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Pseudomonas biofilm matrix composition and niche biology

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Abstract

Biofilms are a predominant form of growth for bacteria in the environment and in the clinic. Critical for biofilm development are adherence, proliferation, and dispersion phases. Each of these stages includes reinforcement by, or modulation of, the extracellular matrix. *Pseudomonas aeruginosa* has been a model organism for the study of biofilm formation. Additionally, other *Pseudomonas* species utilize biofilm formation during plant colonization and environmental persistence. Pseudomonads produce several biofilm matrix molecules, including polysaccharides, nucleic acids, and proteins. Accessory matrix components shown to aid biofilm formation and adaptability under varying conditions are also produced by pseudomonads. Adaptation facilitated by biofilm formation allows for selection of genetic variants with unique and distinguishable colony morphology. Examples include rugose small-colony variants and wrinkly spreaders (WS), which over produce Psl/Pel or cellulose, respectively, and mucoid bacteria that over produce alginate. The well-documented emergence of these variants suggests that pseudomonads take advantage of matrix-building subpopulations conferring specific benefits for the entire population. This review will focus on various polysaccharides as well as additional *Pseudomonas* biofilm matrix components. Discussions will center on structure–function relationships, regulation, and the role of individual matrix molecules in niche biology.

Keywords

polysaccharide; eDNA; alginate; levan; Psl; Pel

Introduction

Cooperation among communities has significant biological advantages for individual members. Herds of mammals, flocks of birds, schools of fish, or colonies of insects are all prime examples of sociobiology existing to afford members of the population advantages they would not solely achieve. While in many cases the organisms that reside amidst a community can survive some period of time without their counterparts, life becomes simpler with the protection of the group. Prominent examples include protection from predators, adverse environmental conditions, and starvation. The latter is especially important for

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weaker members of the society who easily gain access to a consistent nutrient supply. In one of life's simplest forms, microorganisms are being increasingly studied in the context of community organization. Similar to the advantages that herds, schools, and flocks acquire, bacterial biofilms confer protection from external pressures while maintaining persistence from harsh environmental conditions (Klausen Costerton, Stewart & Costerton, 2001). Interestingly, cells within biofilm have diverse gene expression although the community is comprised of clonal members (Boles & Singh, 2008). Examples of polymicrobial biofilms are prevalent, adding an additional layer of complexity to the cooperative and competitive nature within biofilms. Sociobiology of community microorganisms is not a novel concept but proposed to explain heterogeneity in biofilm. While many advantages of sociobiology exist, members failing to provide a community asset, described as ‘selfish cheaters’ who take advantage of the community lifestyle regardless of input, also seem to be present (West *et al.*, 2006). This review will discuss the components used by the predominant bacterial biofilm-forming genus *Pseudomonas* to build and maintain fundamental biofilm communities. Greater detail regarding the sociobiology perspective of biofilm cells can be gleamed from West *et al.*, (2006) discussion. Specifically, production of virulence-relevant polysaccharides by *Pseudomonas aeruginosa* used for biofilm matrix molecules will be covered in greatest detail.

The pseudomonads are ubiquitous environmental organisms, occupying several niches. While some *Pseudomonas* species have the propensity to cause disease, others simply reside in their natural habitat. Frequently in either pathogenic or environmental conditions, the bacteria exist attached to a surface and encased in some form of polymeric substances, characteristic of a biofilm. Biofilm formation by *P. aeruginosa* is of particular interest because of its clinical relevance (Donlan & Costerton, 2002), but other *Pseudomonas* spp. have also been studied in conditions such as plant tissue (Osman *et al.*, 1986; Fakhr, *et al.*, 1999; Preston *et al.*, 2001), soil (Schnider-Keel, *et al.*, 2001; Dechesne *et al.*, 2010) or fresh water streams (Costerton *et al.*, 1987). Comparison of *Pseudomonas* spp. biofilm formation strategies will aid a more complete understanding of the individual species’ unique and conserved mechanisms.

Biofilm-forming organisms rely on extracellular polymeric substance (EPS), also known as matrix, which is essential for colonization of surfaces and volumes (Sutherland, 2001a, b; Flemming *et al.*, 2007). Biofilm development is highlighted in Box 1; where the *P. aeruginosa* biofilm paradigm describes individual bacteria cells initiating adherence to a substratum followed by clonal propagation, matrix building, and eventual biofilm maturation (Costerton *et al.*, 1995; Costerton *et al.*, 1999; Stoodley *et al.*, 2002). Finally, biofilm populations release or disperse small aggregates or even individual cells for seeding of uncolonized sites and reinitiating the biofilm lifecycle (Costerton *et al.*, 1995; Ma *et al.*, 2009; Monds & O'Toole, 2009). Biofilm maturity is hallmarked by the presence of ‘capped’ mushroom-shaped structures (Box 1). The flat biofilm occurs during early surface colonization and propagation. Dense microcolonies initiate stalk formation that eventually forms the capped mushroom structures that are characteristic of *P. aeruginosa* biofilm (Box 1). Cell surface factors have been identified, which allow for initial interaction with surfaces and structure formation including pili (Klausen *et al.*, 2003a, b), flagella (O'Toole & Kolter,

1998; Klausen *et al.*, 2003a, b), proteins (Monds *et al.*, 2007; Newell *et al.*, 2009; Borlee *et al.*, 2010), and extracellular polysaccha-rides (Nivens *et al.*, 2001; Wozniak *et al.*, 2003; Friedman & Kolter, 2004a, b; Jackson *et al.*, 2004; Matsukawa & Greenberg, 2004; Ma *et al.*, 2006; Ryder *et al.*, 2007; Starkey *et al.*, 2009; Byrd *et al.*, 2010). These factors are commonly categorized as biofilm matrix components. Interestingly, in recent years, the predominance of nucleic acids among biofilm EPS has lead to the investigation of the importance of DNA in stabilizing the biofilm matrix (Whitchurch *et al.*, 2002; Webb *et al.*, 2003; Allesen-Holm *et al.*, 2006; Yang, *et al.*, 2007). In most cases, one or two of these components are the most abundant in the biofilm matrix at a given stage of biofilm development or in individual strains, although it is common to have accessory function from other components. Predominantly, *P. aeruginosa* requires polysaccha-ride in its biofilm matrix at several developmental stages, while taking advantage of nucleic acids during later maturation stages (Klausen *et al.*, 2003a, b; Webb *et al.*, 2003; Allesen-Holm *et al.*, 2006; Yang *et al.*, 2007; Ma *et al.*, 2009). Two distinct classes of *Pseudomonas* polysaccharides have been shown to have a role in biofilm formation. Capsular polysaccharides maintain characteristics of protective dynamic polymers that decorate the exterior of one or more cells. Alternatively, aggregative polysaccharides offer structural integrity and interact with additional matrix components. Capsular polysaccharides provide a coat around a bacterium, while aggregative polysaccharides do not. Both capsular and aggregative polysaccharides will be the primary focus of this review as well as accessory biofilm matrix molecules influencing the function of *Pseudomonas* biofilms. The functions of each of these biofilm matrix components will also be discussed in the context of roles they play within the habitat that the microorganism resides (i.e. niche biology).

Capsular polysaccharides

Pseudomonas spp. has been isolated from diverse environmental conditions, and characterization of these isolates reveals that polysaccharide production is important for colonization of these niches (Osman *et al.*, 1986; Keith & Bender, 1999; Gal *et al.*, 2003; Laue *et al.*, 2006; Chang *et al.*, 2007; Li *et al.*, 2010). Furthermore, the production of polysaccharides by biofilm-forming organisms supports colonization by facilitating aggregation, adherence, and surface tolerance (Osman *et al.*, 1986; Penaloza-Vazquez *et al.*, 1997; Keith *et al.*, 2003; Laue *et al.*, 2006). Most notably, *P. aeruginosa* produces alginate, and because of its correlation with adverse clinical outcomes, it has been closely monitored and investigated (Boucher *et al.*, 1997; Hentzer *et al.*, 2001; Nivens *et al.*, 2001; Bjarnsholt, *et al.*, 2009). Surveys of non-*aeruginosa* pseudomonads including phytopathogens have revealed that they produce alginate similar to what has been described for *P. aeruginosa* (Schnider-Keel *et al.*, 2001; Laue *et al.*, 2006). Additionally, various species produce levan, which also aids colonization and biofilm persistence (Laue *et al.*, 2006). Levan has deviations in structure and composition that suggests it has unique functions distinct from those of alginate (Sutherland, 2001a, b).

Capsular polysaccharides: alginate

Molecular structure

Alginate is an important matrix molecule for *Pseudomonas* biofilm formation by providing more than structural stability (Hentzer *et al.*, 2001; Nivens *et al.*, 2001). Specifically, alginate is a high molecular weight, acetylated polymer with nonrepetitive monomers of β-1,4 linked L-guluronic and D-mannuronic acids (Fig. 1a) (Evans & Linker, 1973). It is normally O-acetylated at the 2 and/or 3 position(s) on the D-mannuronate residues (Osman, *et al.*, 1986). Both β-1,4 and β-1,3 linkages typically confer considerable rigidity (Fig. 1a) compared to β-1,2 and β-1,6 linkages found commonly in dextrans (Sutherland, 2001a, b). Alginate and the biosynthetic enzymes are well conserved across pseudomonads (Penaloza-Vazquez *et al.*, 1997; Fakhr *et al.*, 1999; Li *et al.*, 2010).

Regulatory factors

Concurrent with the identification of the alginate-related biofilm phenotype was the identification that specific genetic events lead to the overproduction of alginate in mucoid strains (Lam *et al.*, 1980; Deretic *et al.*, 1993; Martin *et al.*, 1993; DeVries & Ohman, 1994; Mathee, *et al.*, 1999; Hentzer *et al.*, 2001; Qiu *et al.*, 2007). Mucoid conversion occurs primarily through a selective process in patients with lungs cystic fibrosis (CF). CF is a complicated disease that has many manifestations, not the least of which is the buildup of dehydrated mucus in the airway allowing colonization of pathogens such as *P. aeruginosa*. It is also within the CF lung where spontaneous mutations arise allowing for successful competition amidst the rest of the population. The chronic inflamma-tory state of the CF lung likely facilitates mucoid conversion through reactive oxygen intermediates increasing DNA damage of *P. aeruginosa* (Deretic *et al.*, 1993; Martin *et al.*, 1993; Mathee *et al.*, 1999). The overwhelming majority of mucoid strains from the CF airway possess mutations in *muc*A that results in a protein that is defective in interacting with the sigma factor (AlgT/U) controlling alginate expression (Martin *et al.*, 1993). Under nonmucoid conditions, MucA sequesters with the sigma factor AlgT/U. Degradation or truncation of MucA results in a loss of the ability to interact with AlgT/U, allowing it to potentiate alginate production and ultimately a conversion to a mucoid phenotype (Martin *et al.*, 1993; Mathee *et al.*, 1997). While the prevalence of *muc*A mutants is noteworthy, especially *muc*A22 being the most abundant, stable non-*muc*A mutations have also been isolated that result in an identical mucoid phenotype (Martin *et al.*, 1993; Boucher *et al.*, 2000). Alginate regulation is complex, and perturbation of other regulatory factors, in addition to AlgT/U, may result in a similar phenotype.

The mutations resulting in constitutive *alg* operon expression and mucoid conversion can be recapitulated *in vitro* using specific strategies, including hydrogen peroxide-induced DNA damage (Mathee *et al.*, 1999). Recent work has linked mucoid conversion mediated by oxidative stress with the SOS response and the error-prone DNA polymerase DinB (Sanders *et al.*, 2006; Moyano *et al.*, 2007). The frequency of which *P. aeruginosa* is able to adapt to its surroundings and persist is striking, and this also suggests that alginate provides an advantage during infections, especially in the CF lung (Hogardt & Heesemann, 2010). Alginate-aided protection of biofilm is important for colonization of surfaces by

Pseudomonas environmental and phytopathogen species (Keith & Bender, 1999; Yu *et al.*, 1999; Keith *et al.*, 2003; Spiers *et al.*, 2003). The virtually identical organization and conservation of the alginate biosynthesis loci in *Pseudomonas putida and Pseudomonas syringae* suggests that alginate overproduction in the non-*aeruginosa* pseudomonads is likely regulated similarly to *P. aeruginosa* (Penaloza-Vazquez *et al.*, 1997; Fakhr *et al.*, 1999; Li *et al.*, 2010). One significant caveat is that the 5′ regulatory sequences of the *P. syringae algD* region, including the AlgT/U promoter regions, lack similarity to the *P. aeruginosa algD* promoter region (Fakhr *et al.*, 1999). This could help explain some of the divergent environmental conditions affecting alginate production by *P. syringae* such as copper exposure (Kidambi *et al.*, 1995; Penaloza-Vazquez *et al.*, 1997). The *algC* gene, located distant from the *alg* operon and encoding a phosphomannomutase, is also essential for alginate biosynthesis (Zielinski, *et al.*, 1990; Zielinski *et al.*, 1991; Zielinski *et al.*, 1992). Regardless of the high level of alginate gene conservation, it is unclear whether environmental or phytopathogenic strains undergo similar mucoid conversion processes as seen in *P. aeruginosa*. However, it was noted that plant tissue damage is associated with a hypersensitive response (HR), correlated with increased *algD* expression by *P. syringae* (Keith *et al.*, 2003). The HR is generally characterized by brown spots with dead plant cells at the site of infection, often associated with some form of programmed cell death (Heath, 2000). HR is also characterized to have an abundance of reactive oxygen species, perhaps similar to the chronic inflammatory response, which facilitates mucoid conversion of *P. aeruginosa* at relatively high frequency (Mathee *et al.*, 1999). Perhaps, a similar mechanism for alginate overproduction occurs during colonization of *P. syringae* on plant tissue.

Function in biofilm matrix

Early work investigating *P. aeruginosa* biofilms centered on mucoid strains capable of overproducing alginate (Ohman & Chakrabarty, 1982; Sá-Correia *et al.*, 1987; Deretic *et al.*, 1989; Pedersen *et al.*, 1992; Deretic *et al.*, 1993). Mucoid strains, commonly isolated from chronically infected patients with CF, generate enough alginate that it drips or runs off the culture dishes. The alginate polysaccharide makes colonies appear slimly compared to nonmucoid strains (Fig. 2); it is evident that they are encased in this viscous material (Hogardt & Heesemann, 2010). Alginate was once assumed to be the sole vital biofilm matrix component for *P. aeruginosa*, and this was likely due to its abundance and perceived protective capacity (Evans & Linker, 1973; Hoyle *et al.*, 1993). More recent studies have challenged this paradigm.

Structural stability is necessary for biofilm formation especially under high shear stress situations. Robust biofilm integrity relates to the ability to adhere to surfaces, either biotic or abiotic, and/or aggregation to neighboring biofilm cells (Hentzer *et al.*, 2001; Webb *et al.*, 2003; Yang *et al.*, 2007; Ma *et al.*, 2009). However, biofilm matrix components have functions in addition to simple structural capacity. Capsular polysaccharides like alginate and levan possess sufficient properties for water and nutrient retention, respectively. Alginate water retention has not been extensively studied, but it is predicted to be similar to polysaccharides like hyaluronic acid which can bind up to 1 kg of water g^{-1} polysaccharide (Sutherland, 2001a, b). Solubility of alginate is likely affected by the acetylation because acetyl groups inhibit interaction between polymer chains and cations, therefore enhancing

gel formation (Sutherland, 2001a, b). These factors contribute to the highly viscous nature of alginate. Removal of *O*-acetyl residues alters the physical properties of alginate, resulting in increased binding of divalent cations and reduced polysaccharide solubility (Sutherland, 2001a, b). Alginate-containing biofilms have an obvious ability to survive harsh environments. Surviving desiccation stress is a limiting factor of the bacterial soil life cycle (Chang *et al.*, 2007).

Role in niche biology

The contribution of alginate to persistence and immune evasion has been described (Ramphal & Vishwanath, 1987; Pier *et al.*, 2001; Leid *et al.*, 2005). Studies indicated that alginate production by mucoid strains conferred recalcitrance to antimicrobials and opsonophagocytosis (Schwarzmann & Boring, 1971; Simpson *et al.*, 1988; Simpson *et al.*, 1989; Simpson *et al.*, 1993). Indeed, in support of this hypothesis, chronically infected patients with CF have circulating antibodies against alginate; however, it was noted that these antibodies fail to promote opsonic killing of *P. aeruginosa* in vitro (Pier *et al.*, 2004). Alginate also has the ability to scavenge free radicals released by neutrophils and activated macrophages in vitro that are commonly able to kill bacteria (Simpson *et al.*, 1989; Simpson *et al.*, 1993; Govan & Deretic, 1996; Pier, 1998). Alternative studies found that mucoid strains and their revertant nonmucoid counterparts showed little evidence of variations in susceptibility to intrapulmonary killing by immune effectors or antibiotics (Blackwood & Pennington, 1981). In addition, a more recent survey comparing mucoid clinical isolates to nonmucoid clinical isolates confirms the finding that overproduction of alginate conferred no increased persistence in a murine lung infection model (Bragonzi *et al.*, 2009). An explanation might be that clinical isolates revert to a nonmucoid morphology before or during infection in the mouse lungs used to assay for enhanced mucoid persistence. Alternatively, differences in lung infection models, microorganism and mouse strain backgrounds, or infectious doses used could account for these seemingly disparate findings. The nature of *P. aeruginosa* pathoadaptability makes determination of the direct contribution individual polysaccharides such as alginate have on persistence and immune evasion challenging. Clinical sputum samples from chronically infected patients with CF commonly include a mixture of both mucoid and nonmucoid strains. Along with polysaccharide production changes in response to AlgT/U activation, several additional loci are also regulated (Firoved *et al.*, 2002). Perhaps, additional AlgT/ U-regulated factors contribute to the pathogenesis of mucoid strains, thereby clouding the individual role of alginate in some studies. *In vitro* model systems are helpful, but contain inherent flaws and are often limited to simple comparisons with individual strains. Regardless, the effect of alginate on individual aspects of the immune system needs to be investigated with the best models available. Alginate, in addition to other virulence factors present and participating in active infections, aids *P. aeruginosa* survival in the face of immune mediators and a chronic inflammatory state.

Pseudomonas aeruginosa-related CF infection is not the only situation where pseudomonads take advantage of alginate production. Evidence suggests in most situations that EPS production and biofilm formation generate a buffering zone for bacteria to maintain a controlled environment. An *algU* mutant strain of *Pseudomonas fluorescens* CHAO was

more sensitive to osmotic stress and desiccation in vitro or in soil compared to the parental or *mucA* mutant strains (Schnider-Keel *et al.*, 2001). Mucoid variants of *P. fluorescens* have enhanced adherence to plant roots, indicating a supplementary role for alginate in phytopathogenic biofilm formation (Bianciotto *et al.*, 2001). Overall, it is clear that alginate produced under biofilm-relevant conditions aids environmental persistence and pathoadaptability, and further examination of precise mechanisms may aid what is known about *P. aeruginosa* biofilm-mediated capacities.

Capsular polysaccharides: levan

Molecular structure

In addition to alginate, the capsular polysaccharide levan is produced by a subset of pseudomonads, notably by the phytopathogen, *P. syringae* (Osman *et al.*, 1986). Levan is a high molecular mass β-2,6 polyfructan with extensive branching through β-2,1 linkages (Fig. 1b) (Laue *et al.*, 2006). Levan is produced exclusively from sucrose catalyzed by an extracellular levansucrase (Osman *et al.*, 1986) (Li & Ullrich, 2001).

Regulatory factors

The function of levan has been predominantly studied among *P. syringae* strains; however, production of levan has been identified in other *Pseudomonas* spp. (Kang, *et al.*, 1998; Gonzalez *et al.*, 2003; Scarpellini *et al.*, 2004; Pagès *et al.*, 2007; Ivanova *et al.*, 2009; Visnapuu *et al.*, 2009). *Pseudomonas syringae* pv. Glycinea PG4180 possesses three genes *lscA*, *lscB*, and *lscC*, comprising the levansucrase biosynthetic operon, responsible for levan production (Osman *et al.*, 1986). However, *lscB* and *lscC* appear to be primarily responsible for levansucrase functionality (Li & Ullrich, 2001). Compartmentalization of LscB in the extracellular and LscC in the periplasmic space was identified, while that of LscA was not identified in any protein fraction and thus not associated with levansucrase activities in *P. syringae* pv. Glycinea PG4180 (Li & Ullrich, 2001). Recent evidence suggests that levan production is regulated by LadS, which shares homology with the senor kinase-producing *ladS* locus of *P. aeruginosa* (Records & Gross, 2010). The specifics of levan production and levansucrase expression remain to be uncovered, but sensing external stimuli appears to be critical.

Biofilm matrix function

Based on spatiotemporal expression of levansucrase within biofilms, Laue *et al.* (2006) speculated that levan functions as a *P. syringae* storage molecule possibly protecting against starvation. They also showed that levan was not responsible for maintenance of biofilm structure, but that voids and blebs within the biofilm contained increased concentrations of levan, presumably as nutrient stores (Laue *et al.*, 2006). This is an interesting finding that would provide an environmental organism a readily available nutrient store for occasions when other nutrients become depleted. The accumulation of levan in cell-free areas of biofilm had previously been suggested as a similar mechanism for nutrient retention in the oral cavity by *Streptococcus mutans* (Burne *et al.*, 1996). Alternatively, observations of *Pseudomonas brassicacearum* adaptation to cadmium toxicity revealed that levan was downregulated by a subpopulation of cells, while a cadmium efflux pump and alginate were

unregulated in response to cadmium exposure (Pagès *et al.*, 2007). Together, these examples suggest that while alginate and levan share functional and regulatory properties, each possesses unique abilities that soil-borne pseudomonads take advantage of when necessary.

Aggregative polysaccharides

Continued biofilm studies in *P. aeruginosa* revealed that nonmucoid strains were adept at biofilm formation regardless of alginate production. In fact, several reports showed that alginate was not essential for biofilm formation in *P. aeruginosa* (Wozniak *et al.*, 2003; Stapper, *et al.*, 2004). Nonmucoid strains, harboring a native mucA gene while expressing low to absent levels of alginate, were able to produce mature robust biofilms (Wozniak *et al.*, 2003). The lack of biofilm dependence on alginate led to a search for alternative polysaccharides that mediated biofilm formation in nonmucoid strains of *P. aeruginosa*. Matsukawa and Greenberg identified three alternative loci in *P. aeruginosa* PAO1, which they determined to have potential to generate polysaccharide matrix components (Matsukawa & Greenberg, 2004). Only one of the loci, termed *polysaccharide synthesis locus* (*psl*), was found to have relevance for aiding biofilm integrity (Matsukawa & Greenberg, 2004). Disruptions within the other two loci identified were similar to the parental strain in adherence and biofilm formation experiments (Matsukawa & Greenberg, 2004). Additionally, a different search identified a fourth locus later termed *pel* based on an obvious pellicle formation defect. Static and flow cell biofilms of PA14 Δ*pel* also showed a defect in biofilm formation in *P. aeruginosa* PA14 (Friedman and Kolter, 2004a, b). Investigations of nonmucoid biofilms following these reports have been focused on the importance of *psl* and *pel* in biofilm formation.

Aggregative polysaccharides: Psl

Molecular structure

Initial predictions were that Psl contained galactose, D-mannose, D-glucose, and L-rhamnose (Wozniak *et al.*, 2003; Friedman & Kolter, 2004a, b; Matsukawa & Greenberg, 2004; Ma *et al.*, 2007). More accurate biochemical determinations of the Psl polysaccharide have since been made. Psl is composed of a pentasaccharide repeating distinct from other known polysaccharides. Psl contains D-mannose, D-glucose, and L-rhamnose (Fig. 1d) (Byrd *et al.*, 2009). Psl is made from the sugar nucleotide pool of precursors including GDP-D-mannose, UDP-D-glucose, and dTDP-L-rhamnose (Byrd *et al.*, 2009). Psl is commonly found in at least two forms: a high molecular weight cell–associated component and a relatively smaller soluble form of Psl that can be isolated from cell-free culture supernatant (Byrd *et al.*, 2009). Greater details regarding Psl biosynthesis can be found in the recent review from Franklin *et al.* (2011).

Regulatory factors

The psl locus in *P. aeruginosa* contains 15 co-transcribed genes with homology to known carbohydrate biosynthesis genes (Fig. 3) (Friedman & Kolter, 2004a, b; Jackson *et al.*, 2004; Matsukawa & Greenberg, 2004). However, only 11 of the 15 *psl* genes are required to produce an adherent Psl-dependent biofilm (Byrd *et al.*, 2009). One explainable exception is *pslB*, which shares redundant function with *wbpW*, a gene producing an enzyme with

analogous function. The last three genes of the operon, *pslMNO*, are also not necessary for biofilm matrix functions (Byrd *et al.*, 2009). The predicted functions of each of the *psl* products are depicted in Fig. 3 as well.

Induction of *psl* occurs in response to high intracellular levels of bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) (Starkey *et al.*, 2009; Borlee *et al.*, 2010), an important intracellular signaling molecule in the bacterial world (Hengge, 2009). While the extent of c-di-GMP transcriptional effects remains to be elucidated, some transcriptional and posttranscriptional layers of *psl* regulation have been described involving RpoS and RsmA, respectively (Irie *et al.*, 2010). RpoS, a stationary-phase σ-factor, induces *psl* transcription in response to changes in global physiology. Alternatively, the RNA-binding protein RsmA represses Psl translation through binding to the 5′ untranslated region of *psl* mRNA. RsmA repression of *psl* does not appear to be responsive to changes of growth phase (Irie *et al.*, 2010).

Biofilm matrix function

While Psl is found in a larger cell-associated form and a smaller soluble form, the mechanism yielding the smaller form of Psl is not clear; possibly, it is a product of cleavage or breakdown of high molecular weight Psl. Alternatively, the processing of the larger and the smaller versions may be generated through a currently unappreciated mechanism. Breakdown of Psl could facilitate functions away from producing cell such as matrix building for biofilm cell-to-cell association, adherence to a surface, or even cell signaling. The cell-associated Psl can be visualized using scanning electron microscopy (SEM) where isolated blebs of Psl are seen on the surface of *P. aeruginosa* PAO1 (Byrd *et al.*, 2010). Psl is extremely important for initial adherence of sessile cells to both biotic and abiotic substrates (Ma *et al.*, 2006; Byrd *et al.*, 2009; Byrd *et al.*, 2010). Mature biofilms also take advantage of Psl for aiding structural stability and architecture (Ma *et al.*, 2009). Psl also facilitates protection from innate immune effectors, complement and neutrophils (Mishra *et al.*, 2012). Effects of Psl on immunity are likely to be intensified in biofilm where Psl is abundant. Ongoing investigations are focused on the role of both forms of Psl and its impact on single cells or biofilm communities. Regardless of the nature of Psl, it provides *P. aeruginosa* with an effective tool to establish a persistent biofilm infection.

Role in niche biology

The importance of Psl is particularly evident in rugose small-colony variants (RSCV) of *P. aeruginosa*. Converse from well-characterized mucoid *P. aeruginosa* strains producing massive quantities of alginate, overproduction of aggregative polysaccharides results in the rugose small-colony phenotype (Fig. 2). The RSCV phenotype was found to be prevalent in 33 of 86 *P. aeruginosa*-positive patients with CF over a 2-year period (Haussler *et al.*, 1999). Stable variants that display a small, wrinkly colony phenotype on agar compared to wild-type PAO1 (Fig. 2) have been isolated from *in vitro* and *in vivo* biofilms (D'Argenio *et al.*, 2002; Drenkard & Ausubel, 2002; Haussler *et al.*, 2003; Kirisits *et al.*, 2005; Starkey *et al.*, 2009; Borlee *et al.*, 2010). It is unclear why RSCV are so frequently isolated from *in vivo* and *in vitro* biofilms; perhaps, there is a selective role for aggregation during biofilm formation. Nonetheless, when biofilms of PAO1 and RSCV are mixed in various ratios,

RSCV populations stabilize and are consistently found to represent *c*. 1/3 of the final biofilm population (Kirisits *et al.*, 2005). This implies that the RSCV phenotype may have specific role or even a competitive advantage in the biofilm.

Diverse genotypic RSCV have been isolated, but all classes share the overabundance of accumulated intracellular c-di-GMP (Hickman *et al.*, 2005; Starkey *et al.*, 2009). MJK8 is a member of one specific class of RSCV, which has a mutation in the *wsp* operon resulting in a constitutively active diguanylate cyclase, WspR (Starkey *et al.*, 2009). The increased c-di-GMP results in a decrease in motility and increased aggregation (Guvener & Harwood, 2007; Starkey *et al.*, 2009). Additionally, Lee *et al.* (2007) described the requirement of c-di-GMP for the production of exopolysaccharides in *P. aeruginosa* biofilm. Exopolysaccharide biofilm matrix molecules Psl and Pel are induced in response to high intracellular c-di-GMP levels (Hickman *et al.*, 2005; Guvener & Harwood, 2007; Starkey *et al.*, 2009; Borlee *et al.*, 2010). Investigation of the individual impact of enhanced Psl or Pel expression reveals that overexpression of either polysaccharide, but especially Psl, results in enhanced aggregation and biofilm formation. Wild-type, *psl* mutant, and *psl* overexpression strains were grown in a rolling culture tube overnight with Congo red supplemented media. Abundant aggregation and biofilm are noticeable in the tubes when Psl is over-expressed (Fig. 5). Conversely, wild type or strains lacking Psl resulted in moderate to absent adherence (Fig. 5). Artificially inducing *psl* with an arabinose-inducible promoter intensely mimics the RSCV aggregation pheno-type, which has a unique propensity to aggregate, thereby facilitating biofilm formation. Psl may also be important for mediating similar biofilm adherence during colonization of immunocompromised patients.

While the majority of evidence describing the function of aggregative polysaccharides in biofilm formation has been made studying *P. aeruginosa*, the *psl* operon is also conserved throughout several other pseudomonads (Fig. 3) (Nelson *et al.*, 2002; Buell *et al.*, 2003; Vodovar *et al.*, 2006; Winsor *et al.*, 2009). For example, *P. fluorescens* B728a harbors 12 of the 15 genes in the *psl* operon, missing only L, M, and O genes (Fig. 3). Interestingly, the only allele among those that was shown to be necessary for Psl production was *pslL* (Byrd *et al.*, 2009). However, an additional acyl transferase, Psyr3310, was found between *pslJ* and *pslK* in the genome that may be fulfilling the normal function of pslL in *P. aeruginosa* strains (Stover *et al.*, 2000; Feil *et al.*, 2005; Winsor, *et al.*, 2009). Moreover, *P. fluorescens* Pf5, *P. syringae* strains 1448A, DC3000, and *Pseudomonas mendocina* ymp share the similar *psl* operon organization (Fig. 3) (Stover *et al.*, 2000; Feil *et al.*, 2005; Joardar *et al.*, 2005; Paulsen *et al.*, 2005; Winsor *et al.*, 2009). Other *psl* genes that are well conserved in the strains examined include *pslA, pslD, pslH,* and *pslN* (Fig. 3). Conversely, the majority of the *psl* operon is missing in *P. fluorescens* Pf0-1 (Winsor *et al.*, 2009), but unlike *P. aeruginosa*, which is limited in biofilm formation without *psl* components, it may have additional matrix components that play a compensatory role. Psl is not necessary for biofilms produced by *P. aeruginosa* PA14, which does not have *pslABCD* genes (Fig. 3) (Friedman & Kolter, 2004a, b; Lee *et al.*, 2006).

Aggregative polysaccharides: Pel

Molecular structure

Another polysaccharide-producing locus, *pel*, is required for biofilm formation in *P. aeruginosa* PA14 (Friedman & Kolter, 2004a, b). Unlike the Psl polysaccharide, whose structure has been predicted (Fig. 1) (Byrd *et al.*, 2009), the composition and nature of Pel polysaccharide are undefined. Some hints regarding the nature of Pel exist, including comparison of an air–liquid (A–L) biofilm matrix composition of PA14 and its *pel* mutant, which revealed that the mutant exhibited reduced glucose levels compared to the parental strain (Friedman & Kolter, 2004a, b). A recent survey of polysaccharides produced by *P. aeruginosa* PA14 did not provide the Pel structure (Coulon *et al.*, 2010), but alluded to an alternate functional role for Pel.

Regulatory factors

The *pel* locus is comprised of a 7-gene operon producing proteins with predicted polysaccharide biosynthesis functions (Fig. 4) (Friedman & Kolter, 2004a, b). Similar to Psl, Pel is produced maximally when intracellular c-di-GMP levels are elevated (Lee *et al.*, 2007). Surprisingly, unlike the *psl* operon, the *pel* operon is poorly conserved in *P. syringae*, *P. putida*, and *P. mendocina* (Fig. 4). The pel operon also appears to lack some genes necessary for complete biosynthesis of an exopolysaccharide, implying that the Pel machinery may be functioning with other polysaccharide enzymes (Franklin *et al.*, 2011). Intriguingly, recent evidence suggests that there may be cross talk between factors affecting Pel production and Psl production. Ghafoor *et al.* (2011) show that greater Pel production occurs in the absence of Psl. Possible explanations may be that there is cross talk in the regulation of the polysaccharide loci or competition for precursors that account for limited production of individual polysaccha-rides. The latter has been proposed previously to affect production of polysaccharides like alginate (Pham *et al.*, 2004; Rehm, 2010).

Biofilm matrix function

Interestingly, strain PA14 relies greatly on the Pel polysaccharide compared to other *P. aeruginosa* strains with fully intact *psl* loci (Fig. 3). The biofilm defect in a *P. aeruginosa* PA14 *pel* transposon insertion mutant was identified through a screen for poor A–L biofilm formation (Friedman & Kolter, 2004a, b). The original observation led to further investigating the importance of Pel during biofilm development. In addition to the formation of an A–L or pellicle biofilm, Pel promotes aggregative properties in broth culture. In PA14, or when overexpressed in PAO1 regardless of Psl expression, Pel aids adherence to culture tubes and aggregation in broth culture (Fig. 5). Generally, when both *psl* and *pel* are intact, it seems that Psl is predominant while Pel has only limited impact on biofilm phenotypes. However, in the instances where the *psl* operon is absent or disrupted, such as it is in PA14, or when c-di-GMP is maximally elevated, Pel has a clearer impact on biofilm formation. Yang *et al.* (2011) recently identified conditions in which PAO1 biofilms utilize Pel for greater structural stability in formation of microcolonies. In this instance, Pel and Psl together facilitate compactness of the biofilm and cell-to-cell association. Biofilm matrix dependence on Psl and/or Pel has been discussed in detail (Colvin *et al.*, 2011b). Colvin *et al.* proposed four classes of *P. aeruginosa* strains based on their dependence on aggregative

polysaccharides. Class I strains include those that rely predominantly on Pel for biofilm matrix stability. PA14 was the only identified member of this class, and although an exhaustive search was not carried out, the authors predict that other members of this class would also likely have *psl* mutations rendering them Psl negative. Class II strains rely predominantly on Psl for matrix stability even though they seem to be capable of producing the components necessary to generate the Pel polysaccharide, such as PAO1. Class III strains produce both Psl and Pel at low levels, and disruption of the ability to produce either polysaccharide independently did not dramatically reduce biofilm stability. Class IV matrix strains are Psl and Pel overproducers. Characteristically, this last class makes a rather dynamic matrix that does not rely on a single component, and these strains produce a robust biofilm (Colvin *et al.*, 2011a, b). Future characterization of *P. aeruginosa* isolates will likely provide greater insights into these classes and the functional contributions of either Pel or Psl.

In addition to a structural role of Pel, recent evidence suggests Pel functions to mitigate antibiotic efficiency during biofilm formation (Colvin *et al.*, 2011a, b). Coulon *et al.* (2010) examined a *pel* mutant with regard to modifications in lipopolysaccharide (LPS) *O*-antigen and found greater culture concentrations of soluble *O*-antigen (termed OPS) in the mutant compared to the wild-type strain. One might interrupt those data to indicate that Pel is a modifying element of *O*-antigen or LPS molecules rather than an exopolysaccharide. Nonetheless, the impact of Pel on biofilm stability and adherence is apparent, and continued studies will be required to understand the full nature and function of Pel during biofilm formation.

Role in niche biology

While adherence and overall structural stability of the biofilm have been hallmarks of polysaccharide contribution to biofilm function, more recently, polysaccharide-mediated tolerance to antibiotics and even impacts on cell signaling have been appreciated (Khan *et al.*, 2010; Colvin *et al.*, 2011a, b; Yang *et al.*, 2011). A mutant strain lacking Pel production is more susceptible to tobramycin exposure compared with parental and complemented strains (Colvin *et al.*, 2011a, b). While it is possible that Pel is directly binding tobramycin to inhibit the function of the aminoglycoside, it is also possible that a Pel-dependent biofilm forms in such a way that it further limits amino-glycoside penetration into the biofilm. Perhaps, the nature of the Pel-aided biofilm matrix is key because exposure of stationary-phase cultures to tobramycin failed to show any additional sensitivity of the *pel* mutant (Colvin *et al.*, 2011a). Production of polysaccharides Pel and Psl, and therefore biofilm formation, also seems to affect quorum sensing and downstream extracellular DNA (eDNA) release (Yang *et al.*, 2011). The lack of compactness among initial biofilm colonizers without aggregative polysaccharides could also affect quorum sensing expression and ultimately *Pseudomonas* quinolone signal (PQS)-mediated eDNA release as has been described previously (Klausen *et al.*, 2003a, b; Yang *et al.*, 2007; Barken *et al.*, 2008; Yang *et al.*, 2009). The connection between both polysaccharides Pel and Psl along with eDNA, type IV pili, quorum sensing, and biofilm organization is complex and requires extensive investigation to tease apart individual responsibilities and relationships. As a first step toward understanding these connections, recently, the TbpA (PA3885) protein, responding

to quorum sensing and mitigating c-di-GMP levels in the cell, was shown to be a key component regulating the process of biofilm formation (Ueda & Wood, 2009). The levels of c-di-GMP ultimately impact aggregative polysaccharide production and downstream biofilm formation. Advances similar to this will benefit development of novel strategies to combat biofilm-related infections through targeting the regulation of biofilm formation.

Aggregative polysaccharides: cellulose

Similar to RSCV in *P. aeruginosa*, *P. fluorescens* SBW25 produces analogous variants referred to as a wrinkly spreader (WS) phenotype (Spiers *et al.*, 2002). Characterization of the WS phenotype attributes a mutation in the chemosensory-like regulatory apparatus (*wsp*) for expression of the *wss* operon, whose products participate in the cellulose biosynthetic pathway (Spiers *et al.*, 2002; Spiers *et al.*, 2003). While cellulose-related biofilm function has not been described for *P. putida* and *P. syringae* pv. tomato str. DC3000, the genomes also contain homologs of the *P. fluorescens* SBW25 wss operon (Nelson *et al.*, 2002; Buell *et al.*, 2003). Cellulose production by *P. fluorescens* clearly impacts biofilm formation at the A–L interface (Gal *et al.*, 2003; Spiers *et al.*, 2003). Specifically, acetylation of cellulose is important for normal A–L biofilm formation, because the acetylation-defective mutant WS-18 produced a weak biofilm compared to the WS parental strain (Spiers *et al.*, 2003). The dependence of the WS subpopulation that arises from the wild-type *P. fluorescens* smooth colony during biofilms formed at the A–L interface is strikingly similar to the phenotype of RSCV in *P. aeruginosa*. Both situations depend on the enhanced production of c-di-GMP, often from analogous pathways, and ultimately overexpression of exopolysaccharide (Spiers *et al.*, 2002; Gal *et al.*, 2003; Spiers *et al.*, 2003; Kirisits *et al.*, 2005; Spiers & Rainey, 2005; Starkey *et al.*, 2009). It is intriguing, however, that the formation of natural biofilms by environmentally relevant pseudo-monads takes advantage of cellulose as opposed to Psl. The *psl* locus is widely conserved among pseudomonads that produce cellulose (Fig. 3), but the role of Psl in biofilms made by non-aeruginosa Pseudomonas species has not been described. Interestingly, some pseudomonads, like *P. putida*, lack a cognate *psl* locus and contain novel polysaccharide-producing loci important for biofilm stability (Nilsson *et al.*, 2011). Two of the loci have also been described to be important for cell-to-cell interaction and pellicle formation. *Pseudomonas putida* exopolysaccharide A (*pea*) and bacterial cellulose (*bcs*) were each shown to play a role in biofilm-related phenotypes (Nielsen *et al.*, 2011). The *pea* locus in *P. putida* KT4220 includes genes PP3132–3142, and only PP3142 shares an ortholog group with a *psl* gene, *pslA* (Stover *et al.*, 2000; Nelson *et al.*, 2002; Winsor *et al.*, 2009). Like Psl, the *pea*-produced polysaccharide appears to contain mannose, rhamnose, and glucose and is cellulase sensitive. However, the *pea*-produced polysaccharide was not cross-reactive with antisera raised against *P. aeruginosa* Psl (Nielsen, *et al.*, 2011). The absence of the Pel polysaccharide in several non-*aeruginosa* pseudomonads is also intriguing (Fig. 4). This suggests that while some matrix molecules share functions, others provide unique role in specific niches. Examples exist where polysaccharides have functional substitutes, such as where cellulose appears to substitute for *P. aeruginosa*'s Pel-related functions in pellicle or A–L biofilm formation (Wozniak *et al.*, 2003; Friedman & Kolter, 2004a, b; Ryder *et al.*, 2007). Regardless of the matrix molecule produced by *Pseudomonas* spp., each species uses

one or more of the following polysaccharides: alginate, levan, cellulose, Psl, or Pel, for proper biofilm and microcolony development.

Additional *Pseudomonas* biofilm matrix components

Nucleic acids

Another commonly abundant biofilm matrix building block is nucleic acid. Specifically, DNA is a critical component of the biofilm matrix (Whitchurch *et al.*, 2002; Webb *et al.*, 2003; Allesen-Holm *et al.*, 2006; Rice *et al.*, 2007; Yang *et al.*, 2007; Ma *et al.*, 2009). Initially, the importance of DNA in biofilm was shown by Whitchurch *et al.*, (2002). DNA was mistakenly identified through a carbazole colorimetric assay, commonly used to quantitate uronic acid-containing polysaccharides (Whitchurch *et al.*, 2002). Carbazole binds DNA (Tanious *et al.*, 1997), similar to detection of alginate, highlighting the structural similarity of the two polysaccharide molecules (Fig. 1a and e). The deoxyribose backbone of DNA is a sugar in its most basic sense. Further examination indicated that DNA possesses a structural role in early *P. aeruginosa* biofilms (< 60 h) that were disrupted by exposure to DNase I, while older biofilms were more tolerant to DNase I treatment (Whitchurch *et al.*, 2002). This insight suggests that DNA plays a role during biofilm development but that it is only a contributing component of a more diverse biofilm matrix during later biofilm development. Perhaps, the diverse nature of a mature biofilm matrix is better able to withstand the absence of DNA following DNase I treatment. Not surprisingly, extracellular DNA (eDNA) isolated from *in vitro* biofilms was identified to be derived from *P. aeruginosa* genomic DNA (Allesen-Holm *et al.*, 2006). Allesen-Holm *et al.* (2006) further characterized the eDNA using confocal laser scanning microscopy and produced spectacular images showing organization of DNA in the biofilm matrix. After 48 h of growth, the biofilms appear to contain intense amounts of DNA in the matrix, especially in regions where microcolonies are forming. After 144 h, the biofilms contained proportionally less DNA in the matrix, visualized by nucleic acid-specific staining, but the location of DNA staining was striking (Allesen-Holm *et al.*, 2006). Images captured the presence of DNA in the interior of the stalk after 5 days of growth and intensely concentrated in the interior of the mushroom-shaped cap after 6 days of growth (Allesen-Holm *et al.*, 2006). Time points imaged after tower structures (Box 1) had formed indicated that DNA was located in specific areas of the biofilm, not throughout as it was at 2 days of growth. These data strengthen the conclusion that the biofilm matrix becomes less DNA dependent as the biofilm matures and also explains why DNase I treatment of older biofilms does not disrupt the biofilm adherence. The same authors also indicate that quorum sensing affects extracellular DNA accumulation in the *P. aeruginosa* biofilm (Allesen-Holm *et al.*, 2006). Specifically, it is evident that PQS quorum sensing-controlled DNA release in the dense stalk microcolony is required for cap formation (Yang *et al.*, 2009).

The role of DNA in the specific regions during development of the mushroom-like structures has been investigated further. A model where DNA- and pilin-dependent mushroom capping has been proposed states that twitching motility allows for motile cap-forming populations to move up the matrix of the stalk, along the DNA portion, to form the mushroom cap on the previously formed stalk (Box 1) (Klausen *et al.*, 2003a, b; Barken *et*

al., 2008). Ma *et al.* (2009) also investigated the role of polysaccharides and DNA during biofilm development. Although it appears that both DNA and polysaccharides are abundant in *P. aeruginosa* biofilms, there are divergent roles during tower formation and maturation process (Ma *et al.*, 2009). Psl matrix-containing biofilm was found on the exterior of the cap of the mushroom-shaped structures, just outside of the nucleic acid-specific staining. Psl was markedly absent from the interior of the mushroom cap and was therefore termed a Psl cavity (Ma *et al.*, 2009). Instead, genes controlling cell death and lysis seemed to be aiding Psl cavity formation and proposed to be contributing to DNA release. It is especially noteworthy that the biofilm matrix of pseudomonads is a dynamic process where coordinated changes in matrix structure correlate with biofilm development stages.

Regardless of the presence of an array of matrix molecules, mechanisms of matrix interaction remain under continued investigation. Hypotheses have been generated describing polysaccharides or nucleic acids interacting via surface charge of cells or simple nonspecific ‘trapping’ of biofilm aggregates. However, the spatial and temporal accumulation of DNA and production of polysaccharides during biofilm formation indicate that facilitated organization of cells exists in the biofilm. Identification of the mechanism whereby biofilm matrix molecules and cells interact will aid development of therapies for dispersion and clearance of biofilm infections.

Polysaccharide-containing matrix components

In addition to the predominant polysaccharides, accessory biofilm matrix components have been identified that aid biofilm matrix function. The impact of cyclic β glucans, LPS, and membrane vesicles (MV) has not been widely investigated in *Pseudomonas* spp., but evidence suggests that they have an accessory role with the biofilm matrix (Spiers & Rainey, 2005; Schooling & Beveridge, 2006; Nakamura *et al.*, 2008; Coulon *et al.*, 2010).

The primary responsibility of matrix components is to provide structural integrity to a biofilm, allowing for cell–cell interaction and formation of structures that convey accessibility to nutrients. However, another important role of the biofilm matrix is the potential to inhibit antimicrobials. Originally, it was speculated that the sticky, dense, and thick nature of biofilm cells and matrix inhibited penetration of antibiotics, resulting in the recalcitrance observed (Hoyle *et al.*, 1992). Subsequently, it was determined that fluoroquinolone antibiotics penetrate biofilms readily, while aminoglycosides penetrate more poorly (Walters *et al.*, 2003). Therefore, regardless of the accessibility of antibiotics to cells within the biofilm, sessile bacteria are physiologically distinct from their planktonic counterparts (Anderl *et al.*, 2000; Whiteley *et al.*, 2001). However, excreted factors like polysaccharides abundant in the biofilm matrix interact with aminoglyco-sides (Mah *et al.*, 2003; Sadovskaya *et al.*, 2010). Specifically, expression of a polysaccharide matrix molecule was identified that attributed antibiotic resistance of *P. aeruginosa* biofilm (Mah *et al.*, 2003). The *ndvB* locus, expressed preferentially in biofilms, generates cyclic gly-cans (Mah *et al.*, 2003). The resistance mechanism was proposed originally to rely on the ability of cyclic glycans to form a molecular complex with aminoglycoside antibiotics, abrogating the antibiotic's ability to reach its target (Athanassiou *et al.*, 2003; Mah *et al.*, 2003; Evrard *et al.*, 2004; Sadovskaya *et al.*, 2010). More recently, the Pel polysaccharide in both PAO1

and PA14 strains of *P. aeruginosa* has been shown to aid tolerance to amino-glycoside antibiotics, similar to the role of cyclic glucans (Khan *et al.*, 2010; Colvin *et al.*, 2011a, b). Independent of providing a penetration barrier, other matrix molecules have not been implicated in conferring recalcitrance directly for biofilm-contained cell populations, but continued studies will investigate the nature of biofilm-mediated resistance to antimicrobials.

The importance of LPS as a biofilm matrix molecule, or even its role in adhesion of *Pseudomonas* spp. to a surface, has not been defined completely. However, limited investigations have provided valuable clues into the nature of LPS-mediated biofilm stability. LPS was originally investigated because of its ability to affect surface charge or relative hydrophobicity of the cell (Rocchetta *et al.*, 1999). Furthermore, LPS dictates alterations in attachment, transition to sessile growth, and colony morphology variations in several bacteria (Giwercman *et al.*, 1992; Genevaux *et al.*, 1999; Mireles *et al.*, 2001; Ali *et al.*, 2002; Landini & Zehnder, 2002; Rashid *et al.*, 2003). Specifically, *P. fluorescens* WS variants rely on LPS interactions to aid overall biofilm integrity (Spiers & Rainey, 2005). Although the WS-5 stain possesses a mutation resulting in the loss of proper LPS expression (Gaspar, *et al.*, 2000; Spiers & Rainey, 2005), WS-5 was able to form an A–L biofilm, albeit less robust than the wild-type smooth-colony strain. The phenotype was partially complemented by an LPS-expressing strain (Spiers & Rainey, 2005). Additional investigations of *P. aeruginosa* PAO1 and its LPS-mediated adherence and subsequent biofilm formation strongly suggest that modifications in the LPS core affect adherence. Changes in bacterial mechanical behavior during early biofilm formation as a result of ‘differential LPS core capping’ were correlated with mature biofilm matrix modifications (Lau *et al.*, 2009). Although LPS appears not to be affected by quorum sensing after extended growth (De Kievit *et al.*, 2001), but during early growth stages (< 4 h) (Nakamura *et al.*, 2008), LPS responds to quorum sensing. Typically, LPS is anchored to the bacterial outer membrane through lipid A (Rocchetta *et al.*, 1999), but can be released from the cells and accumulate in the culture media during normal growth (Cadieux *et al.*, 1983; Al-Tahhan *et al.*, 2000). It is likely that cell-free LPS associates with the complex biofilm EPS contributing to the architecture of the biofilm. These predictions are supported by the calculation that excreted polymers, products of cell lysis, and remaining intact cells account for 85–95% of total organic carbon in biofilms (Sutherland, 2001a, b). Continued investigation of the nature of LPS in the biofilm matrix is necessary for a complete understanding of the multifarious biofilm matrix, but it is intriguing to consider abundant LPS molecules as biofilm matrix components.

MV are multifunctional bilayered structures that bleb from the outer membranes of Gram-negative bacteria (Beveridge, 1999; Mashburn & Whiteley, 2005; Schooling & Beveridge, 2006; Mashburn-Warren *et al.*, 2008). Importantly for the context of biofilm formation, MV interact with biofilm matrix components like eDNA (Schooling *et al.*, 2009). MV have been proposed to provide a large portion of LPS in the biofilm matrix (Schooling & Beveridge, 2006) as they retain the intrinsic lipid asymmetry of the outer membrane with most of the LPS positioned within the outer leaflet of the membrane (Beveridge *et al.*, 1997). Additionally, MV blebbing from the outer membrane results in a sampling of the

periplasmic contents. Often, these include proteases, alkaline phosphatase, lipases, proelastase, autolysins, and toxins (Grenier & Mayrand, 1987; Kadurugamuwa & Beveridge, 1995; Fiocca *et al.*, 1999; Kolling & Matthews, 1999; Keenan & Allardyce, 2000; Allan *et al.*, 2003). The natural shedding of amphipathic LPS through MV formation could provide a usable framework for structural contribu tions of LPS that have been suggested (Wozniak *et al.*, 2003; Spiers & Rainey, 2005). While it is apparent now that MV are abundant within a biofilm and confer obvious matrix-related function, additional investigations will more fully reveal the impact of MV for *P. aeruginosa* biofilm. Evidence suggests that formation of MV is controlled by the *las* quorum sensing system early in biofilm formation (Nakamura *et al.*, 2008) and that PQS is required and sufficient for the formation of MV (Mashburn-Warren *et al.*, 2008), suggesting that the las QS mainly regulates MV by PQS activation. Further investigation of quorum sensing and the generation of MV will provide greater insight as to how MV are used by biofilm-forming organisms.

Protein components of the biofilm matrix

In addition to polysaccharides and nucleic acids, the importance of biofilm matrix-related proteins has been increasingly appreciated. Proteins present in the biofilm matrix facilitate roles including surface adherence, interaction with other matrix molecules, and matrix stability. Proteins such as CdrA from *P. aeruginosa* possess carbohydrate-binding capacity, making it an interesting candidate for a mechanism promoting matrix molecule interaction (Borlee *et al.*, 2010). The *cdrAB* locus comprises a two-partner secretion (TPS) system with a large adhesion and its transporter. The operon was named because of its response to cyclic diguanylate where in the presence of high c-di-GMP, robust expression is observed. The prototypical TPS system is the filamentous hemagglutinin (FHA) as well as its FhaC secretion partner from *Bordetella pertussis*. A key identifiable characteristic of CdrA making it a relevant biofilm matrix component is that it interacts with Psl from *P. aeruginosa*. Evidence suggests that CdrA-Psl binding could facilitate specific interactions among Psl molecules and with biofilm cells (Borlee *et al.*, 2010). FHA from *B. pertussis* is an adhesion with multiple galactose-inhibitable binding targets including cilia (Tuomanen *et al.*, 1988). FHA is required for *B. pertussis* to grow in characteristic aggregates (Menozzi *et al.*, 1994). Like FHA, CdrA is predicted to be a rod-shaped protein with a β-helical tertiary structure and exposed integrin-binding motif (Borlee *et al.*, 2010). Ongoing investigations of CdrA will elucidate the specific mechanism, whereby it stabilizes the biofilm matrix through its association with Psl.

Some *Pseudomonas* spp. transition from reversible to irreversible biofilm attachment through the function of LapA on the cell surface (Hinsa *et al.*, 2003). Additionally, LapD provides a unique inside-out signaling mechanism for initiation of adherence, because it contains predicted GGDEF and EAL domains each functioning potentially to synthesize or degrade c-di-GMP, respectively. However, LapD lacks the ability to carry out either of these functions and was thus proposed to act as an effector protein linking intracellular c-di-GMP signaling to the extracellular protein LapA (Hinsa *et al.*, 2003). LapA is a cell-to-cell interconnecting molecule as well as a surface adhesion (Gjermansen *et al.*, 2010). Interestingly, a decrease in intracellular c-di-GMP levels in *P. putida* OUS82 led to dispersal of preformed biofilms that was LapG dependent. The authors of the study conclude that the

LapG component acts on LapA in the biofilm matrix to cause dispersal (Gjermansen *et al.*, 2010). The intricacies of the mechanism and the regulation of the *lap* locus are discussed in greater detail elsewhere, but it is critical to note that *lap* loci have been identified in only *P. fluorescens* and *P. putida* (Hinsa *et al.*, 2003). The Lap components are absent in *P. aeruginosa* and *P. syringae* genomes, indicating that the nature of biofilm formation and irreversible attachment is distinct among various species of Pseudomonas (Hinsa *et al.*, 2003). Interestingly, Pseudomonas species with the *lap* genes are in general lacking genes for synthesis of Pel polysaccharide.

Cytotoxic lectins produced by *Pseudomonas* spp. bind carbohydrates and aid biofilm stability and structure formation (Tielker *et al.*, 2005; Diggle *et al.*, 2006). In addition to virulence capabilities of lectins LecA and LecB in the lungs, individual mutants of either *lecA* or *lecB* exhibit defective biofilm phenotypes (Tielker *et al.*, 2005; Diggle *et al.*, 2006; Chemani *et al.*, 2009). Further characterization identified that LecA has high specificity and affinity for hydrophobic galactosides (Diggle *et al.*, 2006) while LecB binds L-fucose readily (Tielker *et al.*, 2005). The impact of these lectins on *P. aeruginosa* cytotoxicity and biofilm formation led to the examination of LecA-and LecB-specific lectin-inhibiting carbohydrates as a therapeutic strategy (Chemani *et al.*, 2009). Indeed, promising results were seen when co-administration of lectin-inhibiting carbohydrates was given in an animal model of infection. Markedly reduced lung injury and mortality were observed compared to infections without intervening carbohydrates (Chemani *et al.*, 2009).

Cell appendages are also necessary for *Pseudomonas* biofilm formation although they are commonly not regarded as classical biofilm matrix molecules. Both flagella and type IV pili aid in biofilm formation (O'Toole & Kolter, 1998; Klausen *et al.*, 2003a, b). During the biofilm maturation process, microcolonies are developed through clonal expansion of nonmotile cells, which develop the stalk of *P. aeruginosa* mushroom-like structures (Klausen *et al.*, 2003a, b). Formation of the mushroom cap, however, requires the presence of type IV pili. In addition, flagellum-mediated motility along with the chemotaxis systems is required for cap formation (Barken *et al.*, 2008). It is unclear whether flagella expression is mediating swimming or swarming motility, but speculation suggests swarming within the biofilm matrix, resulting in movement of cells into the mushroom cap (Barken *et al.*, 2008). Swarming motility requires biosurfactant production (Kohler *et al.*, 2000; Rashid & Kornberg, 2000), and the *rhlA* mutant displays reduced cap formation in mixed *pilA/rhlA* biofilms because of the lack of rhamnolipid biosurfactant (Pamp & Tolker-Nielsen, 2007). Investigating the interaction between multiple cell surface and matrix components is intriguing because *Pseudomonas* biofilms require multiple steps to produce intricate adherent structures containing sessile cells.

During a screen of a mutant library in a type IV pili-defective strain, Vallet *et al.*, (2001) showed that an additional appendage made from an alternative fimbrial gene cluster encoding a chaperone usher pilus (*cup*) is important for adherence. Fimbrial adhesions other than type IV pili had not been described previous to this work, and the other members of the *cup* loci were also investigated for their potential contribution to biofilm adherence (Vallet *et al.*, 2001). Using conditions permissible for *cup* fimbrial expression and assembly, it was shown to cooperate in cell–cell interactions and microcolony formation (Kulasekara *et al.*,

2005; Ruer *et al.*, 2007). Descriptions of the importance of Cup proteins for biofilm formation also suggest that perhaps the fimbriae are not redundant adherence proteins for the well-characterized type IV pili but that they provide a synergistic or conditional ability of *Pseudomonas* to form biofilms (Vallet *et al.*, 2001; Giraud *et al.*, 2011).

Additional relevant protein matrix molecules are being identified as required for biofilm growth. Recently, a functional amyloid-like protein from a *P. fluorescens* strain was identified. The locus responsible for amyloid production, *fapA-F*, when overexpressed significantly enhanced biofilm adherence (Dueholm *et al.*, 2010). The major fibril subunit, produced from *fapC*, is well conserved even in *P. aeruginosa*. Amyloid production in *P. fluorescens* is similar to *Escherichia coli csgAB*-mediated curli formation (Dueholm *et al.*, 2010). CsgAB is important for adherence and initiation of aggregation of E. coli. The *fapC* component is conserved in *P. aeruginosa* as well; this may represent another tool *Pseudomonas* possesses to efficiently form biofilms regardless of the environmental circumstance.

Rhamnolipids

Rhamnolipids are surface-active glycolipid biosurfactants produced by various bacterial species, but they were originally identified from *P. aeruginosa* (Bergstrom *et al.*, 1946a, b; Abdel-Mawgoud *et al.*, 2010). Rhamnose and lipid moieties comprising rhamnolipids are linked by an O-glycoside (Abel *et al.*, 1978). Originally, cultures of *P. aeruginosa* were found to produce an oily glycolipid named pyolipic acid following growth on glucose (Bergstrom *et al.*, 1946a, b; Bergstrom *et al.*, 1946a, b). Later, the structural subunits were identified as L-rhamnose and *β*-hydroxydecanoic acid (Jarvis, 1949; Hauser & Karnovsky, 1954). The specific biological role for the production of this dynamic biosurfactant has remained elusive. However, *in vivo* models have shown that an *rhlA* mutant of *P. aeruginosa*, which fails to product rhamnolipid, is more rapidly cleared from mice in models of intraperitoneal foreign body infection and pulmonary infection (Van Gennip *et al.*, 2009). Sputum samples from *P. aeruginosa*-infected patients with CF had as much as 8 μg mL^{-1} of rhamnolipids, and the higher rhamnolipid concentration correlates with severity of clinical status (Kownatzki *et al.*, 1987). Importantly, Read *et al.* suggest that these values may be an underestimate of the rhamnolipid concentration in explanted lung tissue secretions, which was 65 μg mL^{-1} (Read *et al.*, 1992). It is unclear how rhamnolipid production leads to greater severity of disease, but *in vitro* biofilm studies suggest that rhamnolipid production affects biofilm formation (Davey *et al.*, 2003; Boles *et al.*, 2005; Lequette & Greenberg, 2005; Glick *et al.*, 2010). Rhamnolipids are toxic to neutrophils, a characteristic also proposed to impact invasiveness and persistence (Alhede *et al.*, 2009). Biosurfactants like rhamnolipid are important for motility-dependent capping of mushroom-shaped towers (Pamp & Tolker-Nielsen, 2007) and overall biofilm matrix remodeling (Davey *et al.*, 2003). Alterations in swarming by *rhlA* mutants lead to defective biofilm formation (Harshey, 2003; Shrout *et al.*, 2006; Overhage *et al.*, 2007; Verstraeten *et al.*, 2008; Yeung *et al.*, 2009). Similar to remodeling that occurs for water channel clearance, it appears that rhamnolipids can facilitate dispersal mechanisms allowing for release of planktonic daughter populations (Boles *et al.*, 2005). Final maturation of a biofilm is described as a transition back to a motile, dispersing state with the objective to seed new sites of infection and

potential biofilm formation (Ma *et al.*, 2009). During the colonization period, rhamnolipid potentially interacts with host cells exhibiting cytotoxicity (McClure & Schiller, 1992, 1996; Haussler *et al.*, 1998). More aggressive released swimming populations may also activate the innate immune system unlike metabolically inert microorganisms in the biofilm state. In addition to releasing cells from the biofilm, rhamnolipids induce shedding of LPSs resulting in enhanced cell surface hydrophobicity, thereby favoring primary interaction with a surface (Zhang & Miller, 1994; Al-Tahhan *et al.*, 2000). Modifying hydrophobicity and surface adherence through rhamnolipid secretion is especially important in environments where fewer nutrient sources are available, facilitating an adaptation to more rugged lifestyle (Neu, 1996; Deziel *et al.*, 2003; Boles *et al.*, 2005). Pseudomonads are particularly adept at acclimating to environmental stress through biofilm formation. Rhamnolipids aid adaptation through increased surface activity, wetting ability, detergency, and other amphipathic-related characteristics (Abdel-Mawgoud *et al.*, 2010). For example, bacteria capable of degrading hydrophobic substances, such as linear alkanes (a nutrient source for *P. aeruginosa*), commonly secrete biosurfactants that facilitate uptake and assimilation of hydrocarbons (Hommel, 1994). Solubility of higher molecular weight *n*-alkanes is less than that of small molecular weight molecules, thereby interfering with bacterial uptake (Abdel-Mawgoud *et al.*, 2010). Therefore, it remains undetermined how *P. aeruginosa* is able to use these high molecular weight hydrophobic compounds as an energy source; perhaps, rhamnolipid production is key in that role. Many other *Pseudomonas* spp. in addition to *P. aeruginosa* produce rhamnolipids (Gunther *et al.*, 2005; Onbasli & Aslim, 2009a, b), but their specific functionality has not been well defined.

Conclusions and perspectives

Pseudomonas spp. can persist under extremely harsh conditions, including the environment and the human body, and this property has been linked to their ability to form biofilms. *Pseudomonas aeruginosa* pathoadaptation, in particularly, has driven a large degree of the early biofilm research relating to alginate and the production of ‘slime’ (Schwarzmann & Boring, 1971; Evans & Linker, 1973; Hussain *et al.*, 1991; Ying *et al.*, 1996; Licking, 1999). Alginate-overproducing mucoid strains confer a selective advantage in their niche, the CF lung. The conversion to, and selection for, mucoid strains within the CF lung continues to be investigated because of the negative clinical outcomes associated with consecutive mucoid-positive sputum cultures. Ultimately, the ability of mucoid strains to persist and evade clearance mechanisms results in patient mortality if aggressive therapies are not deployed. Prior to mucoid conversion, Psl- and Pel-expressing nonmucoid biofilm-producing strains are likely responsible for early intermittent colonization of the CF lung. Although mucoid variants are not found during this period, Pel- and Psl-overproducing variant strains are frequently isolated. CF sputum samples, and even *in vitro* biofilms, produce stable RSCV characterized as class IV (Pel and Psl high producers) matrix-producing strains (Haussler *et al.*, 1999, 2003; Kirisits *et al.*, 2005; Ma *et al.*, 2006; Starkey *et al.*, 2009; Colvin *et al.*, 2011a, b). Both mucoid and RSCV morphotypes highlight the adaptive scenarios and the advantage of rapid evolution for *P. aeruginosa*. Additionally, it highlights the necessity for extensive biofilm formation and robust matrix production. While the importance of biofilm can be appreciated, combating the negative impact of biofilm requires greater elucidation.

Numerous questions remain unanswered surrounding *Pseudomonas* biofilm formation. The most pertinent questions include: What is the true nature of matrix components and how are these molecules interacting in the biofilm? How does biofilm formation occur and why do biofilm cells transition back to a free-swimming state? Finally, how do biofilm components confer recalcitrance to immune and environment antimicrobial factors?

Aggregative polysaccharides Pel and Psl were identified and appreciated more recently than the capsular polysaccharide alginate. Understandably, less is known about the basic nature of the aggregative polysaccharides. Byrd, *et al.*, (2009) determined the polysaccharide repeating units and the general nature of how Psl is synthesized. However, the assembly of Psl on the cell surface and release of Psl to the extracellular environment remain under continued investigation. Similarly, predictions regarding Pel-containing sugars, such as glucose, have been proposed based on the comparison of pellicles formed by wild-type and *pel* mutants (Friedman & Kolter, 2004a, b). Increased evidence of cell association or release of these polysaccharides will also be insightful for full functional determination. Aggregative polysaccharides of emerging importance in environmental pseudomonads also need additional analysis for complete understanding of their capacities and functions.

What has been identified is the major cue signaling nonmucoid pseudomonads to form biofilms, high intra-cellular c-di-GMP levels (Guvener & Harwood, 2007; Lee *et al.*, 2007; Monds *et al.*, 2007; Jonas *et al.*, 2009). The intracellular signaling molecule induces changes including aggregative exopolysaccharide overproduction (Kirisits, *et al.*, 2005; Jenal & Malone, 2006; Ma *et al.*, 2006; Lee *et al.*, 2007; Jonas *et al.*, 2009), downregulation of motility (Kirisits *et al.*, 2005), and enhanced adherence protein production (Monds *et al.*, 2007; Borlee *et al.*, 2010).

Intermittent colonization of CF lungs prior to chronic infection likely occurs because of the exposure to environmental *P. aeruginosa*. Common class IV matrix-producing strains (Colvin *et al.*, 2011a, b), such as RSCV, have an advantage during colonization. Enhanced persistence during initial infection is predicted to be due to polysaccharide-mediated aggregation of the c-di-GMP-overproducing strain MJK8 (Byrd *et al.*, 2011). Continued studies are directed at evaluating the interaction of the *P. aeruginosa* RSCV and host innate immune system. Defining the process of initial and intermittent colonization with nonmucoid strains of *P. aeruginosa* in the CF lung prior to chronic infection will aid avoidance or treatment before chronic infections persist.

Interestingly, reports have described a matrix-free cavity within mature mushroom structures of *P. aeruginosa* biofilms, which also include swimming subpopulations poised for release from the biofilm (Purevdorj-Gage *et al.*, 2005; Ma *et al.*, 2009). Several reports suggest that reversal of c-di-GMP levels from high to low or inhibition of quorum sensing will achieve biofilm dispersal (Hickman *et al.*, 2005; Barraud *et al.*, 2009; Schleheck *et al.*, 2009; Brackman *et al.*, 2011). Understanding what conditions generate subpopulations of the biofilm that convert to a planktonic phenotype may aid in the understanding how to prevent or combat biofilms. Prematurely triggering the regulatory sensors responsible for initiation of the free-swimming subpopulation may result in release of the entire biofilm and be an attractive therapeutic strategy. Emerging evidence elucidating the dispersal agents (e.g. D-

amino acids, cis-2-decenoic acid) offers promise for the future ability to treat devastating biofilm infections (Davies & Marques, 2009; Kolodkin-Gal *et al.*, 2010).

Once chronic mucoid infections are established, treatment with antibiotics becomes extremely challenging. Greater understanding of chronic *P. aeruginosa* infections is essential, especially investigating how mucoid and nonmucoid biofilms confer recalcitrance to host immune factors. *in vitro* models can be used to address this need preliminarily, but mechanisms of evasion need to be tested *in vivo*. Currently, only limited chronic infection models have been established, but models in development hold promise (Welsh *et al.*, 2009; Bragonzi, 2010). Additional novel approaches to studying chronic animal infection are nonetheless warranted; perhaps, models achieving bacterial persistence but not restricted to airway infection would be promising. Regardless of the specific modeling, interpretation of data from diverse models will ultimately aid in deciphering the complexity of biofilm infections in human disease.

What was once a simple ‘slime’ holding bacterial cells together has emerged to be a complex network including a wide diversity of molecules possessing functions in structure, signaling, and control of biofilm formation. For *P. aeruginosa*, biofilm matrix components are diverse and include polysaccharides like alginate, Psl, and Pel as well as nucleic acids and proteins. Less well-described biofilm components include LPS, cyclic β glucans, and MV with emerging importance for proper biofilm matrix function. *Pseudomonas* biofilm matrix components in addition to the ones described here will likely continue to be revealed and add to the abundant diverse nature of biofilm matrix. The theme of diversity in function and survivability is hardwired into *Pseudomonas* spp. and that carries through when discussing biofilm matrix components. Matrix components carry out functions that may be redundant, additive, or even synergistic with other co-produced matrix molecules, but individually, each likely contributes to the organisms’ ability to persist and adapt to environmental conditions.

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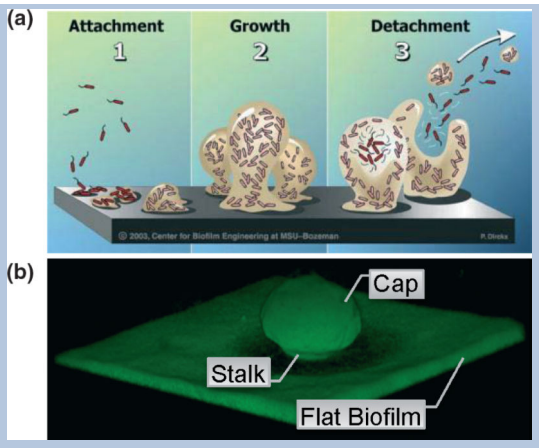
Box 1. Descriptions of biofilm were popularized by the work of Costerton et al beginning more than a quarter century ago. Sessile bacterial populations were identified in several environmental niches (Geesey *et al.*, 1977; Costerton *et al.*, 1987). As the relevance of sessile bacteria in clinical pathologies garnered greater appreciation, a definition of biofilm was proposed as a community of adherent sessile bacteria encased in some form of polymeric matrix (Costerton *et al.*, 1987; Costerton *et al.*, 1994). Greater understanding of biofilm nature lead Pasek and Singh to set forth specific criteria for diagnosis of biofilm infections (Parsek & Singh, 2003). Briefly, (1) bacterial aggregates must be associated with a surface, (2) microscopically bacteria appear encased in a matrix (of host and/or bacteria origin), (3) the infection remains localized as opposed to systemic, (4) the infection is antibiotic recalcitrant, (5) culture negativity is often observed, and (6) chronic: symptoms of infection persist (Parsek & Singh, 2003).

Widely accepted models mimicking biofilm infections include those that can account for some of the above criteria and highlight the biofilm developmental process. Bacterial biofilm developmental stages have been well scrutinized and concluded to consist of key stages (Stoodley *et al.*, 2002). Initially attachment of recently planktonic cells to a surface or location occurs. Then, production of an extra-polymeric substance corresponds with irreversible attachment and structured microcolonies. Finally, the hallmark of biofilm maturation is when a population of cells, often displaying motility characteristics (in *P. aeruginosa*), is released from the biofilm allowing for the cycle to continue (Stoodley *et al.*, 2002; Ma *et al.*, 2009). The structured microcolonies produced by *P. aeruginosa* have been compared to ‘mushroom’ shapes. During biofilm development initial clusters of cells forming microcolonies have been referred to popularly as ‘stalks’ of the mushroom (Klausen *et al.*, 2003a, b). As the biofilm matures further motile sub-populations are important for the formation of ‘caps’ on the stalks thereby completing the mushroom shape (Klausen *et al.*, 2003a, b). A sample image of a *P. aeruginosa* flow-cell biofilm is a classic example of the mushroom-shaped structure.

Psuedomonas biofilm development produces classic structure. (a) The stages of bioflm are represented: (1) Attachment as a transition from planktonic to sessile lifestyle. (2) Growth, during which the recognizable structures form. (3) Detachment and dispersal of cells for seeding new sites of attachment. Image was kindly provided by Montana State University Center for Biofilm Engineering: P. Dirckx. (b) A representative confocal micrograph of *P. aeruginosa* PAO1 biofilm grown in a flow-cell for 3 days.



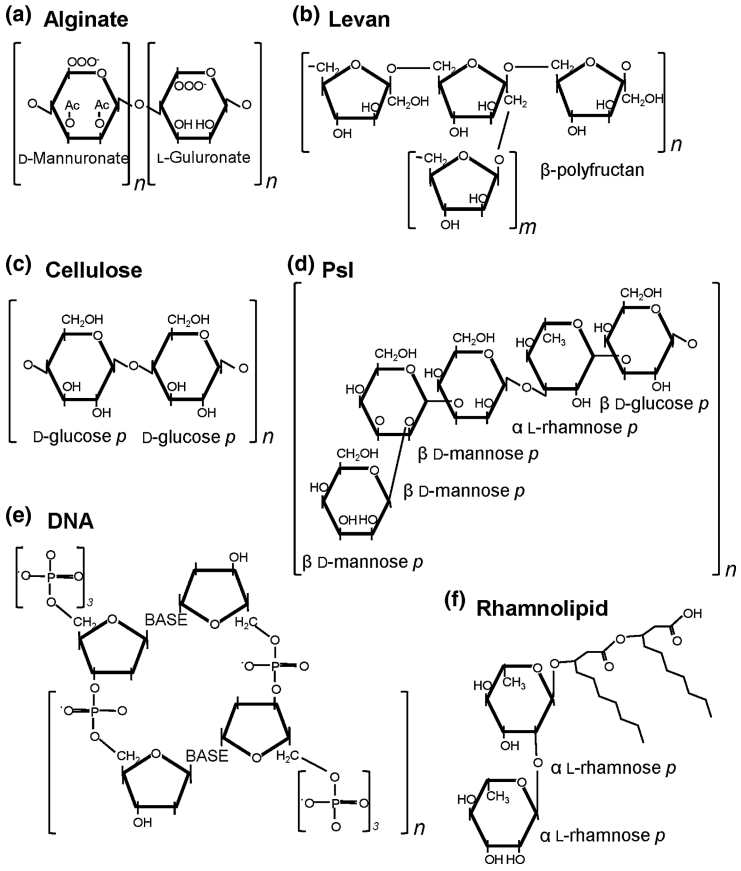


Fig. 1.

Abundant biofilm matrix molecules. Adapted representative chemical structures of (a) alginate, (b) levan, (c) cellulose, (d) Psl, (e) DNA, and (f) rhamnolipid. Brackets depict repeating units of each molecule.

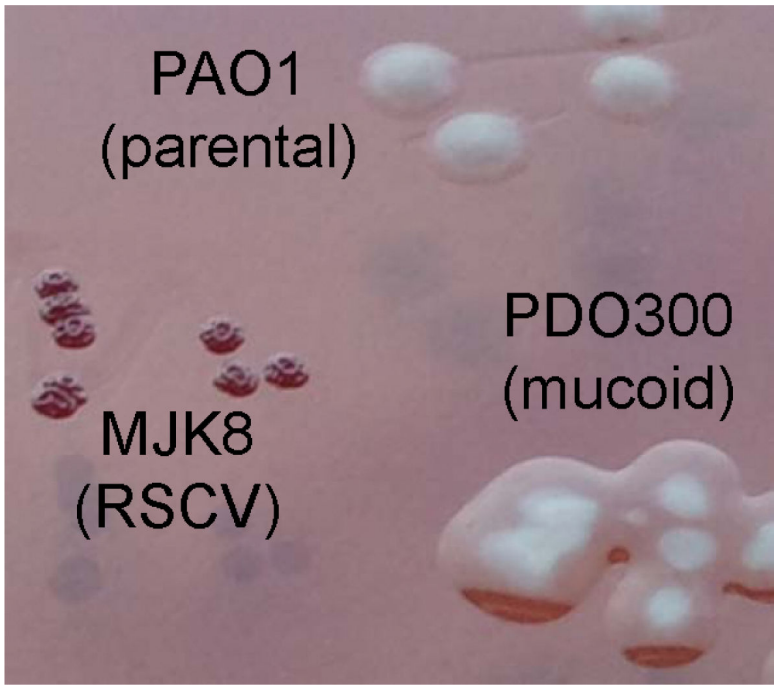


Fig. 2.

Prominent Pseudomonas aeruginosa colony morphology variants. Cultures of PAO1, MJK8 (RSCV), and PDO300 (mucoid) were streaked on VBMM with Congo red. All strains grow at similar rates, yet MJK8 colonies are small and more aggregative and copious overproduction of alginate is obvious from PDO300. PAO1 has well-defined smooth colonies.

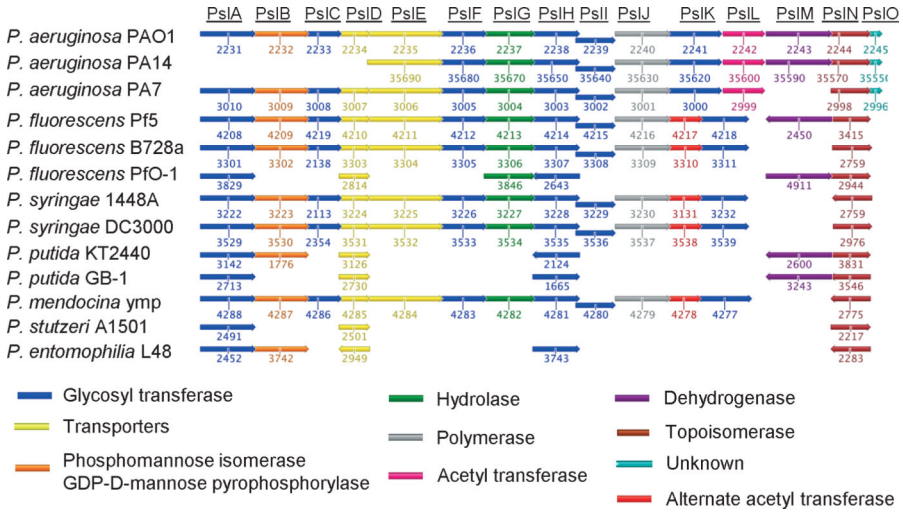


Fig. 3.

Polysaccharide synthesis locus (*psl*) in Pseudomonas spp. Relative gene organization and size are depicted for each pseudomonad and Psl component. The representative PAO1 Psl operon of approximately 18.4 kb is depicted with gene number designations for each comparison species given below individual components. Color coding represents predicted product function of each component. Information was gathered using the *Pseudomonas* database and prepared using the Geneious PRO software package (Stover *et al.*, 2000; Nelson *et al.*, 2002; Buell *et al.*, 2003; Feil *et al.*, 2005; Joardar *et al.*, 2005; Paulsen *et al.*, 2005; Lee *et al.*, 2006; Winsor *et al.*, 2009).

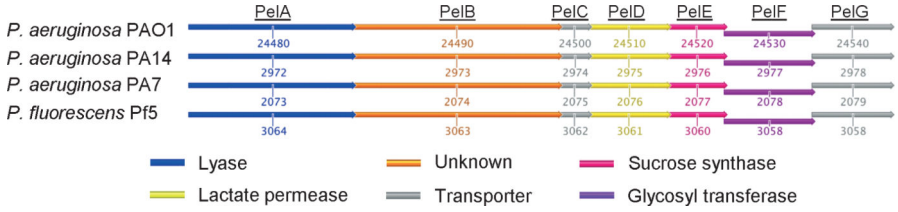


Fig. 4.

Pel polysaccharide synthesis locus among *Pseudomonas* spp. The conservation of individual components of the *pel* operon, which is responsible for Pel biosynthesis, is depicted for *pel*-containing Pseudomonas spp. The representative PAO1 Pel operon of approximately 12.2 kb and the corresponding gene number designation for each comparison species are given below representative loci. Color-coded boxes represent gene product functions as indicated on the top of this figure (Stover *et al.*, 2000; Friedman & Kolter, 2004a, b; Paulsen *et al.*, 2005; Winsor *et al.*, 2009).

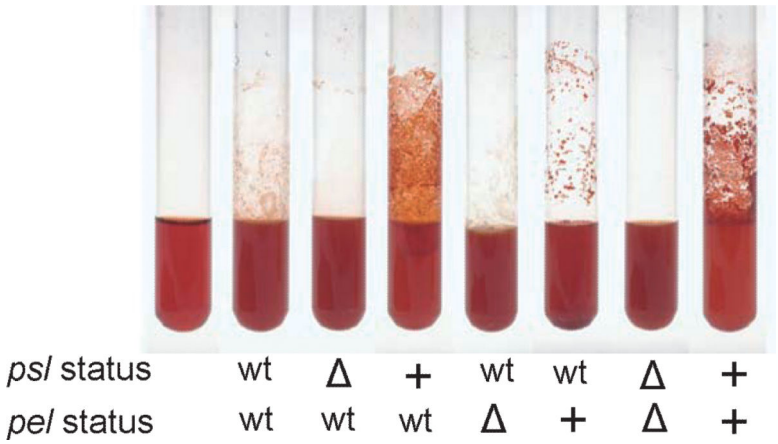


Fig. 5.

Psl and Pel influence aggregation, adherence, and colony morphology. *Pseudomonas aeruginosa* PAO1 strains with wild type, inactivated, or overexpressed status of Psl and Pel were grown in culture tubes containing Congo red to observe aggregation. Polysaccharide status is indicated below each tube.