Self-produced exopolysaccharide is a signal that stimulates biofilm formation in *Pseudomonas aeruginosa*

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Bacteria have a tendency to attach to surfaces and grow as structured communities called biofilms. Chronic biofilm infections are a problem because they tend to resist antibiotic treatment and are difficult to eradicate. Bacterial biofilms have an extracellular matrix that is usually composed of a mixture of polysaccharides, proteins, and nucleic acids. This matrix has long been assumed to play a passive structural and protective role for resident biofilm cells. Here we show that this view is an oversimplification and that the biofilm matrix can play an active role in stimulating its own synthesis. Working with the model biofilm bacterium *Pseudomonas aeruginosa*, we found that Psl, a major biofilm matrix polysaccharide for this species, acts as a signal to stimulate two diguanylate cyclases, SiaD and SadC, to produce the intracellular secondary messenger molecule c-di-GMP. Elevated intracellular concentrations of c-di-GMP then lead to the increased production of Psl and other components of the biofilm. This mechanism represents a unique positive feedback regulatory circuit, where the expression of an extracellular polysaccharide promotes biofilm growth in a manner analogous to autocrine signaling in eukaryotes.

Biofilms consist of microorganisms growing as aggregates; they usually form on surfaces and are responsible for a variety of chronic human infections (1). A hallmark of biofilms is that they are encased by an extracellular matrix, which can be composed of a mixture of DNA, fatty acids, proteins, and polysaccharides (2). The matrix has long been thought to function passively as an extracellular scaffold that holds the community together and occasionally protects resident cells from antimicrobials (3).

In many bacterial species, the intracellular signaling molecule, c-di-GMP, stimulates the synthesis of biofilm matrix components, particularly polysaccharides and proteins (4, 5). A common observation is that cells with elevated levels of intracellular c-di-GMP form thicker and more robust biofilms. *Pseudomonas aeruginosa*, an opportunistic pathogen that causes biofilm-associated chronic diseases in humans, is also a model organism for studying c-di-GMP signaling and biofilms. In *P. aeruginosa*, c-di-GMP stimulates the synthesis of two different extracellular polysaccharides, Pel and Psl, as well as the extracellular matrix adhesin protein CdrA (6–9). C-di-GMP-mediated control occurs at transcriptional and post-translational levels. High c-di-GMP relieves transcriptional repression at the *pel*, *psl*, and *cdrA* promoters by binding to the regulator FleQ (7, 10). Binding of c-di-GMP by PelD, a protein in the Pel biosynthetic complex, is also required for Pel biosynthesis (11). Additionally, the transcriptional repressor RsmA regulates translation of the *psl* transcript (12).

Recent work has focused on environmental signals that modulate intracellular c-di-GMP. Many c-di-GMP synthesis (diguanylate cyclase) and degrading (phosphodiesterase) enzymes harbor domains known to sense signals such as light, oxygen, and nutrients (13, 14). However, few specific instances of environmental signals that modulate the activity of these enzymes have

been identified. In *P. aeruginosa*, the diguanylate cyclases SiaD and WspR are activated upon exposure to SDS or contact with solid surfaces, respectively (15–17). In *Escherichia coli*, the activity of the DosCP cyclase/phosphodiesterase pair is modulated by oxygen availability (18), whereas diffusible signal factor was shown to activate c-di-GMP phosphodiesterase activity through the two-component regulatory system RpfG/C in *Xanthomonas campestris* (19). In addition, blue light was demonstrated to activate the c-di-GMP phosphodiesterase activity of BlrP1 in *Klebsiella pneumoniae* (20).

Here, we identify a unique function of the biofilm matrix. In addition to playing a major structural role in biofilms (6, 21–23), we found that the self-produced matrix exopolysaccharide Psl acts as a signal to stimulate the two diguanylate cyclases SadC and SiaD to produce c-di-GMP. Because elevated c-di-GMP further stimulates matrix polysaccharide production, this regulatory circuit constitutes a unique feed-forward loop. Our findings indicate that the biofilm matrix is a dynamic structure that has signaling properties in some ways similar to the multifunctional extracellular matrix of eukaryotic tissues (24, 25).

Results

Psl Signals Cells to Produce c-di-GMP. Our previous work demonstrated that the posttranscriptional regulatory protein RsmA inhibits Psl expression in *P. aeruginosa* strain PAO1 (12). An *rsmA* mutant strain had increased Psl production and formed rugose small colonies (called rugose small-colony variants or RSCVs). Strains that exhibit an RSCV phenotype have two- to threefold higher levels of c-di-GMP than strains that form smooth colonies (26). As expected, a $\Delta rsmA$ strain had elevated c-di-GMP relative to the wild-type PAO1 strain (Fig. S1). When we introduced a *psl* mutation into a $\Delta rsmA$ strain, c-di-GMP levels returned to wild-type levels. This surprising observation suggested that elevated c-di-GMP in a $\Delta rsmA$ strain is dependent on Psl (Fig. S1).

To test the hypothesis that Psl directly stimulates c-di-GMP production, we made use of a *P. aeruginosa* strain (PAO1 $P_{BAD-psl}$) in which *psl* expression is under the control of an arabinose-inducible promoter. Upon addition of L-arabinose to the culture medium, intracellular c-di-GMP increased two- to threefold

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Fig. 1. Psl stimulates increases in intracellular c-di-GMP. (A) An arabinose-inducible PAO1 $P_{BAD-psl}$ strain had elevated levels of intracellular c-di-GMP when *psl* was transcriptionally induced (α -Psl immunoblot shown in *Inset*) by the addition of 1% L-arabinose. * $P < 0.01$. (B) Overexpression of Psl, Pel, or CdrAB resulted in cellular autoaggregation, but increased levels of intracellular c-di-GMP were observed only in a strain that overexpresses Psl. * $P \leq 0.01$; ** $P > 0.8$. (C) Exogenous addition of Psl stimulated increased intracellular c-di-GMP. * $P < 0.01$.

compared with cells grown in the absence of L-arabinose (Fig. 1A). As shown in Fig. 1B, cells with increased Psl expression also autoaggregated in liquid culture; from this, it seemed possible that autoaggregation rather than Psl was stimulating c-di-GMP synthesis. To investigate, we tested two other strains that also autoaggregate, but in a Psl-independent manner, due to overexpression of Pel polysaccharide or CdrA adhesin. These two strains also formed clumps in liquid culture, but only the Psl-overproducing strain had elevated intracellular c-di-GMP (Fig. 1B). We concluded that autoaggregation per se does not stimulate increased c-di-GMP production. Next we added purified Psl exogenously to cells and found that such cells had twofold higher levels of intracellular c-di-GMP (Fig. 1C). These data indicate that Psl is acting as a signal directing cells to produce c-di-GMP.

It is possible that cells are sensing a physical attribute of Psl rather than Psl itself. For example, the presence of a polymer can impact the osmolarity, ionic properties, and viscosity of the environment (2, 3). To test this possibility, we exposed cells to a variety of polymers commonly used to modulate the physicochemical environment. Addition of varying amounts of sucrose,

dextran, PEG8000, and the neutral polysaccharide methylcellulose to cells all failed to stimulate c-di-GMP production (Fig. S2A). These results suggest that signaling does not result from indirect effects of Psl on the physicochemical environment outside the cell.

The Psl polymer consists of linked glucose, rhamnose, and mannose moieties in a pentameric repeat (27). Exogenous addition of these monomeric sugars did not elicit a c-di-GMP response (Fig. S2C). Moreover, purified Psl pretreated with Psl-reactive polyclonal antiserum failed to stimulate a c-di-GMP response when added exogenously to cells (Fig. 2). Psl was previously shown to be degraded by cellulase treatment (22). Cellulase-treated Psl also failed to stimulate the c-di-GMP response (Fig. 2). Collectively, our data suggest that cells specifically respond to extracellular Psl to produce c-di-GMP.

Psl Signaling Involves Two Diguanylate Cyclases: SadC and SiaD. The combined activities of cellular diguanylate cyclases and phosphodiesterases determine the overall intracellular concentration of c-di-GMP (4, 5). We hypothesized that Psl was stimulating the activity of a diguanylate cyclase or inhibiting the activity of a phosphodiesterase, either of which would result in increased cellular c-di-GMP levels. This is a complicated question to address, because *P. aeruginosa* strain PAO1 has 41 genes that encode proteins with predicted cyclase or phosphodiesterase activities (13).

We initially targeted our analysis to three diguanylate cyclases that have been strongly linked to *P. aeruginosa* biofilm formation: SadC, SiaD, and WspR (15, 16, 28). Using directed mutagenesis, we demonstrated that SadC and SiaD both contribute to Psl-induced c-di-GMP production (Fig. 3A). WspR, which is involved in c-di-GMP synthesis in response to surface contact (15, 17), had no role in the Psl response (Fig. S3). We concluded that SadC and SiaD, but not WspR, are important for the cellular response to Psl.

SadC is a membrane-bound diguanylate cyclase and is important for initial attachment during biofilm formation (28). The SiaABCD proteins form a transmembrane signal transduction complex that responds to SDS to activate c-di-GMP synthesis by SiaD (16). It is possible that Psl acts by inducing transcription of the *sadC* and/or *siaD* genes. However, quantitative real-time PCR showed that overexpression of Psl did not influence the levels of the *sadC* or *siaD* transcripts (Fig. S4).

We also tested whether the surface-exposed sensor protein of the SiaABCD complex, SiaA, is necessary for Psl signaling. We found that a *siaA* mutation had the same phenotype as the *siaD* mutant strain (Fig. S5), which suggests that SiaA is also somehow

Fig. 2. Coincubation of Psl with α -Psl antiserum or cellulase suppressed c-di-GMP production. * $P \leq 0.02$.

Fig. 3. Stimulation of c-di-GMP production by Psl is dependent on the diguanylate cyclases SadC and SiaD. (A) Exogenous addition of Psl stimulated intracellular c-di-GMP production in a SadC- and SiaD-dependent manner. $*P \leq 0.02$; $**P > 0.1$. (B) Induction of *cdrA* and *pslA* transcription in response to exogenous addition of Psl (Left). A $\Delta sadC \Delta siaD$ strain failed to respond to Psl addition (Right).

involved in sensing Psl. We are currently investigating the possibility that SadC and/or SiaA may directly bind Psl.

Biofilm-Associated Psl Signals Planktonic Cells to Produce c-di-GMP.

Cells respond to high levels of c-di-GMP by increasing transcription of the *psl* and *cdrA* genes (7). This regulation is controlled by FleQ, whose repression of *cdrA* and *psl* gene expression is relieved by high intracellular c-di-GMP (7, 10). The Psl-stimulated increase in cellular c-di-GMP was also seen in a $\Delta fleQ$ mutant strain, verifying that Psl signaling is FleQ independent (Fig. S6). As expected, a Psl-mediated increase in c-di-GMP resulted in two- to threefold higher levels of *cdrA* and *pslA* transcripts. Transcript levels remained low in a $\Delta sadC \Delta siaD$ double mutant strain exposed to Psl (Fig. 3B). Based on these results, we constructed a transcriptional *pcdrA::gfp* fusion plasmid as a tool for monitoring Psl sensing. GFP fluorescence was very low in a wild-type (*pcdrA::gfp*) reporter strain growing planktonically (Fig. S7). When this strain was treated with exogenous Psl, GFP fluorescence significantly increased (Fig. S7A and B), consistent with the data presented in Fig. 1C. Next we assessed whether cells that were producing Psl could induce a response in Psl-deficient recipient cells; to do this we mixed two strains together and grew them in shaken liquid culture. One strain (deficient for Psl production) harbored the *pcdrA::gfp* reporter plasmid, and the other strain was either a Psl producer or a *psl* mutant negative control strain. In liquid cultures, GFP fluorescence in the reporter strain was induced when it was cocultured with the Psl overexpressing strain (Fig. 4A and B), indicating that Psl signaling can occur *in trans*.

These observations lead us to speculate on the ecological role of Psl signaling. One scenario is that a free-swimming *P. aeruginosa* cell that lands upon an established *P. aeruginosa* biofilm would first encounter the surrounding Psl-rich matrix that encases the biofilm cells (6, 22). The Psl in the matrix could signal the planktonic cell to produce c-di-GMP, increase matrix production, and subsequently facilitate the integration of the planktonic cell into the biofilm community. To experimentally address this concept, we performed colonization assays. We introduced the PAO1 (*pcdrA::gfp*) reporter cells as planktonic cells and allowed them to attach to preestablished biofilms containing either a Psl-rich or Psl-deficient matrix. As we predicted, the Psl-producing biofilm stimulated the planktonic

reporter strain to produce c-di-GMP and thus activate expression of GFP (Fig. 4C). Transactivation of the planktonic reporter strain was only observed when these cells attached to an established biofilm with a Psl-rich matrix (Fig. 4C; Fig. S8). As expected, GFP expression was not induced in a $\Delta sadC \Delta siaD$ strain carrying the reporter construct (Fig. 4C).

Discussion

Here we demonstrate a unique signaling role for a component of the biofilm matrix. This finding challenges the long-held view of the matrix as a passive extracellular mesh. A signaling role for extracellular polysaccharides is not known to be a common feature in bacterial species. Outside of the Nod factor signals (sugar oligomers produced by rhizobial bacteria) sensed by plants, there are few known instances of exopolysaccharide signaling in bacterial species. Aguilar et al. (29) recently reported that matrix production is linked to control of sporulation in the Gram-positive species *Bacillus subtilis*. It was unclear if this control results from the production of exopolysaccharide, something associated with the matrix, or is simply due to changes in the physicochemical environment (29). However, the widespread importance of extracellular polysaccharides in the biofilm matrix produced by other species raises the possibility that this type of signaling is commonplace.

In *Salmonella enterica* Typhimurium, the RsmA homolog CsrA represses the translation of mRNAs encoding diguanylate cyclases (30). Thus, we initially thought that our *rsmA* mutant strain had elevated c-di-GMP due to increased expression of a diguanylate cyclase. However, our results indicate that the extracellular polysaccharide Psl triggers the activities, not the expression (Figs. S1 and S4) of two diguanylate cyclases: SadC and SiaD (Fig. 3A). The precise mechanisms of the Psl-induced signal transduction cascades are currently under investigation. In particular, understanding the nature of the interaction of Psl with an as-yet-unknown receptor on the cell surface is an important aspect of the signal transduction events.

Upon extensive analyses of SadC and SiaABCD proteins using bioinformatics software, including Phyre 2 (31), we found no common structural/domain features of SadC, SiaA, or SiaD that would suggest a common Psl-binding motif. SadC has several transmembrane domains (four or five) on its N terminus with a GGDEF (c-di-GMP cyclase) domain located toward its C terminus. The GGDEF domain of SiaD covers most of the protein length, and no other relevant structural homologies were found. SiaA, predicted to be the surface receptor protein of SiaABCD complex, appears to have a methyl-accepting chemotaxis protein-like structure in its N terminus, and its C terminus appears to be a response regulator-like serine/threonine phosphatase. There is no evidence that SadC or SiaABCD require Psl to function. SadC overexpression was previously shown to stimulate cyclase activity in the absence of Psl (28). In contrast, overexpression of SiaD did not stimulate c-di-GMP synthesis in the presence or absence of Psl (13).

There are several lines of evidence that Psl signaling is specific. Highly purified samples of Psl (devoid of LPS and proteins) stimulated signaling (Fig. S7A and B). Additionally, pretreatment of Psl samples with anti-Psl antiserum or cellulase diminished signaling activity (Fig. 2). Unfortunately, difficulties with generating significant quantities of highly pure Psl, as well as the semiquantitative nature of Psl Western blots, limited our ability to quantify the amount of Psl required for signaling to occur. We also cannot rule out that a fragment of Psl enters the cell and stimulates c-di-GMP production. Although the monomeric sugars that comprise Psl do not stimulate the response (Fig. S2C), more complex Psl-derived oligosaccharide fragments might stimulate signaling by binding to an external receptor, or by entering the

Fig. 4. Psl stimulates c-di-GMP production in neighboring cells *in trans*. (A) Micrograph image of a $\Delta pel \Delta psl$ (*pcdRA::gfp*) reporter strain cocultured with a mCherry constitutive Psl donor strain Δpel P_{BAD} -*psl* in the presence of 1% L-arabinose (Right). A mCherry constitutive $\Delta pel \Delta psl$ strain was the negative control (Left). The reporter strain cocultured with the Psl-producing strain was induced in GFP fluorescence. GFP (green) images were overlaid with mCherry (red) images. (B) Flow cytometry analyses of cocultures. mCherry-positive cells were gated out and the plot is for GFP fluorescence. Reporter strains cocultured with the Psl donor strain resulted in a population shift to increased GFP fluorescence (gray curve). (C) Established biofilms (3 d old) were exposed to cells carrying a *pcdRA::gfp* plasmid. GFP fluorescence was induced in incoming cells that attached to biofilms containing Psl, but not when they attached to Pel-only-containing biofilms. A $\Delta wspF$ mutation was introduced into the P_{BAD} -*pel* strain background to maintain equally high c-di-GMP levels for all established biofilms. The GFP induction was dependent on SadC and SiaD, because a $\Delta sadC \Delta siaD$ strain failed to elicit GFP fluorescence upon contacting a Psl-rich biofilm.

cell. The predicted inner membrane localization of SadC (32) and SiaA is compatible with both possibilities.

The fact that Psl can act *in trans* in *P. aeruginosa* is probably significant, as it allows Psl to amplify matrix production by the local community as a feed-forward signal. The biofilm matrix could be considered a “community good,” and therefore it is not surprising that there are intercellular signaling mechanisms that might coordinate its production by the community. The biofilm matrix is also a therapeutic target. Our findings suggest that this signaling pathway could be exploited for future antibiofilm-directed therapies against *P. aeruginosa* chronic infections.

Materials and Methods

Bacterial Strains and Growth Conditions. The bacterial strains used in this study are listed in Table S1. *E. coli* and *P. aeruginosa* strains were propagated in LB or Vogel–Bonner minimal medium at 37 °C unless otherwise specified.

C-di-GMP Extraction and Liquid Chromatography MS/MS Measurements. Our quantitative analysis protocol for intracellular c-di-GMP was modified from a previously published protocol (7). We used 2-chloro-AMP as an internal standard compound. All experiments were performed on independent biological quadruplicates. Liquid chromatography MS/MS measurements were performed using an Acuity UPLC with a Synergi 4 μ Hydro RP 80A column and a C18 Guard Cartridge (Phenomenex) on a Premier XL triple-quadrupole electrospray mass spectrometer (Waters). The m/z 691 > 152 transition was used for c-di-GMP and 382 > 170 for 2-chloro-AMP. The cone voltages and collision energies were 40 V/30 eV and 35 V/20 eV, respectively.

Psl Polysaccharide Purification. Psl polysaccharides were extracted from cultures and purified as previously described (27). A detailed protocol for this and for the preparation of highly purified Psl that was used for the experiments in [Fig. S7 A and B](#) is given in [SI Materials and Methods](#).

Quantitative Real-Time PCR. RNA was extracted from log-phase bacterial cultures, cDNA synthesized, and real-time PCR assays carried out as previously described in biological quadruplicates (6). Primer sequences used in this study are listed in [Table S2](#).

Flow Cytometry Sample Preparation and Analyses. Cells were fixed in 2% (vol/vol) paraformaldehyde before being analyzed by flow cytometry (up to 1 million events) on a FACSaria II (BD Biosciences). Data acquired with the FACSaria II were further processed and analyzed using FlowJo software (Tree Star).

Flow Cell Biofilm Cultures and Microscopy. Flow cell experiments were performed as previously described (6). Reporter strains were allowed to attach to preformed biofilm for 1 h without flow. Flow was resumed for 24 h, and the biofilm was stained using 300 μ L of 30 μ M SYTO 62 (Invitrogen).

Detailed methods are described in *SI Materials and Methods*.

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1. Parsek MR, Singh PK (2003) Bacterial biofilms: An emerging link to disease pathogenesis. *Annu Rev Microbiol* 57:677–701.
2. Sutherland IW (2001) The biofilm matrix—an immobilized but dynamic microbial environment. *Trends Microbiol* 9(5):222–227.
3. Flemming HC, Wingender J (2010) The biofilm matrix. *Nat Rev Microbiol* 8(9):623–633.
4. Cotter PA, Stibitz S (2007) c-di-GMP-mediated regulation of virulence and biofilm formation. *Curr Opin Microbiol* 10(1):17–23.

5. Hengge R (2009) Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* 7(4): 263–273.
6. Borlee BR, et al. (2010) *Pseudomonas aeruginosa* uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. *Mol Microbiol* 75(4): 827–842.
7. Hickman JW, Harwood CS (2008) Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Mol Microbiol* 69(2): 376–389.
8. Hickman JW, Tifrea DF, Harwood CS (2005) A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proc Natl Acad Sci USA* 102(40):14422–14427.
9. Ueda A, Wood TK (2009) Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). *PLoS Pathog* 5(6):e1000483.
10. Baraquet C, Murakami K, Parsek MR, Harwood CS (2012) The FleQ protein from *Pseudomonas aeruginosa* functions as both a repressor and an activator to control gene expression from the *pel* operon promoter in response to c-di-GMP. *Nucleic Acids Res* 40(15):7207–7218.
11. Lee VT, et al. (2007) A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. *Mol Microbiol* 65(6):1474–1484.
12. Irie Y, et al. (2010) *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. *Mol Microbiol* 78(1):158–172.
13. Kulasakara H, et al. (2006) Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. *Proc Natl Acad Sci USA* 103(8):2839–2844.
14. Mills E, Pultz IS, Kulasekara HD, Miller SI (2011) The bacterial second messenger c-di-GMP: Mechanisms of signalling. *Cell Microbiol* 13(8):1122–1129.
15. Güvener ZT, Harwood CS (2007) Subcellular location characteristics of the *Pseudomonas aeruginosa* GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces. *Mol Microbiol* 66(6):1459–1473.
16. Klebensberger J, Birkenmaier A, Geffers R, Kjelleberg S, Philipp B (2009) SiaA and SiaD are essential for inducing autoaggregation as a specific response to detergent stress in *Pseudomonas aeruginosa*. *Environ Microbiol* 11(12):3073–3086.
17. O'Connor JR, Kuwada NJ, Huangyutitham V, Wiggins PA, Harwood CS (2012) Surface sensing and lateral subcellular localization of WspA, the receptor in a chemosensory-like system leading to c-di-GMP production. *Mol Microbiol* 86(3):720–729.
18. Tuckerman JR, et al. (2009) An oxygen-sensing diguanylate cyclase and phosphodiesterase couple for c-di-GMP control. *Biochemistry* 48(41):9764–9774.
19. Ryan RP, et al. (2006) Cell-cell signaling in *Xanthomonas campestris* involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. *Proc Natl Acad Sci USA* 103(17):6712–6717.
20. Barends TR, et al. (2009) Structure and mechanism of a bacterial light-regulated cyclic nucleotide phosphodiesterase. *Nature* 459(7249):1015–1018.
21. Colvin KM, et al. (2012) The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. *Environ Microbiol* 14(8): 1913–1928.
22. Ma L, et al. (2009) Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathog* 5(3):e1000354.
23. Ma L, Jackson KD, Landry RM, Parsek MR, Wozniak DJ (2006) Analysis of *Pseudomonas aeruginosa* conditional psl variants reveals roles for the psl polysaccharide in adhesion and maintaining biofilm structure postattachment. *J Bacteriol* 188(23): 8213–8221.
24. Kim SH, Turnbull J, Guimond S (2011) Extracellular matrix and cell signalling: The dynamic cooperation of integrin, proteoglycan and growth factor receptor. *J Endocrinol* 209(2):139–151.
25. Schaefer L (2010) Extracellular matrix molecules: Endogenous danger signals as new drug targets in kidney diseases. *Curr Opin Pharmacol* 10(2):185–190.
26. Starkey M, et al. (2009) *Pseudomonas aeruginosa* rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. *J Bacteriol* 191(11):3492–3503.
27. Byrd MS, et al. (2009) Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. *Mol Microbiol* 73(4):622–638.
28. Merritt JH, Brothers KM, Kuchma SL, O'Toole GA (2007) SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. *J Bacteriol* 189(22):8154–8164.
29. Aguilar C, Vlamakis H, Guzman A, Losick R, Kolter R (2010) KinD is a checkpoint protein linking spore formation to extracellular-matrix production in *Bacillus subtilis* biofilms. *mBio* 1(1):e00035-10.
30. Jonas K, et al. (2010) Complex regulatory network encompassing the Csr, c-di-GMP and motility systems of *Salmonella Typhimurium*. *Environ Microbiol* 12(2):524–540.
31. Kelley LA, Sternberg MJ (2009) Protein structure prediction on the Web: A case study using the Phyre server. *Nat Protoc* 4(3):363–371.
32. Merritt JH, et al. (2010) Specific control of *Pseudomonas aeruginosa* surface-associated behaviors by two c-di-GMP diguanylate cyclases. *mBio* 1(4):e00183-10.