Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development

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Abstract

During the past decade, there has been a renewed interest in using *P. aeruginosa* as a model system for biofilm development and pathogenesis. Since the biofilm matrix represents a critical interface between the bacterium and the host or its environment, considerable effort has been expended to acquire a more complete understanding of the matrix composition. Here, we focus on recent developments regarding the roles of alginate, Psl, and Pel polysaccharides in the biofilm matrix.

Introduction

The bacterium *Pseudomonas aeruginosa* is a remarkably adept opportunist. If provided a breach in local or systemic immunity, the organism can cause severe, life-threatening infections. These infections persist despite aggressive antimicrobial therapy and a robust inflammatory response. Perhaps the most widely recognized *P. aeruginosa* persistent infection occurs in individuals with the genetic disease cystic fibrosis (CF).

The persistence of *P. aeruginosa* during CF infections has been linked, in part, to its ability to form biofilms [1,2]. Biofilms, which are defined as communities of microorganisms that are attached to a surface, play a significant role in infectious disease [3]. Bacteria within biofilms are usually embedded within a matrix, which can consist of protein, polysaccharide, and nucleic acid. The matrix provides a critical role in the biofilm resistance phenotype. A complete understanding of the organization and composition of the *P. aeruginosa* biofilm matrix may assist in the development of novel therapeutics aimed at disrupting biofilms, which will translate into improved clearance of these infections. Here, we focus on the polysaccharide components of the extracellular polymeric substance (EPS) of *P. aeruginosa* biofilms.

Alginate

The CF lung is initially colonized with nonmucoid *P. aeruginosa* strains, but with time, mucoid variants emerge and become the predominant lung pathogen [4]. The mucoid phenotype is due to the overproduction of alginate, a capsular polysaccharide virulence factor that confers a selective advantage for *P. aeruginosa* in the CF airway. Alginate is a high molecular weight, acetylated polymer composed of non-repetitive monomers of β-1,4 linked L-guluronic and D-mannuronic acids. Alginate-producing variants arise *in vivo* most frequently due to mutations in the negative regulator *mucA* [5]. There is a distinct correlation between the appearance of

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mucoid *P. aeruginosa* and a worsening clinical prognosis for CF patients [4]. Infection of the CF lung causes inflammatory cells to be recruited to the site of infection, where they release reactive oxygen species and cause extensive tissue damage [4,6]. Alginate appears to protect *P. aeruginosa* from the consequences of this inflammation, since it scavenges free radicals released by activated macrophages *in vitro* and appears to provide protection from phagocytic clearance [4,6]. Although antibodies to alginate are found in the sera of chronically infected CF patients, these antibodies fail to mediate opsonic killing of *P. aeruginosa in vitro* [7]. Likely each of these properties contributes to the ability of mucoid *P. aeruginosa* to persist and establish chronic infections in the CF lung. Since aspects regarding the genetics, pathogenesis, and biochemistry of alginate have been reviewed elsewhere [4,8,9], we focus attention on observations regarding the role of alginate in biofilms of mucoid *P. aeruginosa*.

Results from several independent laboratories have shown that overproduction of acetylated alginate leads to significant architectural and morphological changes in the biofilm [10–13]. This translates into increased resistance to antimicrobials [10] or IFN-gamma-mediated killing by cells of the innate immune system [14]. In support of this, Alkawash and colleagues showed that degradation of mucoid *P. aeruginosa* biofilms by alginate lyase led to enhanced killing by gentamicin [15]. Surprisingly however, studies from two of these groups and our work showed that alginate synthesis is not required for biofilm development [11,12,16]. In these studies, alginate proficient and deficient *P. aeruginosa* formed morphologically similar biofilms. However, overproduction of alginate, which occurs primarily as a result of *mucA* mutations, clearly affects resistance properties of the biofilm.

Psl

The above-mentioned studies were performed in alginate overproducing strains (i.e. *mucA* mutants). During the past decade, there has been a renewed interest in using *P. aeruginosa* as a model system for biofilm development and pathogenesis. Most of these studies have been performed with nonmucoid (i.e. *mucA*+) *P. aeruginosa* strains such as PAO1 or PA14, which produce little to no detectable alginate *in vitro*. Furthermore, when the alginate genes were disrupted in PAO1 and PA14, these strains were fully capable of forming biofilms. The biofilms formed by these mutants retained what appeared to be polysaccharide matrix material [16]. This suggested that one or more polysaccharides independent of alginate might be essential for biofilm development in nonmucoid *P. aeruginosa* strains. Several groups initiated studies to identify alternative polysaccharide-encoding genes, and two loci were discovered. The first, *pel* (Fig. 1), was found to be involved in pellicle formation in strain PA14 [17]. The role of *pel* in the biofilm matrix will be discussed below. The second polysaccharide locus designated *psl* (polysaccharide synthesis locus, Fig. 1), is an operon composed of 15 genes encoding the Psl biosynthetic machinery. The *psl* operon was shown to be essential for biofilm formation in strains PAO1 and ZK2870 [18–21]. In these studies, inactivation of the *psl* gene cluster led to a significant defect in cell-surface and cell-cell interactions. Psl is also required for adherence to mucin-coated surfaces and airway epithelial cells; biotic surfaces that are clearly relevant to CF [20]. A study in 2006 utilizing an inducible *psl* construct found that in addition to being required for cell-surface and cell-cell interactions, *psl* is also needed for maintenance of the biofilm structure post-attachment. This led the authors to conclude that Psl functions as a scaffold, holding biofilm cells together in the matrix [20].

Carbohydrate and lectin staining analyses indicate that Psl is a mannose- and galactose-rich polysaccharide, however the precise Psl structure has not been elucidated [19,21,22]. This is an area requiring future research. Overhage and Campisano *et al.* demonstrated that *pslA* and *pslD*, which encode a putative UDP-glucose lipid carrier and exporter, respectively, are essential for biofilm formation in strain PAO1 [23,24]. They also demonstrated that while *psl* is constitutively expressed in planktonic cells, its expression is localized to the center of

developing biofilm microcolonies [24]. This implies that Psl has a role in biofilm differentiation. Our lectin staining studies show Psl is equally distributed in undifferentiated, flat multiple-layer biofilms. However, mature microcolonies reveal peripheral staining of Psl with minimal staining of matrix in the center of the microcolonies. Instead, this region has numerous motile cells representing a biofilm at a developmental stage just prior to dispersion (L. Ma and D.J. Wozniak, unpublished data).

Pel

P. aeruginosa is able to form biofilms not only on mucosal and other solid surfaces but also at the air-liquid interface of standing cultures. The genetic basis for these structures, known as pellicles, was elucidated by screening a PA14 transposon library for pellicle-deficient mutants. This revealed a seven-gene operon, named *pel* (Fig. 1), which is necessary for maintenance of biofilm structure in strain PA14 [17]. The authors hypothesized that *pel* is involved in the production of an extracellular matrix material. To determine the nature of this *pel*-associated matrix material, mutants lacking one or more *pel* genes were evaluated for biofilm initiation, colony morphology, and mature biofilm integrity. While biofilm initiation *per se* was not significantly affected in PA14 *pel* mutants, the colony morphology was affected as well as the ability of these cells to bind Congo red [17]. Carbohydrate and linkage analyses provide evidence that *pel* encodes a glucose-rich matrix polysaccharide polymer, which does not appear to be cellulose [17,19]. As with Psl, the Pel structure is unknown and further biochemical analyses of Pel polysaccharide is necessary.

In a similar study using a non-piliated PAK strain, a transposon screen for nonadherent mutants generated several mutations that mapped to the *pel* locus [25]. The authors suggested that any role for *pel* in attachment might not be observed because type IV pili may compensate for a lack of *pel* during attachment [25]. As predicted, biofilm initiation was significantly reduced in non-piliated PAK *pel* mutants. Clearly the roles of *pel* and type IV pili in the initial attachment process will need to be delineated.

Genes *pelA-G* (Fig. 1) are highly conserved in other *P. aeruginosa* strains, including the common laboratory strain PAO1 [17]. The Gram-negative plant pathogen *Ralstonia solanacearum* contains a homologous gene cluster that, when mutated, resulted in a biofilm-defective phenotype similar to that observed in *P. aeruginosa pel* mutants [25]. Putative functions in polysaccharide processing have been assigned to most of the *pel* genes, (Fig. 1) and [17,19,25,26].

Thus, the *pel* locus produces a glucose-rich matrix polysaccharide that is essential for pellicle formation and biofilm structure in *P. aeruginosa* strains PA14 and PAK. The *pel*-encoded polysaccharide is biochemically and genetically distinct from Psl. To date, no immunological or lectin reagents are available to probe Pel expression or localization in developing *P. aeruginosa* biofilms.

Regulation of Psl and Pel polysaccharide

Currently, little is known regarding the regulation of Psl and Pel but several seemingly disparate findings have begun to shed some light on this issue. D'Argenio *et al*. performed transposon mutagenesis of strain PAO1 and isolated a number of colonies that exhibited a "wrinkly" colony phenotype [27]. Genetic analysis of these mutants revealed that mutations in the *wspF* gene lead to the hyperaggregative phenotype. The *wsp* locus was first described in *P. fluorescens* and appears to encode a chemosensory system involved in the <u>w</u>rinkly <u>s</u>preader <u>p</u>henotype [28]. Here, WspF controls the methylation state of WspA, which subsequently controls activation of the response regulator WspR. If *wspF* is inactivated, WspA is hypermethylated and WspR is constitutively active.

In a later study, Kirisits *et al.* reported similar hyperaggregative and hyperadherent PAO1-derived colony variants isolated from biofilm reactors [29]. Transcriptional profiling of these variants showed increased *psl* and *pel* expression, when compared with the parental PAO1 strain. Disruption of the *psl* operon in the variant reversed the hyperaggregative and hyperadherent phenotype but colonies still retained a wrinkled phenotype, presumably due to Pel overexpression. Therefore, the authors conclude that *psl*, in addition to *pel* and perhaps other components are expressed at a higher level and responsible for the hyperaggregative and hyperadherent variant phenotype [29]. Similar conclusions were drawn by Friedman and Kolter when analyzing the autoaggregative *P. aeruginosa* variant ZK2870 [19]. In support of this, over-expression of Psl via a p*BAD*-derived promoter system is sufficient to convert *P. aeruginosa* to a phenotype that resembles the above-mentioned autoaggregative variants [20].

It is reasonable to assume that the wrinkled colonies in the aforementioned D'Argenio study [27] also overexpress *psl* and *pel* and that this overexpression is caused by activation of WspR. In fact, a later study by Hickman *et al.* showed an increase in *psl* and *pel* expression in a *wspF* mutant [30]. This study further evaluated the effect of the Wsp system by investigating the role of WspR as a diguanylate cyclase. Proteins with this activity generate cyclic-di-GMP, a small signaling molecule involved in many cellular processes [31]. The levels of cellular c-di-GMP appear to correlate with the biofilm forming ability of *P. aeruginosa*. When WspR was constitutively activated, as seen in a *wspF* mutant, c-di-GMP levels were high and biofilm formation was greater than in the wild type strain. As seen in the above-mentioned autoaggregative variants, a transcriptional profile of *wspF* mutant bacteria revealed elevated levels of *psl* and *pel* transcription, when compared with the parental strain. This study also elegantly illustrated that when c-di-GMP was degraded, biofilm formation decreased substantially. This strengthens the relationship between *psl* and *pel* expression, the Wsp chemosensory system, and cellular levels of c-di-GMP.

Another regulatory system controlling *psl* and *pel* expression is the GacS/GacA/*rsmZ* system. In a study of two-component systems in strain PAK, a mutation in *retS*, encoding a hybrid sensor kinase/response regulator, elevated *psl* and *pel* expression resulting in enhanced biofilm formation [32]. A second round of transposon mutagenesis in the *retS* strain revealed that mutations in the GacS/GacA/*rsmZ* regulatory pathway reversed the *retS* phenotype. GacS and GacA are a sensor-regulator two-component pair and *rsmZ* is a small regulatory RNA that represses the activity of the posttranscriptional RNA binding protein RsmA. The model for how this system functions is as follows [33]: signals that activate RetS repress expression of *rsmZ* whereas signals that activate GacS induce *rsmZ* expression. When *rsmZ* levels are high, RsmA is inactive and this results in increased *psl* and *pel* expression. The opposite is true: low levels of *rsmZ* favor repression of *pel* and *pel.*. More recent work identified LadS, encoding a hybrid sensor kinase, which also modulates *rsmZ* levels [34] and therefore indirectly affects *psl* and *pel* expression.

Conclusions and perspectives

Since discovery of the Psl and Pel polysaccharides four years ago, much progress has been made in our understanding of the *P. aeruginosa* biofilm matrix. This has forced us to reconsider the notion that all *P. aeruginosa* biofilms are composed of alginate. We also must consider that the relationship between biofilm formation by nonmucoid strains and those in which conversion to mucoidy has occurred does not appear to be a change in the alginate levels in the biofilm matrix, but a fundamental change in its carbohydrate constituents. In the context of CF infections, the discovery of alternative polysaccharides that contribute to biofilm formation in nonmucoid strains, which are believed to be the first to colonize, implies that during initial infection, biofilm formation may precede the switch to mucoidy. Clearly the

potential role of Psl and Pel in *P. aeruginosa* pathogenesis is an area needing further investigation.

Future studies aimed at an understanding of individual *psl* and *pel* gene functions and regulation of their expression will provide more information about the selective advantage of individual *P. aeruginosa* polysaccharides. Additionally, the potential overlapping or redundant functions of the Pel and Psl polysaccharides of nonmucoid *P. aeruginosa* is an area in need of study. A seminal question is whether individual cells have the capacity to produce more than one polysaccharide simultaneously. Do mucoid biofilms also contain Pel and/or Psl? Collectively, the regulation studies illustrate that *psl* and *pel* appear to be coordinately regulated and that c-di-GMP levels play a critical role in this control. The challenge in the coming years will be to discern how the Wsp and RsmA/*rsmZ* pathways are integrated to control the acute vs. biofilm lifestyle of *P. aeruginosa*. Finally, the role of polysaccharide-independent components, such as extracellular DNA, LPS, membrane vesicles, lectins, and *cup* fimbriae must be placed in context with emerging data regarding alginate, Psl, and Pel polysaccharides.

Since Psl and Pel polysaccharides are expressed on planktonic *P. aeruginosa* cells, play a critical role in biofilm development, and are conserved among *P. aeruginosa* strains examined, these polysaccharides clearly represent attractive targets for agents aimed at disrupting the matrix. As chronic infections due to mucoid *P. aeruginosa* are almost impossible to eradicate, targeting Psl and Pel may allow us to prevent initial infection or enhance the clearance of an existing nonmucoid infection. Once a better understanding of the chemical nature of the polysaccharide matrix and its link to colonization and pathogenesis of *P. aeruginosa* develops, this may be feasible.

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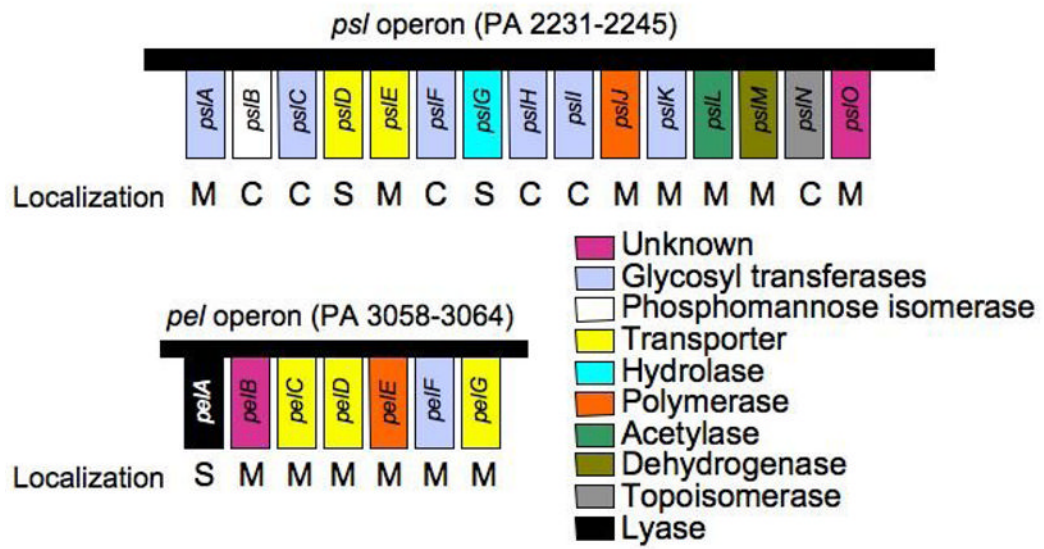**Fig. 1.**
Structure of *psl* and *pel* operons. Putative functions and localization of Psl and Pel enzymes are shown (M-membrane, C-cytoplasm, S-secreted).