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P. aeruginosa Biofilms in CF Infection

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Abstract *Pseudomonas aeruginosa* is an opportunistic pathogen of immunocompromised hosts. In cystic fibrosis (CF), *P. aeruginosa* causes acute and chronic lung infections that result in significant morbidity and mortality. *P. aeruginosa* possesses several traits that contribute to its ability to colonize and persist in acute and chronic infections. These include high resistance to antimicrobials, ability to form biofilms, plethora of virulence products, and metabolic versatility. In *P. aeruginosa*, a cell-to-cell communication process termed quorum sensing (QS) regulates many of these factors that contribute to its pathogenesis. Recent evidence suggests that the CF lung environment presents a specialized niche for *P. aeruginosa*. The relationship of *P. aeruginosa* QS, biofilm formation, and the CF lung environment is discussed.

Keywords *Pseudomonas aeruginosa* · Biofilms · Cystic fibrosis · Quorum sensing

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative microorganism found in many environments, such as soil and water. *P. aeruginosa* is also an opportunistic pathogen implicated in respiratory infections, urinary tract infections, gastrointestinal infections, keratitis, otitis media, and bacteremia. *P. aeruginosa* is found in an estimated 10–20% of all hospital-acquired infections [1]. Patients with compromised host defenses, such as those infected with human immunodeficiency virus, burn patients, or those with cystic fibrosis (CF), are susceptible to *P. aeruginosa* infections. These infections are often difficult to treat using conventional antibiotic therapies.

P. aeruginosa possesses a large genetic diversity that contributes to its ability to persist in the environment and to its pathogenesis. *P. aeruginosa* is able to metabolize various carbon and nitrogen sources and can grow in either an aerobic or anaerobic environment using several terminal electron acceptors. *P. aeruginosa* is intrinsically resistant to many antimicrobials, in part due to its multiple efflux systems. *P. aeruginosa* also produces numerous virulence factors, including secreted factors, such as elastase, proteases, phospholipase C, hydrogen cyanide, exotoxin A, and exoenzyme S, as well as cell-associated factors, such as lipopolysaccharide, flagella, and pili.

In *P. aeruginosa*, expression, production, and/or secretion of many virulence factors are controlled in a cell density-dependent manner known as quorum sensing (QS). Many bacteria, including *P. aeruginosa*, use QS to coordinate population behavior by producing, sensing, and responding to diffusible signaling molecules, termed autoinducers. Two complete QS systems, the *las* and *rhl* systems, have been described and well-studied in *P. aeruginosa*. These systems consist of the transcriptional regulatory proteins LasR (in the *las* system) and RhlR (in the *rhl* system), or R-proteins, and their cognate autoinducer signal molecules *N*-(3-oxododecanoyl) homoserine lactone (3O-C_{12}-HSL) in the *las* system and *N*-butyryl homoserine lactone (C_4-HSL) in the *rhl* system. Each R-protein possesses an autoinducer-binding domain as well as a

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deoxyribonucleic acid (DNA)-binding domain. The binding of the R-protein to its cognate autoinducer molecule produces a R-protein–autoinducer complex that modulates target gene expression, presumably by binding to conserved DNA elements termed *las* boxes located upstream of the translational start site of QS-regulated genes [2]. The *las* and *rhl* systems are not independent and are intertwined in a hierarchical manner, with the *las* system exerting control over the *rhl* system [3, 4]. *P. aeruginosa* also produces a third signal molecule, 2-heptyl-3-hydroxy-4-quinolone or the *Pseudomonas* quinolone signal (PQS), that links the *las* and *rhl* systems [5, 6]. Incredibly, transcriptome analyses have determined that between 6% and 10% of the *P. aeruginosa* genome is regulated by the *las* and/or *rhl* systems [7–9].

P. aeruginosa also readily forms communities of cells encased in an extracellular matrix, consisting of secreted proteins, polysaccharides, nucleic acids, and cellular debris, attached to abiotic or biotic surfaces [10]. These communities, known as biofilms, are of clinical relevance as the awareness of the prevalence of biofilm-centered infections increases. Classic examples include biofilm-mediated infections that occur on medical devices such as intravascular catheters, urinary catheters, orthopedic devices, and dialysis machines [11, 12]. More recently, there is evidence that chronic infections, such as recurrent ear infections (otitis media) and lung infections in CF patients, are caused by biofilm-dwelling bacteria [13–15]. The CF lung provides a particularly attractive environment for *P. aeruginosa* colonization and chronic lung infection and is the major causative agent of morbidity and mortality in CF patients [16]. Biofilms are now thought to be involved in 65–80% of all microbial infections [17, 18].

Biofilm growth is thought to offer a competitive advantage versus free-floating or planktonic growth. Bacteria in biofilms are more recalcitrant to disruption or killing by surfactants, biocides, grazing predators, and host defenses [12]. It is important to note that the biofilm mode of growth is often associated with antimicrobial resistance [12]. Studies indicate that bacteria growing in biofilms are up to 1,000 times more resistant to antimicrobial challenge versus planktonically grown cells [12]. As the knowledge of the importance of biofilms in infection increases, perception has altered research seeking to discover new therapies to treat these infections by necessitating an understanding of the mechanisms that govern biofilm development and its role in bacterial pathogenesis.

P. aeruginosa biofilm development and its relation to quorum sensing

P. aeruginosa biofilm formation has been divided into various stages, including bacterial attachment and irreversible adhesion to a substratum, microcolony development, maturation of the biofilm demonstrated by the appearance of large structures resembling mushrooms and stalks with water channels, and dispersion of bacterial cells from the biofilm (Fig. 1a, b) [19]. Heterogeneous microenvironments have been demonstrated to exist within biofilms due to oxygen and nutrient diffusional limitations [20], and recent evidence suggests that bacteria grow slowly within the biofilm [17]. The biofilm is also physiologically heterogeneous since each bacterium within the biofilm matrix experiences differing nutrient gradients. Bacteria within the biofilm are thought to possess a unique phenotype quite different from their planktonic counterparts [17].

The belief that biofilm development results from an organized set of molecular events has led to several studies to understand the mechanisms involved. The idea that biofilm development is a coordinated behavior was strengthened by evidence of a link between *P. aeruginosa* QS and biofilm development [21]. This study determined that *lasI*, which encodes for the synthetase responsible for 3O-C_{12}-HSL production, is critical for mature biofilm formation in *P. aeruginosa* PAO1 [21]. Mutants lacking the *lasI* gene formed flat, thin biofilms with little of the dramatic three-dimensional structures which typify *P. aeruginosa* biofilms grown in a flow-through model system. It is exciting to note that the mutant was also more susceptible to removal by the surfactant sodium dodecyl sulfate (SDS). Further studies have demonstrated that QS mutant biofilms are also more susceptible to antimicrobial challenge and phagocytosis by polymorphonuclear leukocytes [7, 22]. This suggests that QS is not only important in the development of *P. aeruginosa* biofilms but also in its protective role. For example, the QS regulations of catalase and superoxide dismutase have been demonstrated to be important in *P. aeruginosa* PAO1 biofilm resistance to hydrogen peroxide [23]. Additionally, QS inhibitors, including a halogenated furanone synthesized by the red algae *Delisea pulchra* and several derivatives of this molecule, have been shown to not only interfere with *P. aeruginosa* PAO1 biofilm development but to enhance sensitivity to antimicrobial challenge with tobramycin or azithromycin, presumably by interfering with QS signaling [10].

Both flagellar and twitching motility have been demonstrated to be involved in *P. aeruginosa* attachment to surfaces and subsequent biofilm formation [24–26]. In *P. aeruginosa*, twitching motility is mediated by type IV pilin, and mutants in this pilin (Δ*pilA*) were unable to form the elaborate mushroom structures typical of *P. aeruginosa* biofilms. LecB, or the type II *P. aeruginosa* lectin, is also involved in pilus biogenesis [27] and has been reported to be involved in biofilm development [28]. Mutants in *lecB* formed weak biofilms as compared to the wild-type

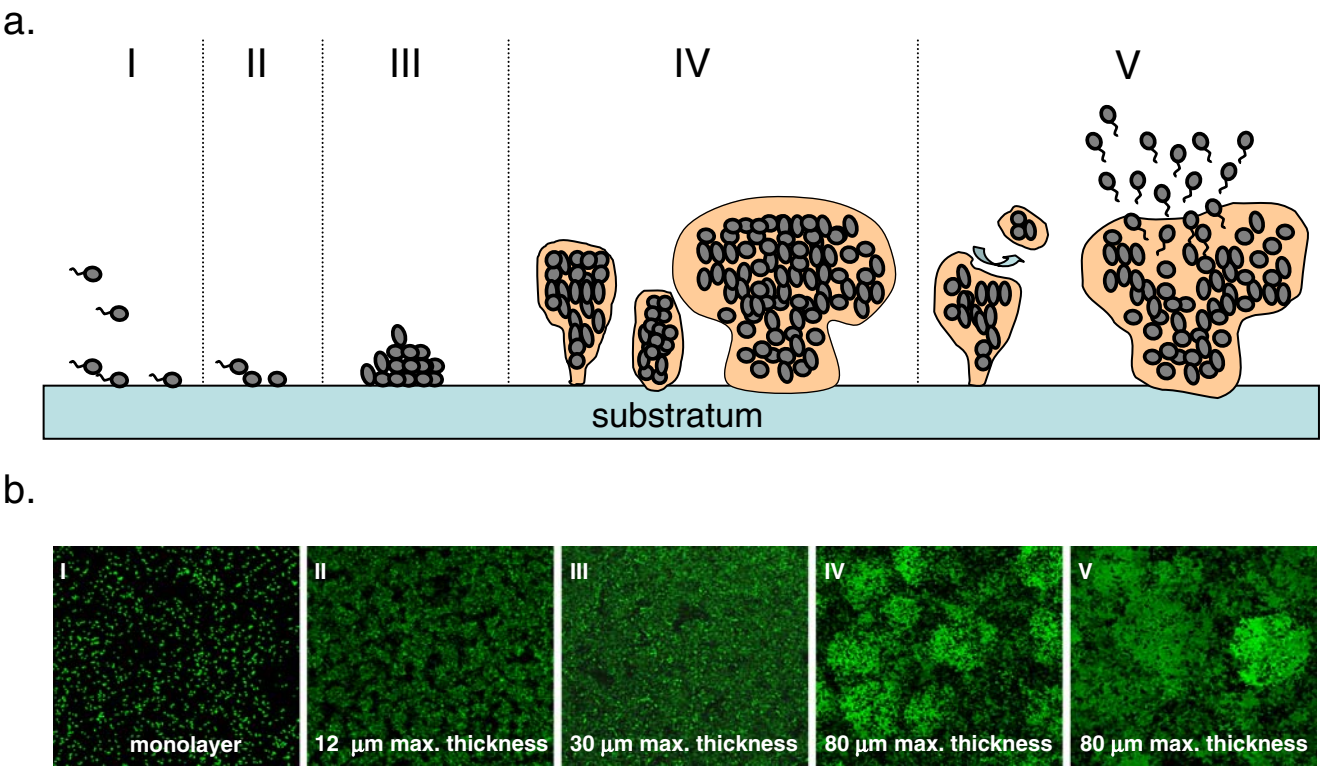

Fig. 1 **a** Model of *P. aeruginosa* biofilm development in a flow-through biofilm system. Bacteria are thought to attach to a surface reversibly (*I*), followed by irreversible attachment (*II*). Microcolony formation occurs (*III*), and complex three-dimensional structures know as stalks and towers form as the biofilm matures (*IV*). Dispersion or detachment of bacteria follows, which can occur by the separation of large pieces of encased bacteria or by a process in which bacteria become motile and tunnel out of the matrix (*V*). Bacteria are represented by *gray ovals*, with or without flagella, and the biofilm matrix in *orange*. **b** *P. aeruginosa* PAO1 expressing green fluorescent protein was grown for 4 days in a minimal media (modified FAB). A two-dimensional projection of the three-dimensional image is shown as a top-down view for 2 h (*I*), 1 day (*II*), 2 days (*III*), 3 days (*IV*), and 4 days (*V*) after inoculation. Maximal thickness of the biofilm is reported for each time period. Biofilm images were collected by a Leica confocal scanning light microscopy using a 488-nm wavelength. Images are at ×400 and represent a 250×250-μm field

P. aeruginosa. LecB is an important virulence determinant in *P. aeruginosa* infections and has been shown in vitro to decrease the ciliary beat frequency of the airway epithelium [28]. Mutations in *lecA*, or the type I *P. aeruginosa* lectin, resulted in thin biofilms as compared to the wild-type *P. aeruginosa* [29]. It is interesting to note that a recent study reported that LecA binds to 3O-C_{12}-HSL [30]. Genes involved in type IV biogenesis and LecA and LecB production are regulated by QS [9, 31].

Genetic analysis has identified several factors that are important for normal *P. aeruginosa* biofilm development. These include genes that encode structural components of the biofilm matrix. Overproduction of alginate, an exopolysaccharide, is the hallmark of the conversion of *P. aeruginosa* to a mucoid phenotype. The appearance of mucoid *P. aeruginosa* in CF infection is usually associated with chronic infection and poor clinical outcome [32, 33]. In mucoid *P. aeruginosa*, alginate is a matrix component in biofilms, and alginate-overproducing bacteria form complex-structured biofilms that are highly resistant to antimicrobials such as tobramycin [34]. Alginate also plays a protective role in *P. aerugniosa* infection and has been demonstrated to scavenge free radicals released by activated macrophages in vitro, to prevent phagocytic clearance, and to protect from defensins [35]. The regulation of alginate production has been well studied in *P. aeruginosa* [35]. However, in nonmucoid bacteria such as *P. aeruginosa* PAO1 and PA14, alginate is not the primary component of the biofilm matrix [36]. Instead, two loci, the *psl* (polysaccharide synthesis locus) in *P. aeruginosa* PAO1 and *pel* genes in *P. aeruginosa* PA14 and PAK [36], have been identified in in vitro assays and shown to be involved in the production of alternative polysaccharides. In PA14 and PAK, the *pel* genes produce a glucose-rich polysaccharide that is critical for pellicle formation and involved in biofilm development [36]. In *P. aeruginosa* PAO1, the *psl* genes have been shown to be important in the initiation and development of biofilms on both abiotic and biotic surfaces, such as mucin-coated surfaces and airway epithelial cells [36]. There is evidence that the *psl* and *pel* genes are regulated by the two-component system GacA/GacS and the small regulatory ribonucleic acid

rsmZ [36]. The GacA/GacS system also positively regulates QS in *P. aeruginosa* PAO1 [37]. It is interesting to note that the gene *pslB* (PA2232) was demonstrated to be QS regulated in the *P. aeruginosa* PAO1 microarray analysis, suggesting there is a link between QS and the expression of the *psl* locus [9]. In *P. aeruginosa* PA14, the *pel* genes have also been shown to be QS regulated [38]. The role of these novel matrix components in nonmucoid biofilm development is of interest as nonmucoid *P. aeruginosa* initially colonizes the CF lung, most likely forming biofilms, followed by a conversion to mucoidy and chronic infection [35]. Although the PSL and PEL polysaccharides are now known to be an important part of the extracellular matrix, alginate may still play a role in the nonmucoid biofilm phenotype. For example, recent experiments have demonstrated that alginate is critical for the protection of *P. aeruginosa* PAO1, PA14, and FRD1 (a muciod *P. aeruginosa* isolate) biofilms to gamma-interferon-mediated macrophage killing in an in vitro assay [39]. There is also evidence that nonmucoid strains produce alginate in vitro under hypoxic conditions, such as those that are thought to exist in the CF lung and in a mouse model of acute pneumonia [35].

Extracellular DNA is also a major component of the biofilm matrix and has been shown to be important in the initial and early development of *P. aeruginosa* biofilms [40, 41]. Exposure of *P. aeruginosa* to DNaseI prevented biofilm development in a flow-through model system and dissolved young biofilms [41]. Further research with QS mutants in *lasI* and *rhlI*, the genes that direct the synthesis of the autoinducers 3O-C_{12}-HSL and C_4-HSL, respectively, determined that QS is important in regulating extracellular DNA release during both planktonic and biofilm growth [42]. Furthermore, PQS, the third QS autoinducer signal, was linked to extracellular DNA release during biofilm development [42]. The extracellular DNA has been localized within the biofilm structure and varies temporally as well as spatially. Early biofilms show large amounts of extracellular DNA on the substratum and coating the microcolonies, while in a mature biofilm, it is present primarily in the stalks of mushroom structures [42]. The source of extracellular DNA has been proposed to be due to either prophage-induced cell lysis or the release of membrane vesicles [42]. Previous work has demonstrated that Pf-1 phage is produced by *P. aeruginosa* PAO1 biofilms [40, 43], and the genes that encode for the Pf-1 phage in *P. aeruginosa* PAO1 have been demonstrated to be QS regulated during planktonic growth [9]. PQS has also been demonstrated to be involved in membrane vesicle formation in *P. aeruginosa* [44]. It is important to note that biofilms that are deficient in extracellular DNA have been shown to be more sensitive to SDS [42]. This suggests that DNase I treatment, such as the current CF treatment regime of inhaled, nebulized recombinant human DNase I, could be important in preventing the establishment of biofilms that are involved in chronic infection.

Several studies have reported that *rhlA*, one of the genes required for rhamnolipid biosynthesis, is important in initial *P. aeruginosa* biofilm development, maturation, and dispersion. Rhamnolipid is well known to be regulated by QS, where the RhlR–C_4-HSL complex activates the transcription of *rhlA* [45]. A recent study by Pamp and Tolker-Nielsen [46] demonstrated that rhamnolipid is essential in the formation of the cap of the mushroom structure in mature biofilms. The study also suggested that *rhlA* is central in microcolony formation, as *rhlA* mutants formed thin, flat biofilms [46]. The deletion of *rhlA* results in thick biofilms with small or nonexistent water channels, suggesting that rhamnolipid is involved in the maintenance of these channels and/or dispersion of bacteria from the biofilm matrix [47–49]. Rhamnolipid has been demonstrated to be required for the dispersion of bacteria from the center of mushroom structures, where bacteria "tunnel" from the center of the macrocolony, and *rhlA* expression is localized to the center of macrocolonies about to undergo dispersion [47–49]. Bacteria that disperse from the biofilm often have altered phenotypes and are known as colony-morphotypic variants [50, 51]. Both *P. aeruginosa* PAO1 and clinical CF isolates have been shown to follow a pattern of biofilm development that includes dispersion, which is dependent upon QS and the presence of Pf-1 phage [50, 51]. Small-colony variants (SCVs) of *P. aeruginosa* PAO1 have been reported to display increased biofilm-forming capability, increased antimicrobial resistance, and enhanced dissemination [51], and SCVs have been isolated from CF patients [1]. It is interesting to note that more diverse morphotypes were reported from biofilm-dispersed CF isolates than PAO1. This may represent an in vivo selective adaptation process that maintains chronic infection.

Several genes encoding regulators have been identified that are important in *P. aeruginosa* biofilm development. These include *gacA*, the global carbon metabolism regulator *crc*, the stationary phase regulator *rpoS*, the three-component regulatory circuit *sadARS*, the response regulator for alginate production *algR*, and a hybrid sensor kinase/response regulator *retS* [52–57]. Mutations in *gacA* and *crc* caused defects in mircocolony formation, resulting in thin biofilms. As described previously, the GacA/GacS/*rsmZ* pathway is involved in the regulation of the extracellular matrix components *psl* and *pel*, which among its other influences may contribute to its regulation of normal biofilm development. Defects in type IV pilin production and twitching motility in *crc* mutants are thought to play a role in its defective biofilm phenotype [55].

AlgR has been previously shown to be required for full virulence in both acute septicemia and pneumonia infection

models. In vitro *algR* mutant biofilms display normal early biofilm development but a defect in mature biofilm development resulting in thin, patchy biofilms [52]. AlgR is also involved in type IV-mediated twitching motility, which may contribute to its defective biofilm phenotype [53]. However, further study has demonstrated that AlgR specifically represses the *rhl* QS system and is responsible for the observed mutant phenotype [52]. The dependence of AlgR regulation of the *rhl* system was found to be surface contact or biofilm dependent. Excess production of rhamnolipid, a *rhl*-regulated product, was observed in *algR* deletion biofilms [52]. Excess rhamnolipid production has been previously correlated for dispersion in biofilm development, resulting in biofilms with a similar phenotype as the *algR* deletion mutants, suggesting that this is the reason for the defect in biofilm maturation [47–49].

In contrast, *rpoS* mutants form thick biofilms in vitro studies [56]. Transcriptome analysis of planktonic bacteria demonstrated that *rpoS* regulates expression of nearly 800 genes in stationary phase growth [58]. It is interesting to note that there is a circular link between *rpoS* and QS regulation, and microarray analysis of the *rpoS* regulon in *P. aeruginosa* PAO1 revealed that 40% of *rpoS*-regulated genes are also QS regulated [58]. However, it is currently unclear which of these genes may contribute to its aberrant biofilm phenotype.

A *retS* mutant also formed more robust biofilms in contrast to wild-type *P. aeruginosa* [57]. Microarray analysis of a *retS* mutant also indicated that it is involved in the regulation of both the *psl* and *pel* loci, which likely is responsible for its enhanced biofilm formation in vitro [57]. The regulator *retS* appears to be involved in QS-mediated phenotypes including autoinducer production via the GacA/GacS/*rsmZ* pathway [57]. This regulator appears to activate genes implicated in acute phase infection, such as the type III secretion system, type IV pilin, and secreted virulence molecules including exotoxin A and lipase [57]. The study authors suggest that this gene is involved in mediating the transition of the *P. aeruginosa* phenotype from an acute to chronic phase infection [57].

Similarly, the *sadARS* system appears to regulate virulence factors, such as the type III secretion system, in addition to being crucial for normal *P. aeruginosa* biofilm formation [59]. Mutation in this loci results in biofilms with abnormal maturation, where the maintenance of the complex three-dimensional water channels is defective. The gene *sadA* has been previously demonstrated to be QS regulated [9]. It is interesting to note that SadR possesses an glutamate–alanine–leucine domain, which has been linked with a phospodiesterase activity that degrades *bis*-(3′,5′)-cyclic-dimeric-guanosine monophosphate (cyclic di-GMP), producing guanosine monophosphate [59]. Increasing cyclic di-GMP levels have been reported to promote biofilm development [60, 61], and several recent studies have revealed a role for cyclic di-GMP in *P. aeruginosa* biofilm formation [62–66]. In *P. aeruginosa* PAO1, decreased cyclic di-GMP levels inhibited biofilm formation, while increased cyclic di-GMP levels promoted biofilm formation in a flow-through system [63]. Protein receptors for cyclic di-GMP have been identified that regulate the production of biofilm extracellular matrix components [65, 66]. For example, cyclic-di-GMP has been demonstrated to bind to one of the proteins in the *pel* operon, PelD, and modulate production of the extrapolysaccharide matrix protein PEL in *P. aeruginosa* PA14 [65]. Cyclic di-GMP has also been demonstrated to be required for alginate biosynthesis in mucoid *P. aeruginosa*, where the protein Alg44 binds cyclic di-GMP and then activates polymerization or transport of the alginate polysaccharide [66]. A role for cyclic di-GMP has also been proposed in biofilm dispersion. Recently, a novel regulator, *bdlA*, has been reported to be involved in the nutrient-induced dispersion of *P. aeruginosa* PAO1 biofilms [67]. BdlA has been proposed to transduce environmental signals that modulate the intracellular levels of cyclic di-GMP, thereby promoting biofilm dispersion [67].

Although there is clear evidence for the role of QS in biofilm development (Fig. 2), other studies have been performed with varying conclusions as to the relative importance of QS in biofilms [10]. These inconsistencies may be attributed to the different conditions, including hydrodynamics and media, and/or *P. aeruginosa* strains utilized in these studies. Nevertheless, several genes that have been identified as QS regulated, including *rhlA*, *rpoS*, *sadA*, and those in the denitrification pathway, have been shown to be important in *P. aeruginosa* biofilm development, maintenance, and dispersion [10, 59, 68, 69]. A recent study of *P. aeruginosa* biofilms in the mouse thermal injury model suggested that QS was not important in the formation of biofilms in acute infection [70]; however, several previous studies have clearly demonstrated a role for QS in this model [71–74]. Further investigation is clearly needed to precisely define the role of QS in both in vitro and in vivo biofilm development.

The CF lung and *P. aeruginosa* biofilms

There are several lines of evidence that support the notion that *P. aeruginosa* exists in a biofilm in the CF lung. These include microscopic analysis of CF sputum, increased antimicrobial resistance of *P. aeruginosa* in the CF lung, and the ratio of the *P. aeruginosa* autoinducer molecules, 3O-C_{12}-HSL and C_4-HSL, in sputum from CF patients mirrors those of biofilm-grown *P. aeruginosa* [75, 76]. The environment of the CF lung is thought to promote

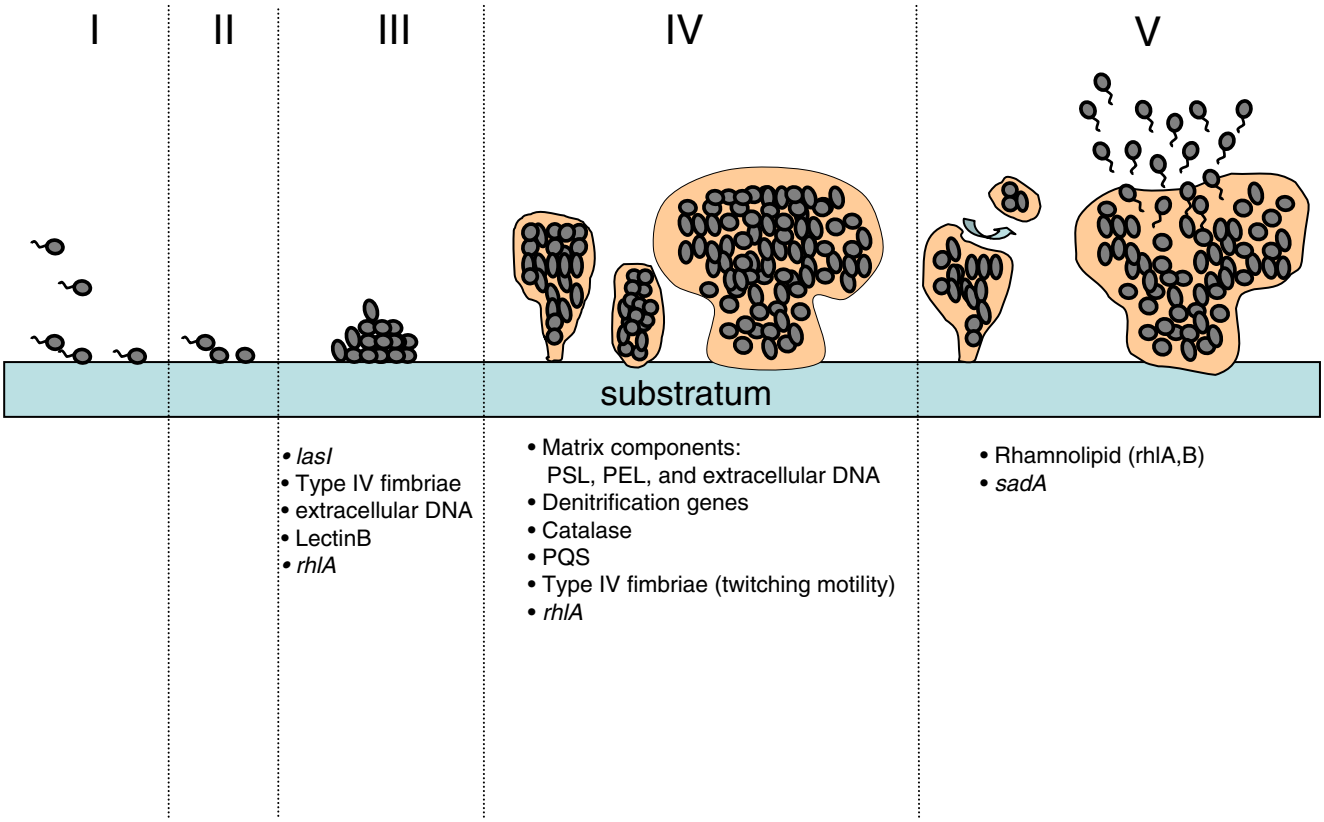

Fig. 2 Genes and factors implicated during *P. aeruginosa* biofilm development that are QS regulated. Genes and factors are listed below the developmental stage of biofilm formation in which they have been reported to be important

P. aeruginosa biofilm formation. Mutations in the CF transmembrane regulator (CFTR) cause a defective function of CFTR in airway epithelium and submucosal glands, leading to decreased mucociliary clearance and altered airway surface liquid (ASL) concentrations [77]. In addition, the nitric oxide (NO) system, involved in bacterial killing, is impaired in CF patients as reflected by a reduction in expression of the inducible NO synthase (iNOS) responsible for NO production in neutrophils, macrophages, and epithelial cells [16].

The ability of *P. aeruginosa* to grow anaerobically is believed to be important in biofilm development in the CF lung [76, 78]. Worlitzsch et al. [79] reported that oxygen gradients exist in thick mucus plugs present in the lower airway of CF patients. As neutrophils infiltrate and mucus secretion increases, metabolically active bacteria and epithelial cells consume the available oxygen, and the surroundings quickly become oxygen limited [79]. The conversion of *P. aerugiosa* to a mucoid phenotype during the progression of chronic CF lung disease may further restrict the diffusion of oxygen and contribute to the microaerobic or anaerobic environments in CF infections. Recent data suggest that anaerobic environments promote the conversion of nonmucoid *P. aeruginosa* to mucoidy [79]. CF airway mucus has been demonstrated to support anaerobic growth of *P. aeruginosa* [79]. *P. aeruginosa* is able to grow anaerobically using the alternative electron acceptors nitrate, nitrite, and nitrous oxide through a respiratory process termed denitrification [80]. Nitrate and nitrite concentrations in CF sputum are able to support anaerobic respiration of *P. aeruginosa* [79, 81–83]. In the absence of nitrate or nitrite, arginine can be catabolized to ornithine using substrate-level phosphorylation and used as an energy source for anaerobic growth [84, 85]. *P. aeruginosa* can also use pyruvate to sustain long-term survival, but this fermentation process can not support growth of the bacterium, under hypoxic conditions [86]. Anaerobic conditions promote biofilm development in both *P. aeruginosa* PAO1 and in CF isolates, suggesting that *P. aeruginosa* readily adapts and perhaps favors anaerobic biofilm growth in vivo [87].

Anaerobiosis is not only important in *P. aeruginosa* survival in the CF lung environment but also within the biofilm itself. Within an aerobically grown biofilm, oxygen depletion has been reported to occur within 30 μm of the surface of the biofilm [20]. As *P. aeruginosa* forms biofilms in the CF mucus, the bacteria undergo a switch from an aerobic to an anaerobic metabolism [78]. *P. aeruginosa* existing within the biofilms experience oxygen gradients, in which the base and the center of the biofilm are anaerobic

[20, 79, 88]. Genes involved in denitrification have been identified as QS regulated, suggesting a link between anaerobic metabolism and QS [9]. A further study reported that during anaerobic biofilm growth of *P. aeruginosa*, the *rhl* QS system was required for proper balance of the denitrification pathway [89]. Dysregulation of the pathway resulted in the accumulation of toxic intermediates, such as nitrite and nitrous oxide, which caused cell death to occur within the biofilm.

It is interesting to note that nitrous oxide has been demonstrated to be a signal involved in *P. aeruginosa* biofilm dispersal [90]. Exposure of *P. aeruginosa* biofilms to NO in combination with antimicrobials or SDS enhanced the activity of these agents. In addition, recent data suggest that mucoid *P. aeruginosa* CF isolates are exquisitely sensitive to nitrite due to an inability to remove the toxic accumulation of NO [91]. The fact that iNOS activity decreases as the chronic CF infection progresses may contribute to the ability of *P. aeruginosa* to persist as a biofilm.

The thick mucus present in the airways of CF lungs provides a diffusion-restricted environment. A recent study demonstrated that in ASL, *P. aeruginosa* PAO1 formed macrocolonies resembling biofilms dependent upon the concentration of solids [92]. In normal ASL, PAO1 failed to form macrocolonies. However, in concentrated ASL mimicking ASL found in CF patients, PAO1 readily formed biofilms under either aerobic or anaerobic conditions. Furthermore, the formation of these macrocolonies was dependent upon a functional *las* QS system. The mutation in *lasI* abrogated macrocolony formation. Diffusion studies demonstrated that concentrated ASL greatly restricted the movement of 3O-C_{12}-HSL, the product of the *lasI* gene. The constrained microenvironment in the CF ASL is thought to promote *P. aeruginosa* biofilm formation by concentrating 3O-C_{12}-HSL and activating QS-regulated factors involved in biofilm development.

Quorum sensing in *P. aeruginosa* pathogenesis and implications in vivo

Both in vitro and in vivo models have clearly demonstrated the importance of QS in *P. aeruginosa* pathogenesis. Numerous models of infection, including plant, invertebrate, and animal models, have supported the premise that QS contributes to *P. aeruginosa* pathogenesis [71–74, 93–101]. In animal models of lung pathogenesis, including the mouse model of acute pneumonia, the mouse model of chronic lung infection, and the rat chronic lung infection model, *lasR*, *lasI*, and *rhlI* have been demonstrated to be required for virulence [73, 93, 102–105]. Other QS-regulated factors, such as type IV pilin biosynthesis and type III secretion pathways, have been clearly linked to *P. aeruginosa* pathogenesis [9, 98].

Evidence exists that *P. aeruginosa* QS is active in CF patients. Several studies have reported the presence of transcripts for known QS regulators and QS-regulated genes in CF patient samples, suggesting a role for QS in vivo. Transcripts for *lasR*, *lasI*, *lasA*, *lasB*, and *toxA* have been detected in sputa samples from chronically infected CF patients [106, 107]. There appeared to be a correlation of *lasR* to the expression *lasI*, *lasR*, *lasA*, *lasB*, and *toxA*, and as *lasR* is known to regulate expression of these genes in vitro, this relationship suggested that *lasR* also regulates these genes in vivo [106]. A further study reported a statistically significant correlation between *lasR* and *algD* transcription [106]. This suggested that *lasR* might to some extent regulate *algD* or, alternatively, be activated by a common environmental trigger in vivo. AlgD catalyzes the first step in alginate biosynthesis, which is responsible for the mucoid phenotype often observed in clinical isolates from *P. aeruginosa* chronically infected CF patients [108]. The autoinducer molecules 3O-C_{12}-HSL, C_4-HSL, and PQS are also present in CF sputa samples, and PQS has been detected in bronchoalveolar lavage fluid and muco-

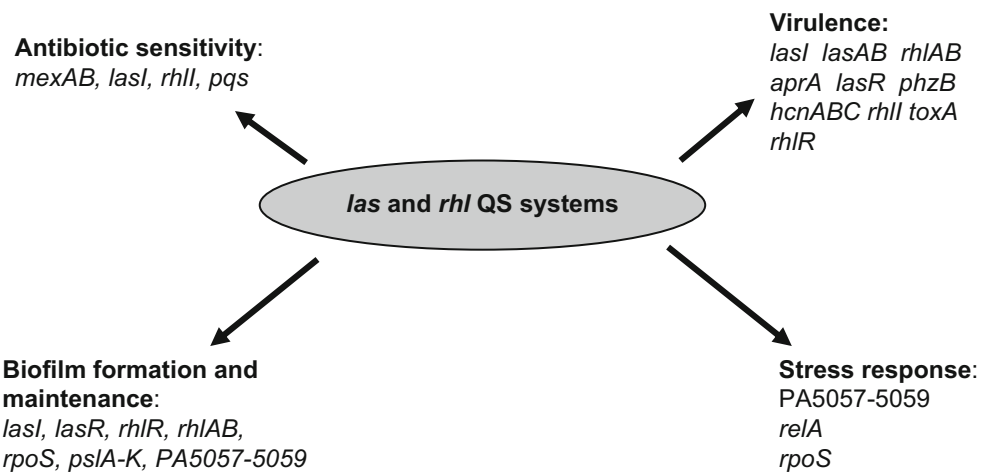

Fig. 3 Quorum sensing regulates multiple phenotypes in *P. aeruginosa*. Several genes that have been identified to contribute to each phenotype are listed

purulent fluid from distal airways of end-stage lungs removed at transplant in CF patients [106, 109, 110].

Curiously, the isolation of *P. aeruginosa* from acute and chronic infections, including the infected CF lung, with defects in QS suggests that the role of QS is diminished or perhaps not important in vivo [111, 112]. The dichotomy between the isolation of QS-deficient *P. aeruginosa* from infections and the importance of QS in the infectious process has been a conundrum. This is important as QS is thought to be an attractive target for the development of novel therapeutics [78, 113]. However, a recent study reported that in instances of high QS activity, spontaneous QS mutants, usually in the main regulator *lasR*, occur [112]. Mutants in *lasR* are more resistant to cell lysis and death at high cell densities and at alkaline pH. These "social cheaters" appear to have a competitive advantage over *P. aeruginosa* with intact QS systems. The appearance of QS mutants suggests, in contrast to previous belief, high QS activity due to the metabolic burden QS places on the bacteria.

Summary

P. aeruginosa possesses many traits, including intrinsic antimicrobial resistance, ability to form biofilms, arsenal of virulence products, and metabolic versatility that contribute to its ability to colonize and persist in acute and chronic infections such as those of CF patients. In *P. aeruginosa*, QS regulates many of these phenotypes (Fig. 3). The CF lung presents an ideal niche for *P. aeruginosa*. Dysregulation in mucocilliary clearance and NO-mediated killing are thought to contribute to colonization of *P. aeruginosa* in the CF airways. A restricted microenvironment due to thick airway liquid results in decreased oxygen concentrations and increased localized autoinducer concentrations that have been demonstrated to promote biofilm development. The biofilm mode of growth subsequently provides protection against numerous host factors and antimicrobial challenge. Due to its role in pathogenesis, biofilm development, and antimicrobial resistance, QS represents an attractive target for the development of novel therapeutics. Further understanding of the QS mechanisms involved in these phenotypes will substantially aid in this goal.

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