Chapter 6

Structure-Function Relationships of Rhamnolipid and Exopolysacharide Biosurfactants of *Pseudomonas aeruginosa* as Therapeutic Targets in Cystic Fibrosis Lung Infections

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Abstract

Chronic *Pseudomonas aeruginosa* lung infection is the cause of much morbidity and most of the mortality in cystic fibrosis (CF) patients. The high prevalence of *P. aeruginosa* infections in CF is related to the microbe's large genome and mechanisms of adaptation to the CF lung environment, the host immune system and antibiotic resistance. Among a wide range of *P. aeruginosa* metabolites involved in infection development in CF, the biosurfactant compounds, rhamnolipids (RLs) and exopolysaccharides (EPSs), have important roles in the early stages of *P. aeruginosa* infection in CF. RLs and EPSs are involved in bacterial adhesion, biofilm formation, antibiotic resistance, and impairment of host immune system pathways, as well as in processes such as biofilm maintenance and the mucoid phenotype of *P. aeruginosa*, which lead to development of chronic infection. Due to the proposed roles of RLs and EPSs and the importance of prevention and treatment of *P. aeruginosa* respiratory infections in CF, these compounds are promising targets for patient therapy. In the future, impairment of *P. aeruginosa* quorum sensing (QS) pathways and modification of host respiratory mucus epithelial membranes should be considered as potential approaches in preventing respiratory infections caused by this microbe in CF patients.

Keywords: cystic fibrosis, *Pseudomonas aeruginosa*, biosurfactant, rhamnolipid, exopolysaccharide, biofilm

1. Introduction

Cystic fibrosis is a congenital, recessively inherited disorder. The genetic background of CF development is >1500 mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) on chromosome 7, which lead to malfunction of the chloride channel in CF patients. CF affects a large number of organs and tissues (airways, pancreas, the small intestine, liver, the reproductive tract and sweat glands), resulting in numerous clinical symptoms (viscid mucus, respiratory infections, intestinal malabsorption of fat, diabetes mellitus, meconium ileus, impaired liver function, male infertility and salt loss) [1].

The malfunction of the chloride channel in CF patients leads to impairment of the non-inflammatory defense mechanism of the lower respiratory tract. Therefore, CF patients, from early childhood, suffer recurrent and chronic respiratory tract infections caused by *P. aeruginosa*, *Burkholderia cepaci*, *Achromobacter xylosoxidans*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, nontuberculous *Mycobacteria* and *Aspergillus fumigatus*. In spite of the host inflammatory response and intensive antibiotic therapy, infections persist and lead to respiratory failure requiring lung transplantation or death [1].

Chronic *P. aeruginosa* lung infection is the cause of much of the morbidity and most of the mortality in CF patients. Chronic infection is considered as growth of *P. aeruginosa* from multiple respiratory cultures over a 6-month period [2]. About 80% of adults with CF have chronic *P. aeruginosa* infection [3]. *P. aeruginosa* is able to survive and persist for several decades in the respiratory tract of CF patients, in spite of the defense mechanisms of the host and intensive antibiotic therapy. However, the microbe has adaptive mechanisms, which explain why it can survive in the hostile CF lung for so long. These include phenotype splitting due to mutations (into nonmucoid or mucoid), their different distributions in respiratory and conductive zones in the lungs and switching to a biofilm mode of growth—mucoid phenotype [4–7].

Recent research indicates that chronic *P. aeruginosa* infections are caused by the ability of bacteria to organize themselves into microcolonies regarding to formation of biofilms. In this state, the bacteria are incorporated in a self-produced protective matrix, often with surrounding inflammatory cells, which is very well protected against antibiotics and the host defense [4]. The biosurfactant compounds (RLs and EPSs), due to their structures and physicochemical properties, as well as their interactions and correlation with other metabolites, significantly contribute to colonization, motility and biofilm formation [8–10]. Additionally, the mucoid colony morphology of *P. aeruginosa* is highly correlated with overproduction of alginate (a type of EPS) [8]. Therefore, it is important to consider these biosurfactants and their biosynthetic pathways as possible targets and approaches for CF therapy in order to impair *P. aeruginosa* mechanisms of pathogenicity. Furthermore, cell-to-cell communication and QS signaling pathways together with their genetic aspects, closely related to RL and EPS biosynthesis, are the most significant targets for new therapy approaches in CF treatment [10–13].

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2. *P. aeruginosa* infection in CF

2.1. *P. aeruginosa*

Pseudomonas is ubiquitously present worldwide, being an extremely diverse bacterial genus. Pseudomonads are frequently closely associated with animals and plants; they are common and numerous in a wide range of environmental habitats. Their ability to adapt genetically, producing varying physiological advantages as a response to their pervasiveness, is the subject of much scientific speculation and study. *P. aeruginosa*, as all species that belong to the genus *Pseudomonas*, due to its metabolic diversity, has potential for adaptation, survival and growth in a wide range of environmental conditions [14, 15].

P. aeruginosa produces an arsenal of secondary metabolites, including EPSs, RLs, enzymes (elastase, alkalne protease, exoenzyme S, phospholipase C and hemolysins), pigments and toxins (exotoxin A), using these virulence factors for infecting and colonizing a wide range of hosts (animals, plats, insect and nematodes) and surfaces [12–24]. The major biosurfactant compounds produced by *P. aeruginosa*, RLs and EPSs, are involved in bacterial adherence, biofilm formation and maintenance, which all are necessary for respiratory infection establishment, development and progression in CF patients [4, 8, 12, 13, 16].

2.2. Pathogenesis of *P. aeruginosa* infection in CF

Despite constant exposure to a wide range of microorganisms, CF patients are predisposed to infection by only specific groups of microorganisms [8]. The proximal event in development of CF is mutation of the CFTR gene (see Introduction), but still, it remains unclear how this primary step causes particular infections in CF patients. However, numerous proposed mechanisms are related to CFTR gene mutation, defective CFTR channels and infection development [8]: (1) reduced ion transport; (2) modified salt content in the airway surface liquid; (3) increased levels of acylated glycolipids on the surface of CF airway epithelial cells; (4) defective CFTR exposed on airway epithelial cell membranes become receptors; (5) low levels of antimicrobial compounds (inducible nitric oxide synthase and nitric oxide); and (6) intrinsic hyperinflammation of airways (**Table 1**) [25–36].

The first step in infection of CF airways by *P. aeruginosa* is microbe acquisition [8]. Due to the abundance of *P. aeruginosa* in many natural environments, most individuals acquire *P. aeruginosa* through casual contact with natural bacterial sources, while some individuals acquire *P. aeruginosa* directly or indirectly from other CF patients. Transmission data and genotype/phenotype properties of clinical and environmental *P. aeruginosa* isolates indicate that characterizing the ecology of *P. aeruginosa* originating from natural environments would lead to a better understanding CF epidemiology [8].

Initially, infection of *P. aeruginosa* in CF is usually the result of an alternating series of acquisitions of different isolates and in the first stage of infection, most of the isolates are nonmucoid and highly antibiotic sensitive [8, 37–39]. Eventually, one or two isolates establish themselves and, due to their genetic, phenotypic and physiological changes, develop chronic infection [13, 16, 40].

Mechanism	Effect
Decreased ion transport, which results from defective CFTR channels enhances fluid absorption in the airways	Lowered airways surface liquid and impaired ciliary transport of the mucous layer, which results in defects in microbial clearance
Altered salt content in the airway surface liquid	Inactivation of immune system defenses pathways; defected neutrophils activity
Increased levels of acylated glycolipids on the surface of CF airway epithelial cells due to defective CFTR molecules	Modified glycolipids are receptors for *P. aeruginosa* adherence
Binding of *P. aeruginosa* to defective CFTR molecule exposed on airway epithelial cells membranes	Internalisation of *P. aeruginosa*
Lowered level of antimicrobial compounds	Propensity of individuals to lung infection
Istrinical hyperinflammation of airways	Damage of host cells and disruption of effective microbe clearance

Table 1. Proposed mechanisms of *P. aeruginosa* in development of respiratory infection in CF airways.

2.2.1. P. aeruginosa quorum sensing systems and biofilm

One of the most important factors which facilitate *P. aeruginosa* to colonize and persist in acute and chronic lung infection in CF patients is the ability of this microbe to grow as a biofilm, assembly of which is regulated by a QS system [13, 30, 40].

QS is the mechanism by which bacteria engage in cell-to-cell communication using diffusible molecules based on a critical cell density [41]. QS molecules manage and regulate diverse physiological processes, some of which are interrelated. In *P. aeruginosa*, expression, production and/or secretion of many virulence factors, such as EPSs, RLs, enzymes, pigments production, biofilm formation and antibiotic resistance, are controlled by QS [10, 13, 42]. *P. aeruginosa* possesses two interrelated QS systems, the *las* and *rhl* systems. The *las* system comprises the transcriptional regulatory protein, LasR and its cognate autoinducer, N-(3-oxododecanoyl) homoserine lactone (3O-C_{12}-HSL). The *rhl* system comprises the RhlR transcription regulator protein (also known as R-protein) and N-butyryl homoserine lactone (C_4-HSL), its cognate autoinducer [13]. Additionally, these two systems are not independent but are interlinked in a hierarchical manner (the *las* system directs the *rhl* system). They are linked by a third signal molecule, 2-heptyl-3-hydroxy-4-quinolone, known as the *Pseudomonas* quinolone signal (PQS). PQS is produced under the control of the *pqs* system, which is considered as the third distinct QS system [11, 42]. Interestingly, transcriptome analyses have revealed that between 6 and 10% of the *P. aeruginosa* genome is regulated by the *las* and/or *rhl* systems [13].

Biofilms are matrix-enclosed microbial accretions that adhere to biological or nonbiological surfaces [43]. *P. aeruginosa* biofilms are related to development of different acute and chronic infections, not only in CF patients [16, 44, 45]. Formation of *P. aeruginosa* biofilm occurs in stages: bacterial attachment and irreversible adhesion to surface, microcolony development, biofilm maturation and dispersion of bacterial cells from the biofilm [46]. Heterogeneous microenvironments due to oxygen and nutrient diffusion limitations occur in biofilms and

they lead to physiological and phenotype diversity [47, 48]. Suggested mechanisms of *P. aeruginosa* biofilm formation involve QS signaling, which coordinates and protects biofilm assembly and maintenance [44, 49–52]. The *las I*, which encodes the biosynthetic pathway for 3O-C_{12}-HSL, is critical for biofilm maturation [50]. Heterogeneity of the bacterial population in biofilm is an important characteristic of *P. aeruginosa* pathogenicity and contributes to the microbe's resistance to antimicrobial therapy. In laboratory conditions, *P. aeruginosa* can form two types of biofilm, "flat" and "structured", and alginate-producing isolates (the mucoid phenotype) form complex structured type of biofilm which is resistant to tobramycin [53]. Additionally, the QS system is involved in regulation of several genes such as *rhlA*, *rpoS*, *sad A* and genes in the denitrification pathways. These genes are important for all stages of biofilm development, maintenance, or dispersion: (1) biosynthesis of the biofilm matrix (EPSs, extracellular DNA); (2) biosynthesis of RLs; and (3) other metabolic pathways (not discussed here) [13, 42].

2.2.2. Adaptation mechanisms of P. aeruginosa in CF lungs

The CF lungs are an unfriendly and varied environment for invading bacteria due to the presence of stressors such as osmotic stress of viscous mucus, oxidative and nitrosative stresses of the host responses, sublethal concentrations of antibiotics and other microbes presence. Regarding to the environment of CF lungs, it is a great challenge of *P. aeruginosa* populations to overcome these stressors and persist [54].

It is believed that mechanisms that allow *P. aeruginosa* to cause persistent chronic infection are related to its remarkable potential for adaptation to environmental changes [8, 15]. *P. aeruginosa* adaptations in CF patients' lungs are dynamic and generate subpopulations of bacteria with differing phenotypes [8]. It is thought that primary infection is related to the large *P. aeruginosa* genome, while development of persistent infection is dependent on spontaneous mutations [55, 56]. Mutations are multiple due to different factors such as the presence of hypermutable strains, development of biofilm and downregulation of antioxidant enzymes [57–59]. Environmental conditions in CF airways then further favor specific *P. aeruginosa* phenotypes. This set of adaptations finally leads to development of the subpopulations of bacteria (mentioned above) within the same respiratory tract, which are relatively similar, but which carry unique groups of genes [56, 60, 61]. Some commonly and intensively studied *P. aeruginosa* adaptation mechanisms present during respiratory infections in CF involve: transition to mucoid phenotype, antibiotic resistance, alterations in lipopolysacharride (LPS), loss of type III secretion and motility, auxotrophy, small-colony variants, defects in the QS system and hypermutability [8, 54].

3. Biosurfactants of *P. aeruginosa*—rhamnolipids and exopolysaccharides

Biosurfactants are a group of amphiphilic compounds, comprise a hydrophobic and a hydrophilic moiety and are produced by a range of microorganisms [9, 62]. *Pseudomonas* spp. are the most common producers of biosurfactants [63], with *P. aeruginosa* being the preeminent RL and EPS biosurfactant-producing species [9, 63]. Up to date, a variety of biosurfactants

have been studied, but RLs (glycolipid biosurfactants) and EPSs (polymeric biosurfactants) are currently attracting the most attention, as they are relevant in medicine, environmental protection, food and the pharmaceutical industry [15, 24, 64–66].

3.1. *P. aeruginosa* rhamnolipids

Rhamnolipids comprise one or two L-rhamnose units and one or two units of 3-hydroxy fatty acid. Variations in lipid components contribute to the biodiversity of RLs [9, 67]. Due to their chemical composition, RLs are classified into four homologue groups (**Figure 1**): RL1—mono-rhamno-di-lipidic, RL2—mono-rhamno-mono-lipidic, RL3—di-rhamno-di-lipidic and RL4–di-rhamno-mono-lipidic structures. RL1 and RL3 are usually classified as principal—common RLs, while RL2 and RL4 are classed as atypical–uncommon RLs [68]. The development of sensitive, high throughput analytical techniques, such as soft ionization mass spectrometry, has led to the further discovery of a wide diversity of RL congeners and homologues (about 60) produced in different concentrations by various *Pseudomonas* spp. and other bacteria [9].

3.1.1. Diversity of rhamnolipid structures

RL biosurfactants are always produced as mixtures of different RL congeners, as observed with various *P. aeruginosa* isolates [15, 69–74]. The complexity of the RL mixtures produced depends on various factors such as bacterial isolate origin, type of carbon substrate, culture conditions and isolation procedure and age of the culture and of course, the *P. aeruginosa* isolate itself [15, 23, 63, 72, 75–80]. Despite the number of such factors reported, some particular RL congeners are predominant in all *P. aeruginosa* producer isolates. These are classified as the major RL structures (Rha-C_{10}-C_8, Rha-C_{10}-C_{10}, Rha-C_{10}-C_{12}, Rha-C_{10}-$C_{12:1}$, Rha-Rha-C_{10}-C_8, Rha-Rha-C_{10}-C_{10}, Rha-Rha-C_{10}-C_{12} and Rha-C_{10}-$C_{12:1}$) [23, 72, 81–84]. Other RLs, produced only sometimes or in low abundance, are the minor RL structures [23, 72, 81–84]. Both the major and the minor RL congeners contribute to the complete profile of RLs. In all studies of RL mixtures produced by various *P. aeruginosa* isolates, mono-rhamnolipid Rha–C_{10}–C_{10} and di-rhamnolipid Rha–Rha–C_{10}–C_{10} were the predominant congeners, in spite of the varying compositions produced [23, 72, 81–84].

The presence of different functional groups in RL molecules (the hydrophobic, lipid part and the hydrophilic and carbohydrate part) gives RLs important physicochemical properties. Due to their amphipathic structure, RLs behave as wetting agents, surface active compounds, emulsifiers and detergents. These RL functional groups are, therefore, utilized in enhancing and facilitating bacterial movement, adhesion and contact with surfaces, as well as substrate uptake, or solubilization.

3.1.2. Rhamnolipid biosynthesis and quorum sensing

Biosynthesis of RLs requires three rhamnosyltransferases. The fatty acid dimer moiety in RLs and free 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA) are both synthesized by the rhamnosyltransferase RhlA. Next, dTDP-L-rhamnose is transferred to HAA by the rhamnosyltransferase RhlB, or to a previously generated mono-RL by the rhamnosyltransferase RhlC [85]. HAA precursors are derived from the FASII cycle (bacterial fatty acid synthesis system), while activated L-rhamnose is derived from the glucose moiety of deoxythymidine

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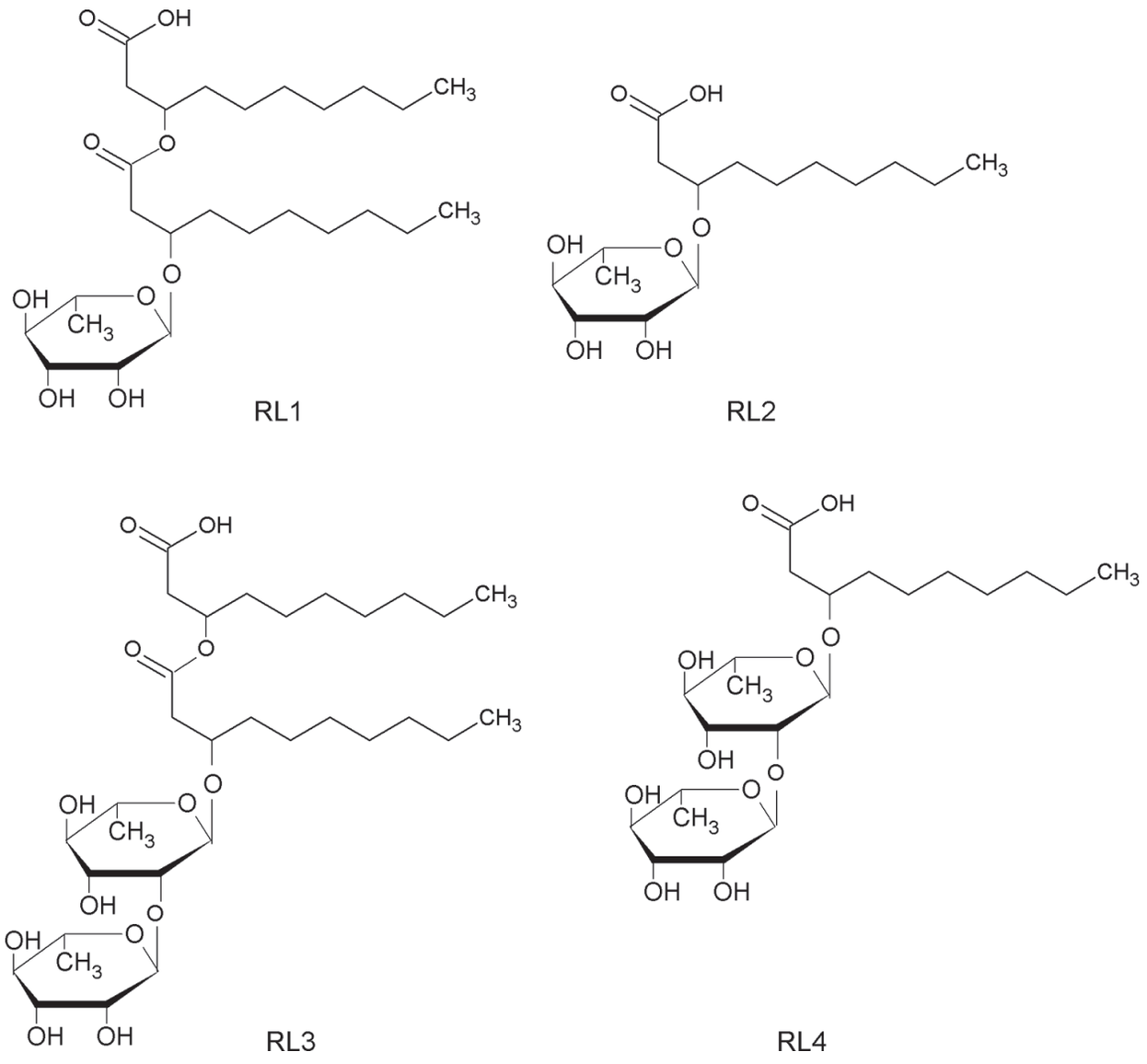

Figure 1. Structure of rhamnolipid congeners: RL1 (mono-rhamno-di-lipidic), RL2 (mono-rhamno-mono-lipidic), RL3 (di-rhamno-di-lipidic) and RL4 (di-rhamno-di-lipidic).

di-phospho (dTDP)-L-rhamnose through several reactions catalyzed by four enzymes that, in *P. aeruginosa,* belong to single operon, *rmlBDA* [11]. dTDP-L-rhamnose has an important role in the regulation of RL biosynthesis, as it is an allosteric regulator for RmlA (which catalyzes transfer of a thymidylmonophosphate nucleotide to glucose-1-phosphate and is a sensor enzyme in this metabolic pathway). Also, this molecule is a precursor for other L-rhamnose containing molecules (LPSs and EPSs). dTDP-L-rhamnose affects the production of mono-RL through coexpression of the operons *rmlBDAC* and *rhlAB*, which are responsible for expression of rhamnosyltransferases RhaA and RhaB [86, 87]. However, in *P. aeruginosa,* the QS system has an essential role in regulation of the *rhlAB* operon and, therefore, in RL biosynthesis.

In Section 2.2.1, we stated that *P. aeruginosa* QS has two interrelated systems, *las* and *rhl*, which are linked by the PQS molecule and that their relationship influences the biosynthesis

of various metabolites. Production of RLs is governed by three *QS* molecules: *Pseudomonas* autoinducer 1 (PAI-1, also known as 3O-C_{12}-HSL), *Pseudomonas* autoinducer 2 (PAI-2, also known as C_4-HSL) and PQS. In *P. aeruginosa*, the *las* operon consists of two transcriptional activator proteins, LasR and LasI, which direct the synthesis of PAI-1. The production of RLs is regulated by the *rhl* system. The synthesis of RLs takes place under the coordinated guidance of the *rhlAB* genes. The *rhl* system consists of the transcriptional activator proteins, RhlR and RhlI, which regulate the synthesis of PAI-2. The transcriptional activator RhlR activates the transcription of *rhlAB* operon and gene *rhlC* (encoding RhlC) [10, 11].

The *rhlAB* operon is clustered on *P. aeruginosa* DNA together with *rhlR* and *rhlI*, which together direct the synthesis of all proteins required for RL production (the rhamnosyltransferases and the transcriptional activators, RhlR and RhlI) [10]. RL synthesis is upregulated and promoted at transcriptional level, related to the QS system, by the Vfr (global virulence regulation) and the *pqs* systems through activation of RhlR expression and *rhlRI* operon, respectively [11]. RasL (repressor of *las* system) and AlgR (biofilm formation) downregulate RL synthesis by repression of LasI and *rhlAB*/*rhlI*, respectively [11]. For instance, increasing bacterial cell density induces the *las* system, resulting in an increased concentration of PAI-1 that binds to the transcriptional activator site LasR and forms the LasR–PAI-1 complex. The LasR–PAI-1 complex induces genes controlled by the *rhl* system, including the regulator gene *rasL*, *rhlR* and *pqsH*, required for PQS production. PQS acts as a link between the *las* and *rhl* systems. The activity of these signals depends on their ability to dissolve in and freely diffuse through aqueous solution [10]. PQS induces the *rhlI* gene, which directs the production of PAI-2 that binds to and activates RhlR (RhlR–PAI-2 complex). The RhlR–PAI-2 complex induces genes for RL production, which are controlled by the *rhl QS* system (operons *rhlAB*, *rhlC*, *rhlI*, *rhlR* and *rhlG*). The RLs produced enhance the solubility of PQS in aqueous solutions and promote cell-to-cell communication. This is important because of the role PQS plays in the *P. aeruginosa* stress response, in conditions related to the CF lung environment (oxidative stress and antimicrobial agents) [88].

In conclusion, in the complex QS network, there is a hierarchy between *las* and *rhl* systems in RLs biosynthesis. Furthermore, RL biosynthesis is regulated at the transcriptional level according to nutritional and environmental conditions, as well as at the posttranscriptional level [11, 42]. However, most of the regulatory mechanisms are not completely understood [11, 42].

3.2. *P. aeruginosa* exopolysaccharides

Pseudomonads have the potential to produce various types of EPSs such as alginate, levan, marginalan and cellulose, as well as different heteropolysaccharides and protein polysaccharides complexes [89]. Nearly all *Pseudomonas* isolates, including *P. aeruginosa*, *Pseudomonas putida* and *Pseudomonas fluorescens* can produce alginate as the main acidic EPS [90–92]. Alginate is composed of β-1,4-D-mannuronic and L-guluronic acids linked via β-1,4-glycosidic bonds [93]. Alginates are also produced by *Azotobacter* isolates and some genera of brown and red algae. In comparison to algal alginates, bacterial alginates are O-acetylated at some of the C-2 and C-3 carbons of the mannuronic acid residues and acetylation occurs

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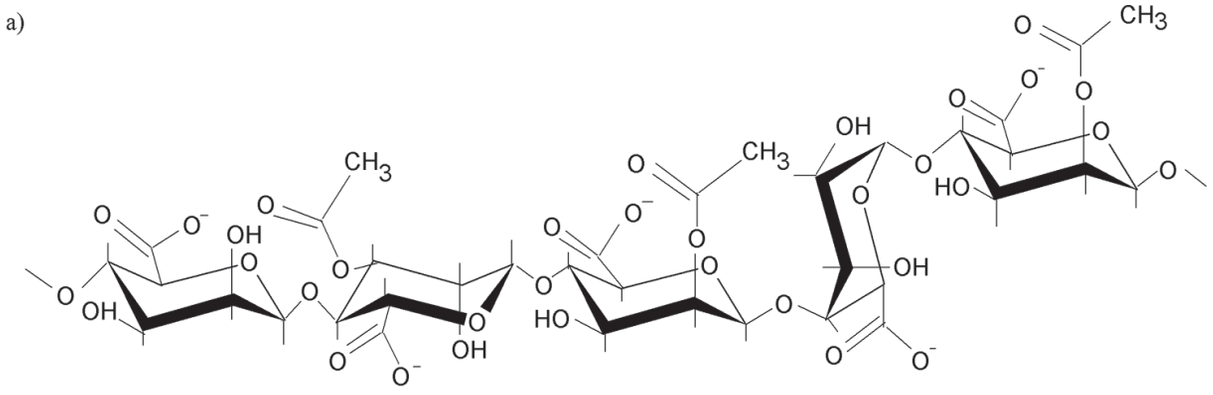
during transport through the periplasm. A high degree of O-acetylation increases the viscosity and flexibility of alginate, as well as its ability to bind water [94].

3.2.1. Diversity of exopolysaccharide structures

P. aeruginosa has the genetic ability to produce at least three polysaccharides: alginate, Psl (polysaccharide synthesis locus) and Pel (pellicle formation locus). Alginate and Psl have different chemical structures (**Figure 2a**) although they have similar biosynthetic mechanisms [89]. In comparison to alginate, a highly O-acetylated linear polymer of 1,4-linked mannuronic acid (M) and guluronic acid (G), Psl is a helicoid polysaccharide composed of a repeating pentamer containing D-mannose, L-rhamnose and D-glucose (**Figure 2b**). The structure of Pel is not completely characterized and it is supposed that it differs from alginate and Psl



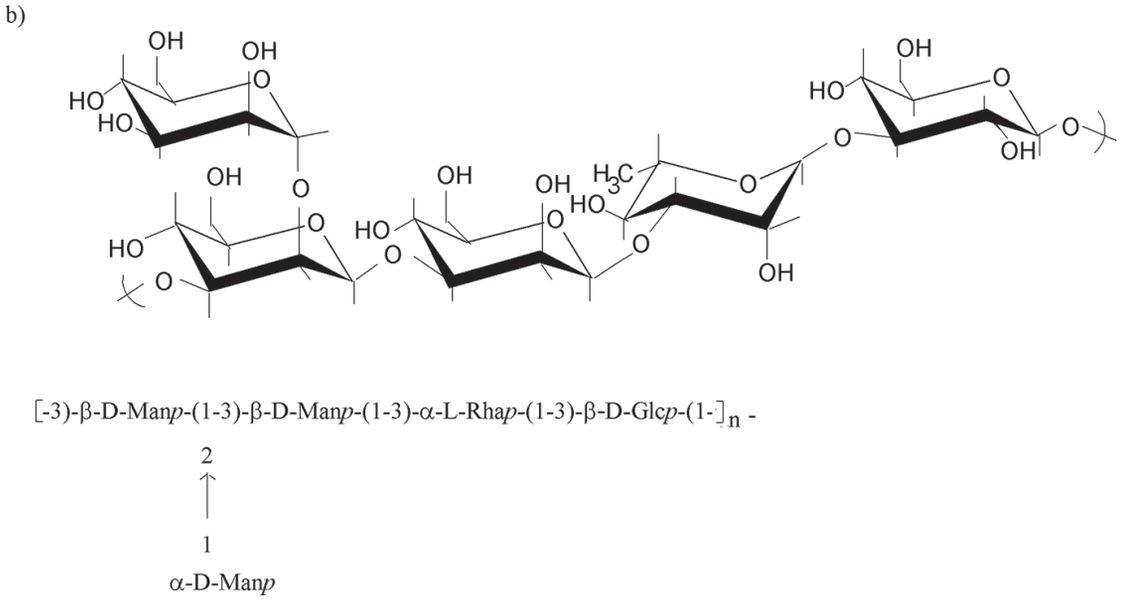


Figure 2. Structures of extracellular polysaccharides produced by *P. aeruginosa*: (a) alginate and (b) exopolysaccharide Psl.

[95]. Pel is proposed to be a glucose-rich polysaccharide, different to cellulose [96]. Each EPS has distinct physiological properties, affecting the cells and the biofilm matrix. While alginate is secreted into the surrounding medium without covalently linking to the cell surface, Psl has helical distribution around the cell surface with a key role in cell-to-cell and cell-to-surface interactions during biofilm formation. Pel forms a connecting matrix allowing it a structured assembly at the air-liquid interface connecting the cells. This matrix could also contain O-antigen-LPS and cyclic glucans [95]. The diversity of EPSs produced by bacterial biofilm subpopulations is one of the proposed *P. aeruginosa* survival strategies for adaptation to environmental changes, as related to the conditions in CF lungs.

3.2.2. Exopolysaccharide biosynthesis and quorum sensing

EPS biosynthesis requires sugar-nucleotide precursors and for alginate production, this is GDP-mannuronate. The enzymes required for GDP-mannuronate production include: (1) the bifunctional enzyme, AlgA which exhibits phosphomannose isomerase (PMI) and GDP-mannose pyrophosphorylase (GMP) activity; (2) AlgC, a phosphomannomutase; and (3) AlgD, which is a GDP mannose dehydrogenase [97–99]. AlgD catalyzes the first step in alginate biosynthesis, which is responsible for the mucoid phenotype often observed in clinical *P. aeruginosa* from chronically infected CF patients [13].

Alginate is first synthesized as a linear homopolymer of D-mannuronic acid residues. The polymer is then modified in the periplasm through selective O-acetylation by the concerted action of AlgI, AlgJ and AlgF and epimerized by AlgG [100, 101]. Alginate has a reasonably random structure (**Figure 2a**). This differentiates alginate from Psl and numerous *E. coli* capsule polysaccharides, the structures of which are more regular, with repeating subunits (**Figure 2b**). The randomness of alginate's structure occurs because during polymerization, AlgG converts D-mannuronic acid residues to L-guluronic acid and critically, either the C-2 and/or C-3 carbons can have acetylated hydroxyl functional groups, which become available for linking the residues.

AlgC appears to be crucial for general EPS biosynthesis, not just alginate, as it is also required for precursor synthesis of Psl, as well as LPSs and RLs [102, 103]. The LasR from the *las* system might, to some extent, regulate expression of *algC* and *algD*, confirming the correlation of QS systems with EPS production [13].

4. Physiological role of *P. aeruginosa* biosurfactants in CF infection

4.1. Physiological role of rhamnolipids and exopolysaccharides

Among proposed functions of RL biosurfactants, related to their physicochemical properties (surface activity, wetting ability, detergency and other amphipathic-related properties), are promotion of the uptake and biodegradation of poorly soluble substrates, immune modulators and virulence factors [9, 15]. Additionally, these molecules are involved in the process of swarming, as surface wetting agents and chemotaxis stimuli and in *P. aeruginosa* biofilm structuring, maturation (the formation of water channels in mature biofilms) and dispersion

[10]. Probably because they do not present the profile of typical or traditional virulence factors, RLs are sometimes not considered significant members of the virulence arsenal of *P. aeruginosa* [9]. However, published data strongly demonstrate their importance as virulence determinants and their significant role in infection establishment and persistence [8, 9].

Physicochemical properties of EPSs, such as surface activity, viscosity, flexibility of molecule, as well as its ability to bind water, protect the microbe from dehydration in the unique CF microenvironment following the switch from nonmucoid to mucoid phenotype [94]. In this regard, the *P. aeruginosa* mucoid phenotype is the most studied adaptation in patients with CF and it is directly proportional to overproduction of EPSs, which is widely considered to be a marker for the transition to chronic infection [8, 54]. Alginates are well studied as compounds associated with biofilm formation and invasion of pathogenic microorganisms. The alginate-containing matrix of mucoid *P. aeruginosa* is thought to allow the formation of protected microcolonies and provide increased resistance to opsonization, phagocytosis and destruction by antibiotics [104]. Alginates also have a protective role in *P. aeruginosa* infection because they scavenge free radicals released by activated macrophages *in vitro*, prevent phagocytic clearance and protect the microorganism from the host defense system [13].

4.2. Rhamnolipids and exopolysaccharides in *P. aeruginosa* biofilm formation

Swarming motility is the rapid and coordinated movement of a bacterial population across a surface, which often results in characteristic flowery, dendritic colony shapes on agar plates [105]. This type of colony movement is related to the production of an extracellular slime layer, mainly composed of EPSs and surface active compounds, which is a pivotal feature of swarming cells, acting as a wetting agent that reduces the surface tension [106]. Several studies suggest that *P. aeruginosa* expresses swarming motility and that it requires flagella and the production of wetting agents (RLs and its lipidic precursors HAAs) [85, 107–109]. Also, HAAs and di-RLs actually modulate the swarming process, as di-RLs and HAAs behave as self-produced chemotactic attractants with opposite activity, while mono-RLs seem to be act solely as wetting agents [107, 109]. Additionally, swarming motility is clearly related to biofilm formation [105].

The importance of swarming motility for biofilm formation indicates that RLs are involved in the process of biofilm formation. Indeed, it was shown that RLs enhance adhesion of planktonic cells in the early stages of biofilm development, when an initial microcolony is formed (**Figure 3**). Proposed mechanisms for RL effects on cell adhesion include regulation of cell-surface hydrophobicity and modification of adhesive interactions, especially when nutritional conditions are changed [85, 110–112]. Also, RLs are involved in later differentiation of the biofilm structure, the detachment and dispersion of *P. aeruginosa* cells, where RLs behave as mediators which disturb cell-to-cell and cell-to-substratum interactions and maintenance of open channels inside the biofilm [111, 113]. Furthermore, regulation of RL production by *P. aeruginosa* is regulated not only in temporal terms, but also in quantifiable terms, because overproduction of RLs disrupts biofilm structure or impedes biofilm formation [113].

EPSs also play an important role in biofilm formation and invasion of pathogenic microorganisms. During biofilm maturation, *P. aeruginosa* begin to excrete EPSs, such that the

bacteria in the mature biofilm are encased in a matrix of EPSs that they have produced [114]. Overproduction of alginate is the main indicator of *P. aeruginosa* converting to the mucoid phenotype and is responsible for the notable microbial resistance to antibiotics as well as defense from the host immune system of CF patients (**Figure 3**). The mucoid phenotype of *P. aeruginosa* produces a great amount of alginate as a result of several genes, including *algD*, which encodes GDP-mannose dehydrogenase, responsible for synthesis of alginate precursor [8, 94]. The alginate-containing matrix of the mucoid phenotype allows the formation of protected microcolonies and provides increased resistance to opsonization, phagocytosis and antibiotics, resulting in persistent infection and a worsening prognosis for CF patients [104].

In the context of immune system pathways, polymorphonuclear leukocytes (PMNs) are considered as the central line of defense in innate immunity and they are produced as a predominant response to infection, especially in CF lungs [115]. When PMNs phagocytose bacteria, the host cells produce highly reactive oxygen species, which kill *P. aeruginosa* or induce mutations in the microbial *mucA* gene. However, the alginate produced by mucoid phenotype *P. aeruginosa* is also an oxygen radical scavenger, helping to protect this pathogen against host inflammatory defense mechanisms [116]. Airway epithelial cells play a crucial role during establishment of respiratory infection because *P. aeruginosa* attaches to and enters respiratory epithelia, producing an immune response in the lung by activating lymphocytes at the site of infection [117].

Surfactant protein A (SP-A) is involved in prevention of alginate-induced *P. aeruginosa* invasion of lung epithelial cells. SP-A plays a part in the innate immunity in the lung, with a

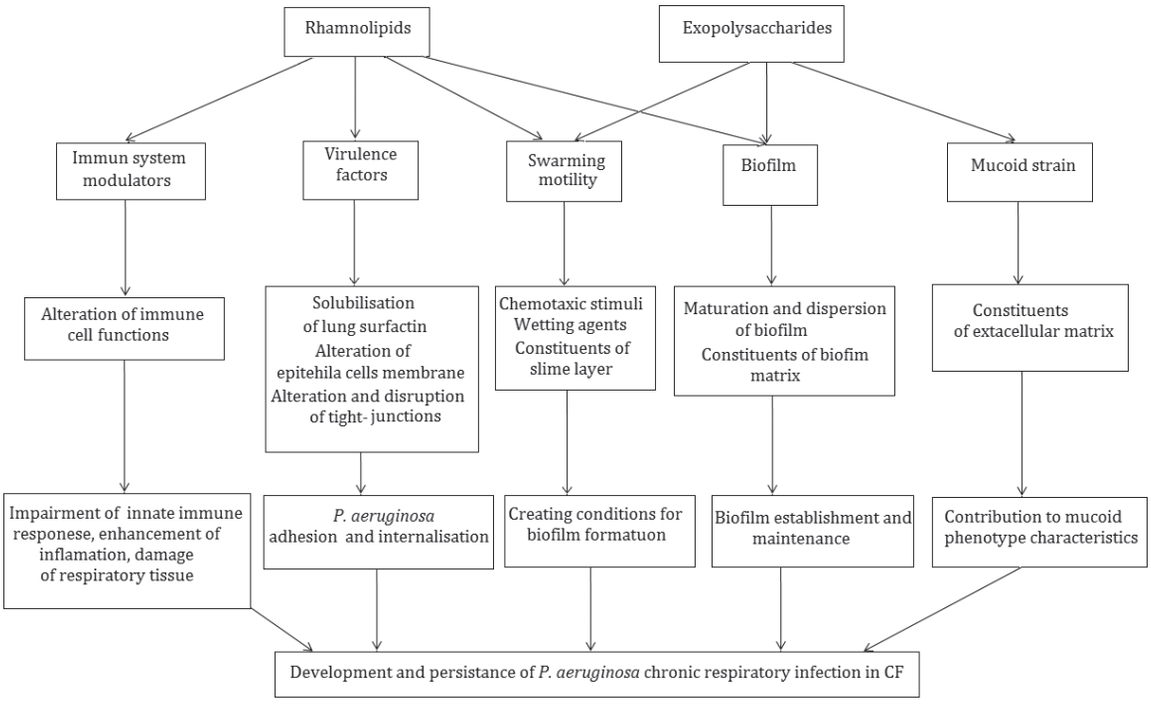


Figure 3. Proposed roles, relations and effects of *P. aeruginosa* biosurfactants RLs and EXPs in development and persistence of chronic respiratory infection in CF patients.

direct role in bacteria opsonization and killing, as well as impairment of bacterial membrane permeabilization [117]. Alginate is surface exposed and levels of SP-A could be crucial in modulating the interaction of *P. aeruginosa* with the epithelial barrier.

4.3. Effect of *P. aeruginosa* rhamnolipids and exopolysaccharides

Respiratory mucosa protects host airways from microbial infection. *P. aeruginosa* and other microbial species capable of causing lung infections have developed mechanisms to overcome this barrier, such as alteration of the apical membrane of epithelial cells or alteration and disruption of tight junctions (TJ) [118]. Proposed mechanisms involve alterations of respiratory epithelial ion transport, inhibition of transcellular ion transport and interference with the normal tracheal ciliary function. Bacterial adherence to the basolateral domain of epithelial cells and internalization are suggested as a potential mechanism of *P. aeruginosa* pathogenicity (**Figure 3**). The physiological pathways of these processes are not still completely clarified, but reports indicate involvement of virulence factors, production of which is controlled by the type III secretion (cytotoxic proteins) and the *las* and *rhl* QS (RLs, elastase) systems [119, 120].

RLs concentration of up to 8 μg/ml was found in the sputum of CF patients infected by *P. aeruginosa* [120], while secretions from a lung removed contained 65 μg/ml RLs [121]. These concentrations of RLs are likely adequate for promotion of *P. aeruginosa* epithelial cell infiltration. Furthermore, this indicates link between elevated levels of RLs and worsening of patient clinical status.

RLs produce damage to the bronchial epithelium and inhibit ciliary function [122–124]. Damage to the bronchial epithelia is related to impairment of the protective layer of lung surfactant in CF patients. Phospholipase C and RLs produced by *P. aeruginosa* can act synergistically to break down lipids and lecithin from lung surfactant [12]. It is believed that RLs, due to their detergency, solubilize the phospholipids in lung surfactant, making them more accessible to cleavage by phospholipase C [12].

The effects of *P. aeruginosa* RLs on the respiratory epithelia function were studied in several animal models [122]. RLs caused ciliostasis and cell membrane damage to rabbit tissue were a secretagogue in cats and inhibited epithelial ion transport in sheep tissue. Additionally, the authors investigated the effect of RLs on mucociliary transport in the anesthetized guinea pig and guinea pig and human respiratory epithelia *in vitro* [122]. Reduction of tracheal mucus velocity (TMV) *in vivo* occurred depending on the applied RL concentration (10 μg of RLs caused cessation of TMV without recovery; 5 μg of RLs reduced TMV by 22.6% over a period of 2 h and 2.5 μg of RLs caused no overall change in TMV). RLs (10 μg) did not disrupt the ultrastructure of guinea pig tracheal epithelium. RL (250 μg/ml) stopped ciliary beating of guinea pig tracheal. Treatment with RL concentration of 100 μg/ml caused immediate slowing of the cilliar beat frequency (CBF) of human nasal brushings, as well as CBF of human nasal turbinate organ culture. Mono- and di-RL had equivalent effects [122]. In addition, RLs stimulate the release of mucus glycoconjugates from feline trachea or human bronchial mucosa [125, 126].

In vitro reconstructed respiratory epithelium was exposed to several *P. aeruginosa* isolates with alterations in genotype: wild type, CF isolates and strains with altered QS system expression

[118]. The authors found that only RL-producing *P. aeruginosa* (those that expressed the *rhl* QS system) was able to infiltrate the epithelia by modulating the permeability of the tissue. The early stages of infection did not correlate with type III secretion and elastase activity [118], in contrast to previous reports [127, 128]. The effect of exogenously applied purified RLs on the epithelial barrier was also studied [118]. The authors used JBR 515, which is commercial mixture of 50% w/v Rha-C_{10}-C_{10} and 50% w/m Rha-Rha-C_{10}-C_{10}. RLs produced by bacteria *in situ* or purified. The applied RLs caused loss of epithelial cell polarity by: incorporation in first, the apical and later, the basolateral epithelial membranes (due to chemical structure); cilia loss; ezrin displacement; and alterations of TJ. The final result was a decrease of transepithelial resistance and higher permeability of respiratory epithelia, without affecting cell viability [118]. After disruption of TJ, paracellular invasion by some *P. aeruginosa*, involving RL deficient strains, was observed, but they were not internalized [118]. This was in contrast to previous reports [129, 130], perhaps due to the *in vitro* conditions used in the studies as difference. Altogether, the importance of RL biosurfactant and the QS system in *P. aeruginosa* invasion of respiratory epithelium is acknowledged, but the exact mechanisms of cell polarity and structure alterations remain unclear.

The effect of RLs on immune system pathways with direct impairment and modulation of immune cell activity is well known [9] (**Figure 3**). RLs are reported to have hemolytic activity on various erythrocyte species; induce direct neutrophil chemotactic activity [130]; enhance the oxidative burst response of monocytes; stimulate and release inflammatory mediators from mast cells and platelets; induce lysis of PMNs; stimulate both chemotaxis and chemokinesis of PMNs (depending on concentration); and enhance production of several interleukins produced by granulocyte-macrophage and nasal epithelial cells (at noncytoxic levels) [131–135]. Furthermore, RLs, especially di-RLs, are cytolytic for human monocyte-derived macrophages and at lower concentrations, they inhibit the phagocytic response of macrophages [136].

The response of *P. aeruginosa* mutants (PAO1 and QS, *rhlA* and *pqsA* deficient) to the presence of PMNs was studied [115]. Previously reported data showed that *in vitro,* PMNs performed their immune function and eliminated QS-deficient *P. aeruginosa* biofilms, although they were incapable of eliminating QS-proficient biofilms [51]. Additionally, purified RLs induced necrosis in PMNs [134]. In biofilm, *P. aeruginosa* (PAO1 wild type) produced increased levels of various virulence factors in response to PMNs, while *P. aeruginosa rhlA* mutant was eliminated by PMNs [115]. Additionally, 2000-fold higher levels of RLs from *P. aeruginosa* PAO1 occurred in biofilm than in surrounding fluid, indicating that RL molecules were grouped around biofilm [115]. Similarly, a *P. aeruginosa rhlA* mutant was cleared more quickly than the wild strain from two *in vivo* mouse models of lung infection [137]. Also, microscopic analysis showed that there were no intact PMNs in close contact with outer layers of biofilm. This correlated with microscopic investigations of *P. aeruginosa* infected *ex vivo* tissues samples from CF lungs, where PMNs were located peripherally [115]. The RLs isolated in this study were a mixture of mono- and di-RL congeners (Rha-C_{10}-C_{10}, Rha-C_{10}-C_{12}, Rha-C_{10}-$C_{12\Delta}$ and respective di-RL derivates) [137]. Van et al. [137] proposed that RLs have a role as a protective mechanism in biofilm resistance to phagocytosis and supported a "launch a shield" model, where

RLs surround the biofilm and on contact destroy PMNs. This study [137], in correlation with previous reports about QS regulation of bacterial response to PMNs [50, 134] showed that *P. aeruginosa pqsA* mutant was unable to respond to exposure to PMNs by increasing RL production and that there was impairment of the QS hierarchy. These studies show that RLs probably contribute to the inflammatory-related tissue damage observed in lungs of CF patients, which involves complex and tight regulation by the QS system. RL production, though, is not continued because it affects all host cells, not only immune cells and high levels of RL may create conditions (due to inflammation and host tissue damage) which are not favorable for *P. aeruginosa* persistence [137]. This study supports a model by which cross-kingdom-based communication contributes significantly to immunomodulation and evasion and which is one reason studying the infective properties of *P. aeruginosa* is so fascinating.

Modification of membrane LPSs in *P. aeruginosa* is also an important mechanism in the development of chronic infection in CF patients [138–140]. Membrane LPSs in *P. aeruginosa* are composed of three parts: highly acylated lipid A; a central core oligosaccharide bound to lipid A and O-antigen; and a variable polysaccharide composed of repeated units located out from the core [138, 140]. It is not surprising that the structure of LPSs is modified in *P. aeruginosa* isolated from CF patients because of their direct interface position with the pulmonary environment [8]. Compared to normal lipid A, that from CF patients contains more hexa- and hepta-acylated moieties as well as added aminoarabinose, a cationic amino sugar residue which is responsible for resistance to antimicrobials [140]. Acylation levels of lipid A are responsible for LPS recognition by the host and induction of the proinflammatory response, so their modification causes *P. aeruginosa* to be less visible to the host immune system [141]. Also, in CF isolates, O-antigen is lost, due to mutations in genes responsible for O-antigen production. This loss can facilitate chronic persistence in respiratory tracts of CF patients [138–140]. Modification of LPS can directly correlate with overproduction of alginate, which is typical for the mucoid phenotype. Alginate might interact via the carboxylic groups in polyguluronic acid units with modified membrane LPSs in *P. aeruginosa*, across cationic amino sugar aminoarabinose residues. This likely enhances polymerization and facilitates release of EPSs from the membrane. Thus, study of factors that influence increased production of EPSs and RLs, as well as the structure-function relationships of these compounds would likely be of great importance for improved therapy of CF patients [8].

Figure 3 summarizes the proposed roles, relationships and effects of the biosurfactant RLs and EPSs produced by *P. aeruginosa* in the development of chronic respiratory CF infection.

5. Rhamnolipids and exopolysaccharides as targets—current and future perspectives

The importance of biofilm formation and maintenance for the establishment and persistence of *P. aeruginosa* chronic respiratory infection in CF has been discussed in Section 2.2.1. The complex regulation of biofilm development includes the QS network, swarming motility and production of extracellular metabolites and involves significant roles for RLs and EPSs.

Agents	Type	Strategy	Resistance	References
Ticarcillin, Piperacillin Cefrazidime, Cefepime Imipenem, Meropenem Aztreonam	*β*-Lactams	Impairment of biofilm structure and *QS* inhibitors	Antibiotic cleavage by *β*-lactamase enzymes, antibiotic expulsion by encoded efflux mechanisms and reduced drug uptake due to loss of outer membrane porin proteins	[16, 155]
Ciprofloxacin	Fluoroquinolones	*QS* inhibitors	Mutations by DNA gyrase and topoisomerase IV enzymes and efflux systems	[155, 156]
Tobramycin, Gentamicin, Amikacin	Aminoglycosides	Impairment of biofilm structure	Aminoglycoside-modifying enzymes AMEs and rRNA methylases as well as efflux mechanisms	[16, 155, 157]
Patulin, penicillin acid, cis-2 decanoic acid	Bacterial metabolites	Impairment of biofilm structure and *QS* inhibitors	No resistance	[16, 158]
Solenopisin A	Fire ant venom	Impairment of biofilm structure and *QS* inhibitors	No resistance	[16, 154]
Salicylic acid and 4-nitro-pyridine oxide (4-NPO)	Synthetic compounds	Impairment of biofilm structure and *QS* inhibitors	No resistance	[16, 152, 154]
Garlic extract	Natural mixture	Impairment of biofilm structure and *QS* inhibitors	No resistance	[16, 152, 159]
Halogenated furanones from algae *D. pulchra*, Furanone C-30	Synthetic or modifies natural derived furanones	*QS*-inhibitor and *P. aeruginosa* elimination in combination with antibiotics	No resistance	[16, 160]

Table 2. Antibiofilm approaches in therapy of P. *auruginosa* infection of CF patients.

Therefore, a logical approach in preventing and treating chronic *P. aeruginosa* infection in CF patients is focused on antibiofilm strategies. Antibiofilm strategies can take two differing approaches, one common, related to antibiotic therapy and the other novel, related to interruption of QS (**Table 2**). Furthermore, vaccination is proposed as a modern approach to prevent *P. aeruginosa* infection in CF, where virulence factors, such as alginate, have been used as the antigen. However, most vaccines are still in the clinical research phase and have not reached the market [142].

Traditional antibiotic therapy is related to the early colonization period, the only possible phase when *P. aeruginosa* can be eradicated from CF airways [143, 144]. The effectiveness of antibiotics later is significantly reduced due to microbe adaptation mechanisms (membrane changes, efflux system changes, production of various virulence factors and EPS-containing extracellular matrix, mutation and modification of enzymes) [16] (**Table 2**). Furthermore, tobramycin (an aminoglycoside) is the most common antibiotic for *P. aeruginosa* therapy

choice in CF lungs [145]. This is in spite of the fact that alginates produced by the microbe decrease, the movement of aminoglycosides, cationic antimicrobial peptides and quaternary ammonium compounds through *P. aeruginosa* biofilms [27, 146] (**Table 2**). To overcome obstacles related to antibiotic resistance and increase the antimicrobial effects, an inhaled version of tobramycin, as well as liposomal-encased current antibiotics are available. These antibiotic formulations have improved delivery times and provide higher drug concentrations at the site of infection. Additionally, the importance of biofilm formation as having a crucial role in the antibiotic resistance of *P. aeruginosa* (as well as other CF pathogens) is now being recognized. Recent research trends include analysis of biofilm formation in terms of *P. aeruginosa* antibiotic resistance/susceptibility and the potential for antibiotics as efficient therapy agents for biofilm impairment [147–150].

A more novel antibiofilm strategy, QS interruption, is a promising approach for treating CF respiratory infections. In this strategy, the QS system is targeted, due to its regulation of the biosynthesis of RLs and EPSs [151–153]. The QS impairment approach involves identification of molecules which can interrupt QS pathways. Generally, these compounds have one of following mechanisms of activity: blocking production of QS signal molecules, degradation of QS signal molecules or prevention of microbe recognition and response to QS stimuli [16]. Various natural compounds inhibited QS or directly impaired biofilm (**Table 2**) (e.g., garlic extract, metabolites from *Penicillium* spp., salicylic acid, the *P. aeruginosa* metabolite *cis*-2-decanoic acid). Furanones are QS blockers and the furanone produced by *Delisea pulchra and* synthetic furanones, enhanced *P. aeruginosa* elimination in combination with antibiotic therapy [16]. Furanone C-30 repressed 77% of *P. aeruginosa* genes induced by exposure to PMNs [50]. The great advantage of using QS inhibitors in CF therapy is that they are not expected to induce bacterial resistance, because their activity is not closely related to bacterial growth [154].

In the context of the physiological roles of RLs and EPSs discussed in Section 4, these compounds are also promising targets for future strategies in CF therapy related to specific modulation of respiratory mucus [118].

6. Conclusion

RLs and EPSs, biosurfactant molecules, play significant roles in bacterial acquisition, biofilm development and establishment of chronic *P. aeruginosa* infections in CF patients. Specifically, RLs and EPSs are, due to their amphipathic structures and physicochemical properties, involved in processes of respiratory mucus alteration, modulation of immune system defense pathways, biofilm development and maintenance and the *P. aeruginosa* mucoid phenotype. These compounds are responsible for antibiotic resistance and survival and general persistence of *P. aeruginosa* in the specific, dynamic environmental conditions in CF patients' lungs. Consequently, RLs and EPSs are the direct or indirect cause of bad outcomes and high mortality rates among these patients. Currently, therapy generally based on application of antibiotics fails to prevent and treat chronic *P. aeruginosa* infection. Therefore, RLs and EPSs are interesting novel targets for dealing with respiratory infection in CF patients. In addition, the

P. aeruginosa QS system is an important aspect of CF lung infection, as it regulates synthesis of the biosurfactants and other virulence factors, as well as biofilm formation. Future perspectives to prevent and treat *P. aeruginosa* respiratory infections in CF certainly should involve impairment of QS pathways. Finally, further study of potential approaches to modify host respiratory mucus epithelial membranes is required.

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