*, *rhlB* for biosynthesis and *rhlI* and *rhlR* for regulation [90]. It is made up of sugar group (rhamnose) and a lipid/fatty acid group 3-(hydroxyalkanoyloxy) alkanoic acid and has a both hydrophilic and hydrophobic group like any typical biosurfactant [90, 91]. Rhamnolipids production helps *P. aeruginosa* in uptake and metabolism of hydrophobic molecules such as oils, hexadecane for nutritional source and growth [92]. Rhamnolipids (monorhamnolipids) also adhere to *P. aeruginosa* cell membrane (LPS) and plays key role in influencing *P. aeruginosa* cell surface physical property such as increasing cell surface hydrophobicity which aids in bacterial adhesion to substratum and bacterial cell-to-cell aggregation through hydrophobic interactions [93]. Rhamnolipids also lower the surface tension of *P. aeruginosa* cell surface thus aid them in swarming motility to travel across different location within the substratum [93]. It also proven to influence biofilm architecture by establishing and sustaining fluid channels in biofilms for water and oxygen transport [94]. *P. aeruginosa* employs rhamnolipids to their own advantage to eradicate competing bacteria. Binding of rhamnolipids into competing bacterial cell membrane consequently creates pores and increase cell permeability to induce cell lysis [95]. It is also a known virulence element, by binding to epithelial cell membrane it interrupts epithelial cell membrane integration, disrupts epithelial cell junctions, and triggers death in various mammalian cell types includes leukocytes, macrophages [96]. Rhamnolipids biosynthesis by *P. aeruginosa* in infected patients has been associated with escalation in pathogenicity in cystic fibrosis lung, ventilator-associated pneumonia patients [97].

4.5 Pyocyanin a unique virulence factor and its diverse function

P. aeruginosa biosynthesis and secretes a unique secondary metabolite called phenazines. Different types of phenazines are produced by *P. aeruginosa* however, pyocyanin is the most predominant one. Pyocyanin biosynthesis occurs at the later stage in *P. aeruginosa* population density or in biofilm, in laboratory culture it is generally expressed at the late exponential stage via regulation through QS (PQS) system [98]. Pyocyanin production is easily identified by its color, bluish -pure pyocyanin and green color when grown in laboratory in bacterial growth media (e.g. Tryptone Soy broth, Nutrient media, Luria broth, these media are all yellow in color and blue pyocyanin mix with yellow turns green). The two set genes of *phzA1-G1 and phzA2-G2* encrypts initial phenazine molecule (phenazine-1-carboxylic acid, PCA) followed by conversion of PCA to pyocyanin (N-methyl-1-hydroxyphenazine) encoded by genes *phzM* and *phzS* [98]. Pyocyanin production has been associated with the severity of infection and acknowledged as a hyper virulent strain [99]. Analysis of pyocyanin production on variety of clinical and environmental isolates indicates pyocyanin production is very common among all isolates however, the amount of pyocyanin production is depended upon strain phenotype and genotype variations. A study by Fothergill et al. (2007) on strains isolated from different clinical sites (CF, keratitis) and environmental (water) strains indicated that Liverpool epidermic strain (LES) from CF patients (attended Liverpool CF centre in England between years 1995 to 2004) exhibited significantly high pyocyanin production in comparison to keratitis and water isolates [99]. Pyocyanin plays diverse role in establishment of *P. aeruginosa* biofilm formation

including inducing oxidative stress in competing bacteria and outcompete their growth (e.g. *S. aureus*) and fungi (e.g. *candida albicans*) [100, 101]. Pyocyanin promote cell signaling by activating transcription factor SoxR and stimulating various genes expression includes efflux pump genes *mexGHI-opmD*, and *PA2274* (monooxygenase, to control oxidative stress response in *P. aeruginosa*) [102]. By regulating target genes pyocyanin also maintain bacterial fitness, pyocyanin/phenazine deficient mutant (Δ*phz*) showed drastic change in its colony morphology (wrinkled colony), whereas pyocyanin over producing mutant strain (DKN370) remained smooth [103]. Pyocyanin induce oxidative stress and cell death (via H_2O_2 production) in *P. aeruginosa* population in late exponential phase and triggers eDNA production [49]. An interesting discovery by Das et al. 2012 and 2015 revealed that pyocyanin intercalates with DNA and influence *P. aeruginosa* cell surface hydrophobicity and subsequently promote biofilm formation [51, 104].

Pyocyanin has been in limelight in many decades due to its virulence property. In context to *P. aeruginosa* infection in human, pyocyanin production has been linked to increase in virulence and severity of infection [99]. Different studies reported different concentration of pyocyanin to be found in sputum of CF patients from 0.9 to 16.5 μg/ml and 27.3 μg/ml in bronchitis patients sputum and also significantly higher amount (5.3 μg/g) also found in burn wound exudates [105, 106]. In mammalian cells, it declines intracellular cAMP and ATP levels, provoke neutrophils apoptosis, and modulates host immune system [105–108]. Pyocyanin being a zwitter ion (positive and negative charge group and can penetrate into host cell membrane), and redox (electron donating and accepting property) molecule it oxidized cytosol (mammalian intracellular fluid), produces reactive oxygen species (ROS) by diffusing into host cells and undergoes redox reaction to accept electrons from NADPH and donates to molecular oxygen [109, 110]. ROS production leads to the decline in intracellular glutathione (a master antioxidant in mammalian cells essential for cell health and fitness) level which leads to bronchial epithelial cell death and tissue damage [109, 110]. It also impedes chlorine ion (Cl^-) secretion and transport in CF patients' lungs (bronchial epithelial cells) and consequently halt mucous clearance in human airways [111]. In burn wound patients infected with *P. aeruginosa*, pyocyanin production shown to provoke premature senescence and apprehend human fibroblast growth by levying oxidative stress [106, 112]. Mouse model study revealed that exposing pyocyanin to mouse lung airways triggers repress of transcription factors protein FoxA2 expression (essential for tissue development) and consequently leads to over production of host cells (cell hyperplasia) and mucous hypersecretion by [113].

4.6 Siderophore benefits *P. aeruginosa* growth and biofilm formation

Siderophore are small molecules and belongs to the class of "iron-chelating compounds". They are intrinsically secreted by microorganisms primarily for scavenging and uptake of Ferric ion, Fe^{3+} for their own benefits including nutrition, metabolism, growth, and virulence in mammals [114]. For example, Bacillus spp. *(subtilis and anthracis*) biosynthesis primary siderophore (bacillibactin), enterobactin, vibriobactin, yersinibactin, and pyoverdine by *E.coli*, *Vibrio cholerae*, *Yersinia pestis* and *P. aeruginosa* respectively. Pyoverdine is a fluorescent green color compound and its biosynthesis is encoded by the operons of *pvd*. Pyoverdine forages Fe^{3+} from host iron-binding molecules (transferrin) and binds strongly to it thus contribute to pathogenicity in host as shown in the immunocompromised mouse model [115, 116]. Pyoverdine also benefits from *P. aeruginosa* virulence factor protease action in degrading human iron-binding protein (ferritin), thus outcompetes host and scavenges iron [117]. Burn mouse model study have shown that pyoverdine

contribute to severity in infection and mutants deficient in pyoverdine production showed significantly less virulence [116]. Infection model study in *Caenorhabditis elegans*, showed that pyoverdine penetrates host cells and undermines mitochondrial dynamics and triggers hypoxic response thus hinders ATP generation in host [118]. Other features of pyoverdine including communicating molecule to control biosynthesis of virulence proteins in *P. aeruginosa* including exotoxin A and protease [119]. Iron is essential to sustain bacterial growth thus pyoverdine aids in survival of *P. aeruginosa* in infection site, triggers biofilm formation where, pyoverdine deficient mutant strains forms fragile biofilm [120]. *P. aeruginosa* also produces another siderophore molecule called pyochelin, however pyochelin has lower affinity for Fe^{3+} than pyoverdine. However, this pyochelin-iron complex in coordination with pyocyanin undergoes oxidative-reductive reaction and contribute to oxidative damage (via hydroxyl radical formation) and inflammation in host [121, 122]. In CF patients pyochelin found to be involved in inflammation and tissue damage [123].

5. Conclusion

P. aeruginosa ability to easily colonize in host, biofilm formation, synthesis and secretion of virulence factors and causing pathogenicity, evading host immune defense system, and antimicrobial resistance made it a critical pathogen and needs an immediate attention. Secretion of extracellular molecules by *P. aeruginosa* plays a principal role in fitness of bacterial population, establishment of biofilms, infections, and pathogenicity in host. To reduce and eradicate *P. aeruginosa* associated infections development of novel antibiotics or antimicrobial agents, QS inhibiting molecules, virulence factor neutralizing agents, biofilm disrupting enzymes or/and combination treatment strategy with existing antimicrobial agents are of top priority. Further to prevent antimicrobial resistance in bacteria, necessary steps need to be taken by government organization, hospitals/clinics, health care workers and scientist from research institutes to educate children and students in schools, colleges and universities, people from rural places and developing countries about proper hygiene and use and misuse of antibiotics. Also, proper management of antibiotics uses in the agriculture and meat industry need to be implemented. This small steps at every level will help in minimize the spread of antimicrobial resistance in bacteria and will help to cut down catastrophe in both health and economic division and promotes better treatment outcome against infectious diseases.

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