Which Bacterial Biofilm Exopolysaccharide Is Preferred, Psl or Alginate?

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In this month's issue of the *Journal of Bacteriology*, Jones et al. (1) examine the relationship between the *Pseudomonas aeruginosa* chronically produced exopolysaccharide, alginate, and a polysaccharide that is produced during the initial stages of biofilm formation, Psl. Psl, composed of mannose, rhamnose, and glucose, was identified as one of the first exopolysaccharides in nonmucoid biofilm matrices (2). The authors show that AmrZ, a member of the ribbon-helix-helix family of DNA binding proteins, is one of the switches involved in activating alginate expression and repressing Psl expression. Furthermore, the authors demonstrated that altered expression of *amrZ* impacted biofilm structure indicating that the levels of AmrZ are tightly regulated. The authors conclude from these studies that AmrZ is one of the multifunctional regulators that mediate the transition from initial colonization (acute phase) to chronic biofilm formation (alginate production). This study is an important step forward in dissecting the complicated biology of this opportunistic pathogen that uses numerous mechanisms to cause and maintain infections.

There are at least three exopolysaccharides produced by *P. aeruginosa*, and they include the following: (i) alginate, a polysaccharide composed of mannuronic and guluronic acid; (ii) a glucose-rich exopolysaccharide synthesized by enzymes encoded by the *pel* gene cluster (PA3058-PA3064); and (iii) a mannose-, glucose-, and rhamnose-rich exopolysaccharide produced by proteins encoded in the *psl* gene cluster (PA2231-PA2245) (3–5). Each of these polysaccharides is associated with some stage of *P. aeruginosa* biofilm development, and mounting evidence suggests that Pel, Psl, and alginate are involved in different stages of the infectious process. Alginate was the first *P. aeruginosa* exopolysaccharide discovered, associated with the “mucoid” phenotype of *P. aeruginosa* strains isolated from cystic fibrosis (CF) patients (6). Pel was initially described in terms of *P. aeruginosa* growing at an air-liquid interface in cultures grown in static broth (3). The third exopolysaccharide, Psl, was identified from strains that were not capable of producing alginate but were still able to form biofilm structures on solid glass surfaces (2, 4, 7, 8). Alginate is arguably the best characterized exopolysaccharide produced by *P. aeruginosa*, and several excellent reviews have been written on the molecular biology of its production and clinical ramifications (5, 9–13).

The roles of alginate, Psl, and Pel have been examined extensively in biofilms individually (4, 7, 8, 11, 14, 15). Recently, mutants deficient in one or more polysaccharides and their effects on biofilm architecture were examined. Alginate-deficient mutants developed biofilms with a decreased proportion of viable cells and had more surface structures containing extracellular DNA. Deletion of *pslA* and *alg8* resulted in cells that overproduced Pel. Biofilms of cells with either Psl or alginate genes deleted lost the characteristic mushroom-like structure observed in later-stage biofilms. *P. aeruginosa* with *psl* and *pel* genes deleted lost their

ability to form biofilms altogether, indicating the importance of these exopolysaccharides in early stages of biofilm formation. These data show that alginate, Psl, Pel, and extracellular DNA interactively contribute to some aspect of *P. aeruginosa* biofilm architecture (16). The current state of knowledge indicates that Psl and Pel are likely involved with the initial stages of biofilm development (or acute stages of infection), whereas alginate is the “stress” response polysaccharide associated with chronic stages of infection.

The ability of *P. aeruginosa* to switch from the acute phase of infection to the chronic stage of infection involves numerous inputs. For instance, nine DNA binding proteins have been identified to directly interact with *PalgD*, making it challenging to come up with a single model that incorporates all of these proteins (Fig. 1). There are at least three DNA bending proteins involved in *algD* expression, integration host factor (IHF), AmrZ, and Hp-1. These three proteins may or may not bind at the same time to *PalgD* to control its expression. It is likely that these three proteins do interact with *PalgD*, either individually or in some combination, to induce looping of the promoter region under certain physiological conditions that have yet to be determined. The current work by Jones et al. (1) shows that AmrZ is able to act as an activator on *algD* as it represses *pslA*. These data indicate that either an AlgU-dependent gene somehow modifies AmrZ or increases the amount of AmrZ such that it becomes an activator of *PalgD*.

There are also two sigma factors involved in controlling *algD* transcription, AlgU (AlgT, sigma 22) and RpoN, depending upon the mucoid strain that is examined. Consequently, it has been proposed that antagonism is a mechanism by which both are able to initiate *algD* transcription (17) (Fig. 1D). The best-studied sigma factor for alginate production, AlgU, probably activates *algD* transcription with AlgR, AmrZ, and some combination of the other two DNA bending proteins (Fig. 1B). AlgU directly transcribes *algR* and *amrZ*, as well as *algD*, and both AlgU and AlgR are required for alginate production in multiple mucoid strains (18, 19). It appears from the literature that there may be other strains besides *muc-23* strains that are RpoN dependent, but the frequency of these strains among clinical isolates is not known. Since AlgB is an NtrC-like transcriptional regulator, it is likely that AlgB and RpoN work in concert to transcribe *PalgD* under nitrogen-limiting conditions or other physiological conditions distinct

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FIG 1 Transcriptional regulation of alginate biosynthetic genes. (A) The transcription of the major operon (encompassing the *algD*, *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX*, *algL*, *algI*, *algJ*, *algF*, and *algA* genes) containing genes encoding the biosynthetic enzymes and membrane-associated polymerization, modification, and export proteins is regulated by the promoter region of *algD*. Transcriptional regulators (AlgR, AlgB, AmrZ, CysB, and CRP [from *E. coli*]), histone-like proteins (Hp-1 and IHF), and two sigma factors (AlgU/T and RpoN) associate with the DNA and are involved in *PalgD* transcription. The numbers beneath the regulator name indicate the regions of the DNA (relative to the transcriptional start site) that have been found to bind the regulator through experimental evidence. Binding sites of the regulators are shaded with their respective colors. Hp-1 binding regions are underlined. Sigma factor consensus sequences are boxed. (B and C) Models showing AlgU/T and RpoN-dependent transcriptional activation. DNA bending is thought to occur with the aid of IHF and Hp-1. Transcriptional activators in the far upstream region (FUS), such as AlgR (B) or AlgB (C) are then able to activate transcription near the +1 site by interaction with the AlgU-RNAP (RNAP stands for RNA polymerase) or RpoN-RNAP complexes, respectively. (D) *PalgD* transcription is thought to be regulated by sigma factor competition. RpoN and AlgU binding sites overlap, and alginate production is activated depending on the type of stress encountered by the cell (nitrogen-related stress versus cell wall stress). (Reprinted from reference 34.)

from membrane perturbation (Fig. 1C). The position of the AlgB binding site is significantly removed from where a typical NtrC-like activating binding site should be located. However, all of the other DNA bending proteins may compensate for this apparent dislocation.

In addition to the proteins that bind to the *algD* promoter, two intracellular second messenger molecules, bis-(3'-5')-cyclic-dimeric-GMP (c-di-GMP) and cyclic AMP (cAMP), have been discovered to play a role in the production of alginate, Pel, and Psl. Mounting evidence suggests that c-di-GMP positively regulates

the production of biofilm matrix components at the transcriptional and allosteric level in *P. aeruginosa* and other Gram-negative species. The PilZ domain has been recognized as a c-di-GMP binding motif and was identified at the amino-terminal region of Alg44 through bioinformatics by Amikam and Galperin (20). Subsequent studies showed that elevated c-di-GMP levels, through overexpression of diguanylate cyclases, increased alginate production. Conversely, decreasing c-di-GMP pools by overexpression of phosphodiesterases reduced alginate levels (21). These authors localized Alg44 to either the inner or outer membrane fraction, whereas a previous study localized Alg44 to the periplasmic space (22). However, whether Alg44 is a single-pass transmembrane protein or whether it is merely associated with the membrane remains to be determined.

MucR is a membrane-bound diguanylate cyclase and a positive regulator of alginate production in a mucoid *mucA22* derivative of *P. aeruginosa* PAO1, PDO300 (23). Deletion of *mucR* also affected other known c-di-GMP processes in *P. aeruginosa*, including swarming motility, biofilm formation, as well as alginate production. This study additionally showed, through the use of *lacZ* and *phoA* fusions, that the predicted amino-terminal MHYT domain of MucR resides on the inner membrane. MHYT domains are proposed to bind O₂, NO, or CO. Moreover, O₂ and NO are known to stimulate alginate production (24–27). The authors proposed a model for c-di-GMP regulation of alginate production whereby the guanylate cyclase of MucR is stimulated, by an as-yet identified signal, which binds to a predicted MHYT domain in the amino terminus of MucR. Binding to the MucR MHYT domain stimulates the production of c-di-GMP locally, and this increased local c-di-GMP concentration may be sensed by Alg44, resulting in increased alginate production (28).

Evidence supporting the role for cAMP in alginate production is more indirect. The *algD* promoter is sensitive to glucose repression both in *Escherichia coli* and *P. aeruginosa*, indicating that catabolite repression may be operating (or playing a role) in the expression of alginate biosynthesis. One of the mechanisms for control of catabolite repression in *E. coli* is through the DNA binding protein cAMP receptor protein (CRP). The *P. aeruginosa* ortholog of the *E. coli* CRP is Vfr (for virulence factor regulator), which regulates the transcription of *lasR*, the type III secretion system, flagellar genes, and twitching motility (29–32). The *E. coli* cAMP-CRP complex bound the *algD* promoter and activated its transcription (33). These data indicate that Vfr and cAMP may directly control *algD* expression, but no studies have been conducted to examine this in any mucoid *P. aeruginosa* strain.

There are many other regulatory points that overlap the biosynthesis of Psl, Pel, and alginate polysaccharides, including RpoS, quorum sensing, and metabolic precursors used for the building blocks of each polysaccharide. RpoS regulation of Psl expression may indicate that it is also produced in response to certain stress conditions. There is likely an order to the expression of these exopolysaccharides depending upon which stresses are encountered first by *P. aeruginosa*. One model that could be proposed based upon the current literature is that Pel is induced initially by the Las quorum-sensing system, followed by Psl, as the organisms encounter starvation or other stresses. The data from Jones et al. (1) indicate that AmrZ would facilitate the transition from Psl to alginate production by repressing Psl and activating alginate, potentially when membrane-perturbing (AlgU-inducing) conditions ensue. There are likely still more regulatory mechanisms yet to be

discovered. The levels of alginate produced by mucoid mutants of *P. aeruginosa* PAO1 and other nonmucoid clinical isolates (i.e., non-CF isolates) were inversely related to biologically relevant concentrations (e.g., <5 μ M to 100 μ M) of iron present in the medium (J. R. Wiens, Y. Okkotsu, M. J. Schurr, and M. L. Vasil, unpublished results). It will be exciting to follow the developments that emerge from future studies, and hopefully, a therapeutic agent targeted to one or all of these systems will emerge from that knowledge.

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