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c-di-GMP and its effects on biofilm formation and dispersion: a *Pseudomonas aeruginosa* review

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Biofilm Formation and Other Relevant Phenotypes in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative bacterium that has become an indispensable model organism in our quest to understand the A-to-Z of bacterial biofilms (1). It is genetically tractable, has a sequenced and annotated genome (<http://www.pseudomonas.com>), and boasts a number of useful tools (2, 3) that facilitate both *in vivo* and *in vitro* studies. Although it is commonly found as an environmental isolate, it is also an opportunistic pathogen capable of colonizing plants and mammalian hosts, and is particularly significant for its efficient colonization of lungs of cystic fibrosis (CF) patients (4-6). The versatility of *P. aeruginosa* is in large part attributed to a battery of traits that provide it selective advantage(s) across diverse environments. Like many other bacteria, *P. aeruginosa* is capable of transitioning between motile and sessile/biofilm lifestyles, which is believed to contribute to this bacterium's versatility.

Bacteria were once perceived to be simple, single-celled organisms; however, it is quite clear that microbes can participate in a broad range of complex multicellular behaviors, including quorum sensing, the formation of complex spore-forming aggregates by *Myxococcus* and *Bacillus*, swarming motility, and the formation of bacterial biofilms (7-13). Biofilms are defined as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to inert or living surfaces (14). The ability to form a biofilm is a common trait of a diverse array of microbes, including lower order eukaryotes. We now understand that in bacteria the intracellular second messenger molecule – 3',5'-cyclic diguanylic acid, or c-di-GMP (Fig. 1) – appears to control many facets of group behavior, including biofilm formation in *P. aeruginosa*.

c-di-GMP is synthesized from two GTP molecules by diguanylate cyclases (DGC), and is degraded by phosphodiesterases (PDE). The genome of *P. aeruginosa* PAO1 encodes 41 c-di-GMP proteins predicted to participate in c-di-GMP metabolism, while *P. aeruginosa* PA14 has 40 such proteins (15, 16). Interestingly, most of these proteins are linked to various sensory input domains on their N-terminus, including PAS, GAF, and REC domains (17, 18), presumably transducing environmental stimuli to cellular response(s). In fact, c-di-GMP has been implicated in numerous cellular functions including regulation of cell cycle, differentiation, biofilm formation and dispersion, motility, and virulence (19-28), adding credence to this prediction. With regards to biofilm formation in particular, the current general model associates high intracellular levels of c-di-GMP with biofilm formation or a sessile lifestyle, and low c-di-GMP levels are associated with a motile or planktonic

existence (29). While this general pattern holds true in many instances, the relationship between c-di-GMP levels and phenotypic outputs is likely to be much more complex (15, 29).

In the laboratory, biofilm formation is a cyclical process wherein a free-swimming planktonic cell encounters a surface – biotic or abiotic – and initiates cell-to-surface attachment (Fig. 2). The cell initially attaches reversibly by its polar flagellum (30), then subsequently attaches irreversibly along the cell's longitudinal axis, which we believe is the first committed step in biofilm formation. Irreversibly attached cells form the basis of the monolayer upon which mature biofilms are established, which vary in morphology from mushroom-shaped macrocolonies to uniform, thick layers of bacteria. Lastly, biofilm-associated cells are dispersed from the mature biofilm to resume a planktonic lifestyle, which completes the cycle. At this point, it is worth remembering that biofilm formation is dependent on a bevy of cellular factors and coordinated pathways. For example, previous reports have identified flagellar motility (31, 32), twitching motility (mediated by type IV pili) (31), and EPS (exopolysaccharides) (33, 34) as prerequisites to *P. aeruginosa* biofilms. Therefore, understanding the full-scope of biofilm formation requires a comprehensive view into how each component, and its regulation, contributes to the overall process.

Here, we aim to highlight key findings within the context of *P. aeruginosa* biofilm formation focusing on how second messenger molecule c-di-GMP regulates the numerous factors and pathways that contribute to the formation of a mature biofilm by this organism. This chapter will be divided into the various stages of biofilm formation – initial attachment (reversible, then irreversible), maturation, and finally, dispersal. It is important to keep in mind that biofilm formation studies using *P. aeruginosa* as a model have used a variety of different strains, including PA14, PAO1, PAK, and others. Thus, while we have tried to integrate the literature regarding *P. aeruginosa* biofilm formation into a single narrative, it is important to note that there may be examples of strain-specific findings, which we will highlight as appropriate.

Initial attachment: Reversible and Irreversible

Reversible attachment

The first step in biofilm formation is reversible attachment, a step wherein a bacterium first contacts a surface. To overcome surface repulsion, *P. aeruginosa* utilizes flagellar-mediated swimming motility (35). Biosynthesis of the flagellar machinery is tightly regulated in an intricate fashion and involves FleQ, a transcriptional regulator of flagellar expression, which binds to the upstream activation sequence of the *flhA* gene to start the cascade of flagellar gene expression (36). FleQ activity is regulated by at least two distinct methods: 1) sequestration of FleQ by FleN (36, 37) and 2) conformational change of FleQ when bound to c-di-GMP (38). More specifically, it has been shown that both FleN and c-di-GMP can inhibit the ATPase activity of FleQ *in vitro*, which renders FleQ incapable of regulating downstream flagellar genes (39). And in an interesting twist, FleQ may also have an indirect role of modulating c-di-GMP levels since decreased intracellular levels of c-di-GMP were observed in a *fleQ* mutant compared to wild type *P. aeruginosa* PAO1 (38). The redundancy in regulating flagellar biosynthesis, whether FleN- or c-di-GMP-mediated, not

only underscores the importance of flagellum and its motility, but also provides flexibility in controlling their expression.

The flagellar machinery includes the rotor, stator, flagellar hook and filament proteins, along with a number of ring structures that anchor the complex in the membrane and peptidoglycan layers. Flagellar-mediated propulsion requires the stator complex, which in turn generates torque through proton motive force. Recent identification of a “bacterial clutch” protein – YcgR – implies the possibility of controlling flagellar function after this molecular machine is assembled (24, 40–44). YcgR contains a PilZ domain, a common feature among c-di-GMP responsive proteins (21). While the specifics of this mechanism remain under investigation, YcgR is shown to interact with both the motor (FliG) and stator (MotA) proteins of the flagellar machine to reduce motility when c-di-GMP levels are high (40, 42, 44).

Despite the lack of an annotated *ycgR* gene in the *P. aeruginosa* genome database, sequence comparison of *E. coli* *ycgR* with the *P. aeruginosa* PAO1 genome identified *flgZ* (encoded by PA3353) as a potential homolog (45). Like *E. coli* YcgR, FlgZ is a cytoplasmic protein with a predicted PilZ domain. And based on studies in other pseudomonads, FlgZ binds c-di-GMP and co-localizes to the polar flagellar machinery, similar to YcgR (45, 46). Expanding on these findings, follow-up functional analyses should help identify the role(s) of FlgZ in *P. aeruginosa* motility.

Thus, there appear to be two distinct mechanisms that control flagellar motility during early biofilm formation in response to increasing intracellular c-di-GMP: the FleQ-c-di-GMP complex represses transcription of the genes required for flagellar assembly, while YcgR forms a complex with c-di-GMP that modulates flagellar rotation. It is important to note that repression of flagellar function can occur prior to initial, reversible attachment, thereby blocking even the earliest stage in biofilm formation. Alternatively, suppression of flagellar function may occur after reversible attachment, which would stabilize bacterial-surface interactions and promotes the transition to irreversible attachment.

Following reversible attachment, *P. aeruginosa* transitions into an irreversibly attached state and begins its progression towards a mature biofilm. Upon irreversible attachment, the cell encounters a number of changes that lay the groundwork for subsequent steps in biofilm formation. In the following section, we discuss how flagellar motility is modulated once bacteria have engaged a surface as well as mechanisms that may control other types of surface motility, such as pili-mediated twitching motility.

Irreversible attachment.

Once bacteria have begun interacting with a surface, they have three possible fates. First, *P. aeruginosa* can move using twitching motility, powered by type IV pili (TFP), which extend and retract to tug bacterial cells across the surface. Alternatively, *P. aeruginosa* has a second form of motility known as swarming, which utilizes the flagellum, as well as surfactants, to migrate on a substratum. Recent studies by Wong and colleagues have visualized such surface movements in real time (47–50) although the role of c-di-GMP and the control of these behaviors remain to be explored in detail. Third, bacteria can attach “irreversibly” via

the long axis of the cell. This irreversible attachment is much more stable than the reversible attachment discussed above, is the first committed step in biofilm formation, and is typically associated with the so-called “monolayer” stage of biofilm formation (51).

In the following paragraphs, we will focus on the DGCs and PDEs of *P. aeruginosa* that may contribute to controlling irreversible attachment. As previously alluded to, the control of swarming motility, twitching motility, and irreversible attachment are tightly interlinked. For example, while these surface motility mechanisms likely allow *P. aeruginosa* to explore a newly colonized surface environment, suppression of these motility functions is required to allow this microbe to initiate and commit to the more stable, irreversible attachment. We will begin with a discussion of the control of swarming motility, followed by the control of twitching motility.

In the discussion of swimming motility above, we outlined the role of FleQ and YcgR in regulating flagellar-mediated swimming motility. Given the essential role of the flagellum in swarming motility, these same regulators may also impact flagellar-mediated surface motility as well. The role of FleQ and YcgR in modulating swarming has not been examined; however, enzymes involved in c-di-GMP metabolism that impact swarming have been identified. Based on the inverse relationship between c-di-GMP and motility, it is likely that DGC activity (producing c-di-GMP) will repress motility, whereas PDE activity (degrading c-di-GMP) will promote motility.

Consistent with this model, work from Merritt *et al.* and Kuchma *et al.* identified two proteins – a DGC and a PDE – called SadC and BifA, respectively, that inversely impact swarming and biofilm formation. *In vitro* activity assays and quantification of intracellular c-di-GMP levels indicated that the SadC and BifA proteins were indeed DGC and PDE enzymes, respectively (52, 53). The *sadC* mutant is a hyper-swarming strain that is also defective in attachment, and conversely, the *bifA* mutant has a hyper-biofilm phenotype (showing a ~5.5-fold increase in attachment) and is unable to swarm. Thus, SadC and BifA both appear to contribute to control of surface motility, and thereby, are also required to establish irreversible attachment. Interestingly, when assayed for swimming motility, neither *sadC* nor *bifA* mutant was statistically different from that of wild type *P. aeruginosa* PA14, and only the *bifA* mutant showed a reduction in twitching motility (52, 53), which collectively suggest that these DGCs and PDEs preferentially impact swarming motility. Thus, SadC and BifA appear to regulate c-di-GMP levels predominantly for surface behaviors of *P. aeruginosa* PA14.

While identifying mutants with biofilm defects, O'Toole and Kolter discovered mutations in type IV pili (TFP) (31). TFP are polar appendages that can serve as a bacteriophage receptor (54), and as previously mentioned, are required for a mode of surface motility known as twitching (54, 55). The complexity of TFP biosynthesis is well-documented (55), and its expression is partly regulated by c-di-GMP. The FimX protein (encoded by PA4959) has a predicted DGC and a PDE domain. Based on *in vitro* studies and crystal structure, its DGC domain was demonstrated to be degenerate (28, 56). Due to conflicting results, however, whether FimX PDE domain is enzymatically active remains controversial. It is possible that

the binding of c-di-GMP to the PDE domain may implicate FimX as an effector protein rather than an enzyme of c-di-GMP hydrolysis.

Using strain *P. aeruginosa* PA103, the intracellular levels of c-di-GMP in the *fimX* mutant were shown to be on par with wild type. However, suppressor mutant studies using the *fimX* mutant showed that an increase in c-di-GMP by overexpressing DGCs rescued twitching motility and phage sensitivity defects (27). Surprisingly, overexpression of the same DGCs in *P. aeruginosa* PAO1 *fimX* mutants failed to restore twitching motility despite proper pili formation (27). The differences in these findings may be due to differences in strain background, or reflect differential responses to the non-physiological overexpression of DGCs. Regardless of these differences, FimX and its ability to regulate TFP biosynthesis and function remains consistent across *P. aeruginosa* strains.

Currently, the mechanism by which FimX impacts twitching motility is unclear. FimX may act as a canonical PDE and reduce c-di-GMP levels, or alternatively, this protein may act as part of an effector system, binding c-di-GMP and modulating twitching motility. FimX is a polar-localized protein in *P. aeruginosa* PAK (28), and thus may exert its effect on pili biogenesis and function via directly interacting with the pilus machinery. Subsequent studies identified a second c-di-GMP-binding protein, PilZ (encoded by PA2960) that was also defective in twitching motility, and failed to export pilin subunits despite proper expression of the *pilA* gene (21, 57). Therefore, this protein may also regulate pilus assembly. Whether FimX and PilZ interact, co-localize, or compete for c-di-GMP molecules is unclear, but their converging role in regulating pilus assembly warrants further investigation. At the same time, the mechanism(s) by which c-di-GMP regulates pilus assembly and function deserves additional attention given the role of pili in irreversible attachment, as well as in the formation of the mushroom “caps” during biofilm maturation (58).

In addition to repressing surface motility, irreversible attachment also appears to require the production of exopolysaccharides (EPS). Beyond the role of EPS in irreversible attachment, these secreted polysaccharides, together with nucleic acids, proteins and additional factors, comprise the matrix of the mature biofilm – a point discussed in more detail below. Here, we will focus on the role of EPS in irreversible attachment.

Two polysaccharides, Pel and Psl, have been identified in *P. aeruginosa* PAO1, but only Pel is produced in strain *P. aeruginosa* PA14 (33, 34). These two EPS are believed to be structurally different, with Pel thought to be mainly glucose-rich, although the structure of this EPS remains to be definitively established, while Psl is a mannose-rich polysaccharide (34). Colvin *et al.* demonstrated that *P. aeruginosa* PA14 *pel* and *P. aeruginosa* PAO1 *psl* mutants were both arrested at the monolayer stage of biofilm development, with concurrent reduction in accumulated biofilm biomass compared to their respective parental strains (59). These data suggest that either Pel or Psl can promote irreversible attachment. Wong and colleagues recently showed that *P. aeruginosa* PAO1 migrates across a substratum leaving a trail of the Psl polysaccharide, which in turn enhances subsequent attachment, eventually driving microcolony formation (47), a key step in the production of the mature biofilm (51). In contrast, no specific role for the Pel EPS has been assigned. Interestingly, clinical isolates expressing both Pel and Psl have been identified (34, 60),

indicating that the *P. aeruginosa* PA14 may be somewhat unusual in only having one of these EPS biosynthetic operons. Furthermore, no protein adhesin of *P. aeruginosa* has been identified as mediating irreversible attachment analogous to the cell-surface protein LapA of *Pseudomonas fluorescens* (61). All data considered, we speculate that perhaps at early stages of attachment, Pel and/or Psl serve as such an adhesin.

P. aeruginosa PAO1 produces Psl, a mannose-rich polysaccharide. Genetic analysis identified *pslACDEFGHIJKL* to be essential in Psl production and/or secretion, whereas mutations in *pslBMNO* genes failed to exhibit any change in surface-associated Psl and may be non-essential for the production of this polymer (22). The *psl* genes are constitutively expressed in PAO1 (62), indicating that this EPS may be regulated strictly via post-transcriptional mechanisms, at least in this strain and in the laboratory. It is possible that there are unknown environmental signals that drive regulation in the host or environment. To date, no PilZ domain protein has been identified as required for Psl biosynthesis, leaving open the question as to how the production of this EPS is regulated post-transcriptionally by c-di-GMP.

Pel polysaccharide production is dependent on a 7-gene operon in both *P. aeruginosa* PA14 as well as *P. aeruginosa* PAO1. The *pel* genes are transcriptionally regulated by FleQ, which binds at two different sites on the *pelA* promoter region. Although FleQ acts as a repressor in low c-di-GMP environments, upon elevation of c-di-GMP, conformational change induces FleQ-dependent expression of the *pel* operon (38, 63). The PilZ domain protein PelD is crucial for proper Pel production (64). Similar to previously described PilZ domain proteins, PelD binds c-di-GMP (65, 66), and presumably requires activities of DGCs and PDEs as a means to ensure the regulated production of this EPS. To date, several studies have identified potential candidate DGCs and PDEs that may play such a role in EPS regulation.

In strain *P. aeruginosa* PA14, mutation of the *bifA* gene encoding a PDE was shown to boost Pel production, as well as biofilm formation (52). Merritt *et al.* also demonstrated a decrease in Pel-mediated EPS production when the DGC RoeA (encoded by PA1107) was mutated in *P. aeruginosa* PA14 (67). Mutating *roeA* reduced intracellular c-di-GMP levels, but did not alter expression of the *pel* locus (52, 67), suggesting that RoeA likely contributes to the production of the c-di-GMP bound by PelD, although such a connection has not been formally demonstrated. Similarly, the boost in Pel production seen in a *bifA* mutant was not associated with a change in *pel* transcript levels (52). In any event, loss of RoeA function reduced early biofilm formation, and impacted the ability of *P. aeruginosa* PA14 to irreversibly attach to the surface (67).

An interesting observation emerged while analyzing the phenotypes of *bifA*, *roeA*, and *sadC* mutants. While the *bifA* mutant showed high levels of Pel production, the *bifA roeA* double-mutant showed very little Pel EPS production, a phenotype similar to mutating RoeA or the Pel locus (67). In contrast, the *bifA sadC* double-mutant showed levels of Pel production quite similar to the *bifA* mutant. Surprisingly, both the *bifA roeA* and the *bifA sadC* double-mutants showed similar levels of c-di-GMP, which were still ~4-5-fold higher than the measured levels of the wild type *P. aeruginosa* PA14 (67). These

data indicated that quite distinct phenotypes could emerge from strains with similar global c-di-GMP pools, arguing strongly for localized pools of c-di-GMP as a mechanism to confer specific phenotypic outputs. Consistent with this hypothesis, Miller and colleagues, using FRET biosensors, argued for asymmetrical distribution of c-di-GMP pools (68).

Localization studies of RoeA in the bacterium showed discrete patches, perhaps beginning to address a RoeA-specific impact on EPS production. However, the inability to make a functional fluorescent fusion protein to PelD did not allow a clear determination of whether RoeA and PelD were co-localized (67).

It is likely other factors in addition to motility and EPS control irreversible attachment. For example, an in-frame deletion of a dual-domain protein MorA (encoded by PA4601) in strain PAO1 resulted in wild-type swimming motility (69), but reduced biofilm formation (15, 69). The impact of this mutation on swarming or c-di-GMP levels was not examined, thus it is difficult to make any conclusions as to its role in biofilm formation; however, it is likely MorA contributes to irreversible attachment given its decreased biofilm formation in the microtiter dish biofilm assay, a model that identifies early defects in attachment. Interestingly, a *morA* mutant in the small colony variant *P. aeruginosa* 20265 (a small-colony variant of strain *P. aeruginosa* PA14) increased swimming motility (70). The contribution of MorA may be strain specific (PAO1 vs. PA14), or the small-colony variant may uncover a new function of MorA not identified in *P. aeruginosa* PAO1.

Extracellular DNA (eDNA) is also important in early stages of attachment in *P. aeruginosa*. Whitchurch and colleagues demonstrated that degradation of eDNA by DNase I treatment prevented *P. aeruginosa* surface attachment, but this effect was marginal on more mature biofilms (71). On a cellular level, eDNA release facilitated cell-to-cell clumping even among planktonic cells (72). These data suggest that the early release of eDNA may facilitate cell-to-cell clumping and a stable surface attachment leading up to the irreversible attachment. Despite its contribution in biofilm formation, unlike other biofilm-relevant traits discussed thus far, eDNA release has yet to be correlated with c-di-GMP. We reiterate that there has been no single adhesin identified for *P. aeruginosa* to stabilize irreversible surface attachment, but the collective evidence we have provided thus far suggests a possibility wherein EPS, and perhaps eDNA, are regulated by c-di-GMP and contributes to irreversible attachment.

Taken together, it is likely that several c-di-GMP-regulated processes contribute to irreversible attachment and the commitment to a biofilm lifestyle in *P. aeruginosa*. Upon initial reversible attachment, *P. aeruginosa* can immediately transition to irreversible attachment, but also has the capability to move across the substratum by TFP-mediated twitching motility or flagellar-mediated swarming motility. Thus, co-regulation of these three surface behaviors – irreversible attachment, twitching and swarming – are tightly inter-related. We have discussed how a single PDE, BifA, impacts biofilm formation, swarming and twitching motility, but the DGCs SadC and RoeA impact swarming and EPS production, respectively, leading to irreversible attachment. Another example shows that twitching and swarming are linked through the actions of the PilY1 protein and the minor pilins, which are required for both TFP biogenesis and for regulation of swarming motility (73). Accordingly, to establish irreversible attachment and progress to the formation of a

mature biofilm, *P. aeruginosa* must utilize various c-di-GMP-mediated pathways to repress both twitching and swarming motility subsequent to cell-to-surface contact.

Biofilm Maturation: Microcolonies and Macrocolonies

Mature biofilms are enclosed in an extracellular matrix composed of proteins, polysaccharides, nucleic acids, lipids and other cellular components (1). This polymeric matrix forms a protective barrier for biofilm-associated cells from a plethora of threats including, but not limited to, bacteriophage, amoebae, host immune responses, and antibiotics (14). In addition to providing structural rigidity, polymeric substances that comprise the matrix of a biofilm are known to retard the diffusion of molecules including antibiotics (14), likely contributing to one mechanism of antibiotic tolerance associated with biofilms. In this section, we provide a picture of biofilm maturation, that is, the events following initial surface attachment and monolayer formation, and how c-di-GMP contributes to this overall process.

Microcolony formation

The current model in the field postulates that the formation of microcolonies and macrocolonies, and thus mature biofilms, requires the production of the EPS component of the matrix. In general, *P. aeruginosa* relies on three different sugars as a central building block for its matrix: alginate, Pel and Psl. This role in the formation of a mature biofilm for Pel and Psl is in addition to their contribution to irreversible attachment, as described above. We address how Pel and Psl contribute to biofilm maturation below. The third EPS, alginate, likely plays a critical role *in vivo*, particularly in the context of CF; however, there is little evidence that alginate contributes to biofilm formation in the typical *in vitro* models using glass or plastic as a substratum (74) despite the evidence that genes involved in alginate synthesis, *algC*, *algD*, and *algU*, are upregulated when *P. aeruginosa* attaches to a surface (75).

The regulation of alginate, as is the case for Pel and Psl, is linked to c-di-GMP. Studies using *P. aeruginosa* PDO300, a mucoid strain, identified the dual-domain GGDEF/AL protein MucR (encoded by PA1727) as a regulator of mucoidy phenotype (76). Loss of mucoidy observed in a *mucR* mutant indicated that MucR might be a functional DGC. Perhaps not surprisingly, *P. aeruginosa* PAO1 and *P. aeruginosa* PA14 overexpressing MucR overproduced EPS (76), although one must be cautious in interpreting such non-physiological, over expression studies. Alg44, a PilZ domain protein, was subsequently identified (57, 77) and demonstrated to bind c-di-GMP *in vitro* (57), and a *alg44::Gm^R* insertional mutant failed to release alginate to the extracellular milieu (77), findings which are consistent with its expected role in stimulating alginate production. With its demonstrated affinity for c-di-GMP, it is possible that Alg44 responds to c-di-GMP produced by MucR, analogous to a RoeA-PelD hypothesis stated above.

How are Pel and Psl regulated to specifically contribute to the later stages of biofilm formation, in contrast to their potential role in irreversible attachment? One common aspect between Pel and Psl production is their dependence on c-di-GMP. However, there appear to

be several different c-di-GMP-metabolizing proteins that contribute to the temporal control of Pel and Psl production.

The WspR protein (encoded by PA3702) was identified as a regulator of EPS and contributes to biofilm microcolony formation and maturation (78). WspR is a component of a chemotaxis-like system that controls EPS production in a number of pseudomonads, including *P. aeruginosa*. Though the precise signal has yet to be determined, contact with a solid surface seems to be sufficient to trigger regulation through the Wsp system (79, 80). On an agar surface, the Asp70 residue of WspR is phosphorylated, which in turn is associated with subcellular clustering of this protein and induction of its WspR DGC activity (79). The resulting WspR-mediated accumulation of c-di-GMP in turn yields increased EPS production, which can readily be observed via a wrinkly colony morphology (78). This wrinkly colony phenotype requires a functional Pel/Psl system, and presumably the c-di-GMP produced by the Wsp system induces EPS production via increased expression of the *pel/psl* genes and activity of their products.

Interestingly, Irie *et al.* observed an unusual increase in intracellular c-di-GMP when *psl* genes were overexpressed. Subsequent supplementation of purified Psl *in trans* to a *P. aeruginosa* PAO1 culture led to a conclusion that Psl, and not the expression of *psl* genes, stimulated c-di-GMP production. Furthermore, this outcome is mediated by two different DGCs – SiaD (encoded by PA0169) and SadC (81). Thus, c-di-GMP stimulates Psl production, and this EPS in turn stimulates c-di-GMP levels, establishing a positive feedback loop. How might this increased Psl impact biofilm formation? Using high-resolution microscopy and single-cell tracking studies, the stimulatory effect of Psl on biofilm formation appears to act by: 1) promoting incorporation of planktonic cells into biofilms and/or their attachment to a surface, and 2) maintaining micro- and macrocolony structure.

In addition to changes in *psl* gene expression, increase in c-di-GMP also affects *cdrA* expression. The relative levels of *cdrA* transcript in a *wspF* mutant (high intracellular c-di-GMP) was 27-fold higher than that of wild type *P. aeruginosa* PAO1 (82). Originally a 220 kDa protein, it is processed and secreted as a 150 kDa molecule that can bind Psl in the extracellular milieu and mediate auto-aggregation (82). This Psl-binding property is thought to assist biofilm formation, especially en route to a mature biofilm in *P. aeruginosa*. However, it is unlikely that CdrA acts as an early attachment adhesin since *cdrA* and *wspF cdrA* mutants showed no noticeable defects in static microtiter biofilm assays after 20 hours (82). Nevertheless, the increase in *cdrA* expression in biofilm-grown cells (82) implies its significance in the later maturation of this surface-associated group behavior.

EPS appears to play roles in two discrete steps in biofilm formation: irreversible attachment and biofilm maturation. The DGCs/PDEs that control production of polysaccharide are also distinct, with RoeA acting early and WspR participating in later stages in biofilm formation. We suggest that multiple DGCs in *P. aeruginosa* control EPS production, but do so at different stages in this process. For example, perhaps RoeA is required for initial stable surface attachment, but to maintain this attachment, WspR and its surface-dependent activity must be induced to reinforce the attachment decision and maintain EPS production.

Although temporal regulation of *cdrA* has not been explored, its dependence on c-di-GMP suggests a similar reliance on RoeA and/or WspR along with other relevant DGCs and PDEs for controlled expression. The observation that Psl can stimulate c-di-GMP levels also suggests the possibility of a self-reinforcing, and perhaps temporal, maintenance of the biofilm. Thus, we suggest that multiple, serial signals may be required before a bacterium commits to a life on the surface.

Macrocolony formation

How microcolonies transition to macrocolonies is still poorly understood. The mechanism may be just the continued development of a microcolony over time, or *P. aeruginosa* may have evolved explicit mechanisms for inducing macrocolony formation at a particular phase. Alternatively, perhaps maturation of the biofilm is driven by a combination of genetic determinants required for microcolony formation intersecting with, and modified by, physiological factors and constraints.

Examining the localization of Psl during the maturation of a biofilm by *P. aeruginosa* PAO1 illustrates how microcolony and macrocolonies may be distinct. Microcolonies of *P. aeruginosa* PAO1 probed with anti-Psl antibodies showed a uniform distribution of the polysaccharide across the biofilm (83). Based on the data above, initial production of Psl is likely further reinforced via stimulation of SiaD- and SadC-dependent c-di-GMP production. In the larger macrocolonies, however, the expression profile of Psl is much less uniform. In macrocolonies, Psl is primarily expressed on the periphery of the structure, whereas Psl expression in the central regions of the colonies is low or absent (83). Once expressed, Psl in the extracellular matrix is tethered together with the assistance of c-di-GMP-dependent CdrA (82). Moreover, CdrA is responsible for proper morphology of macrocolonies and stabilizing attachment to the substratum that enables the biofilm to withstand any changes in physical parameters of the environment (82). This differential expression of *psl* may be attributed to a relatively higher metabolic activity observed in the periphery of the macrocolony and the lack of necessary energy to make Psl in the heart of the biofilm (84, 85), or Psl may be actively down regulated in the center of the macrocolony because it is only required on the macrocolony surface at this late stage of maturation. For example, the loss of Psl production together with other contributing factors, e.g., rhamnolipid production (86), may enable the formation and maintenance of channels between macrocolonies, which has been postulated as being necessary for transporting nutrients, metabolites, and waste (1, 86). Unfortunately, currently available data do not allow us to readily distinguish between these models.

Bringing it Full Circle: c-di-GMP-Dependent Dispersal

Under appropriate conditions, dispersal enables a controlled release of *P. aeruginosa* cells from a mature biofilm structure back to a planktonic mode of growth. A number of signals have been identified that trigger dispersal of biofilm cells back to the planktonic state. Here, we focus on pathways that utilize c-di-GMP as a key second messenger in the dispersal process.

Yoon and colleagues postulated a role for nitrous oxide (NO) and nitrosative stress in biofilm biology (87). Based on these observations, Webb, Kjelleberg and colleagues postulated a role for NO and related compounds in triggering release of bacteria from a biofilm. Subsequent studies showed that NO could indeed stimulate release of planktonic cells from an established biofilm (88). Sodium nitroprusside (SNP) is a known NO donor, and when added at a sub-lethal concentration of 500 nM to *P. aeruginosa* biofilms, this compound induced dispersion and reduced biofilm surface coverage by approximately 68% compared to wild type (89, 90). These findings supported the conclusion that NO could serve as a dispersal signal. This experiment was performed using an established, mature biofilm, so the underlying mechanism(s) is thought to mimic those operating in a natural dispersion. This dispersion event was subsequently linked to changes in PDE activity (90), that is, SNP promoted PDE activity in a dose-dependent manner (90). Intracellular c-di-GMP levels, in turn, decreased ~45-47% upon exposure to 5 μ M of SNP (90). Additionally, c-di-GMP concentrations of dispersed cells closely resembled those of planktonic cells, which was approximately 10-fold lower than biofilm-associated cells (20). To demonstrate the causality between SNP/NO and biofilm dispersion, PTIO (NO scavenger; 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) was added in conjunction with SNP, and the mixture of NO-generator and NO-sink effectively inhibited dispersion (90), although the impact of PTIO on PDE activity and c-di-GMP level was not explored.

Glutamate is a second molecule known to induce release of planktonic cells from the biofilm, and does so in a process called nutrient-induced dispersion (91). A collection of strains carrying mutations in PDEs was screened to identify enzymes involved in glutamate-mediated dispersion, which led to the identification of two candidates, *dipA* (encoded by PA5017) and *rbdA* (encoded by PA0861). The *dipA*::Tn and *rbdA*::Tn mutants, unlike wild type *P. aeruginosa* PA14 parent strain, maintained an intact biofilm even after treatment with glutamate. Interestingly, under SNP/NO-induced dispersion conditions, these mutants also resisted this dispersion inducer as well (20). Thus, the same mechanism may be responsible for biofilm dispersion under both glutamate- and NO-induced conditions.

Loss of the *dipA* gene, encoding a dual-domain GGDEF-EAL protein, resulted in increased intracellular c-di-GMP (20). The resulting change in c-di-GMP also increased EPS production, likely through a post-transcriptional regulation since both *pelA* and *pslA* expression remained comparable to wild type *P. aeruginosa* PAO1 (20). The *dipA* mutant showed an increase in intracellular c-di-GMP levels (~110 pmol/mg) compared to wild type *P. aeruginosa* PA14 (~80 pmol/mg). A recent report by Roy *et al.* utilized tetracycline-inhibition of protein synthesis to show that expression of *dipA* occurs during mature biofilm stage (4 days) (20). Effects of the *dipA* mutation are strain-specific as disruption of this gene in *P. aeruginosa* PA68 strain resulted in a reduction in swimming (92), implying DipA's role modulating motility and EPS production in this strain. Meanwhile, loss of the *rbdA* gene, which also encodes a dual-domain GGDEF-EAL protein, showed an increase in EPS production by *P. aeruginosa* PAO1 that was largely Pel-dependent (19). The transcriptional analysis of the *pelA* gene in the *rbdA* mutant showed a ~1.5-fold increase in expression compared to wild type *P. aeruginosa* PAO1 (19), suggesting that EPS production was post-transcriptionally regulated. Unfortunately, the specific mechanism underlying

DipA and RbdA's contribution to the control of EPS remains an open question. Also, reflecting on BifA's role in EPS production, comparative analysis of *bifA*, *rbdA*, and *dipA* expression levels during dispersal could be an interesting avenue to explore.

In a separate study investigating biofilm dispersion, the *bdlA* gene was identified. Specifically, the *bdlA* mutant biofilm failed to disperse upon NO exposure, but its intracellular c-di-GMP levels remained unchanged from wild type *P. aeruginosa* PAO1 after NO treatment (93). BdlA lacks domains associated with c-di-GMP metabolism (i.e., GGDEF, EAL, or HD-GYP domains), but has two putative PAS domains, as well as a TarH domain (94), both of which are common signal transduction domains. BdlA is also found in a larger, and smaller, proteolyzed variant. Functionally, both PAS domains are necessary to complement the dispersion phenotype in a *bdlA* mutant biofilm, likely due to a ClpP-dependent cleavage of BdlA between the Met and Ala residues on position 130-131, which are located between the PASa and PASb motifs of BdlA (94). Additionally, phosphorylation on a tyrosine residue (Y238) is necessary to prime BdlA for cleavage; a Y→A substitution variant failed to complement *bdlA* mutant phenotype (94). This post-translational modification of BdlA is consistent with previous reports (91, 93), but how does BdlA fit into the paradigm of c-di-GMP-regulated dispersion? It turns out that low c-di-GMP levels, particularly driven by DipA, impairs proper cleavage of BdlA while an increase in c-di-GMP mediated by the DGC encoded by PA4843 induced BdlA cleavage (94). These set of phenotypes establish a convincing model wherein DipA, expressed during late biofilm maturation stage, regulates intracellular c-di-GMP to promote dispersal via cleavage of BdlA. How the change in intracellular c-di-GMP levels stimulates ClpP-mediated cleavage of BdlA is unknown, as is the mechanism by which cleavage of BdlA stimulates release of bacteria from the biofilm.

Concluding remarks

Since its initial discovery as an allosteric factor regulating cellulose biosynthesis in *Gluconacetobacter xylinus* (known as *Acetobacter xylinum* at the time) (95, 96), the list of functional outputs regulated by c-di-GMP has grown. We have focused this chapter on one of these c-di-GMP-regulated processes, namely, biofilm formation. The majority of DGCs and PDEs encoded in *P. aeruginosa* genome still remain uncharacterized, thus there is still a great deal to be learned about the link between c-di-GMP and biofilm formation in this microbe. In particular, while a number of c-di-GMP metabolizing enzymes have been identified that participate in reversible and irreversible attachment, and biofilm maturation, there is still a significant knowledge gap regarding the c-di-GMP output systems in this organism. Even for the well-characterized Pel system, where c-di-GMP-mediated transcriptional regulation is now well documented, how binding of c-di-GMP by PelD stimulates Pel production is not understood in any detail. Similarly, c-di-GMP mediated control of swimming, swarming and twitching also remains to be elucidated. Thus, despite terrific advances in our understanding of *P. aeruginosa* biofilm formation and the role of c-di-GMP in this process since the last version of this book (indeed there was no chapter on c-di-GMP!) (97), there is still much to learn.

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Figure 1. c-d-GMP: A central regulator of biofilms

Shown is the structure of c-di-GMP (center). This molecule is synthesized from two molecules of GTP by enzymes known as diguanylate cyclases (DGC), which carry a conserved GGDEF domain. c-di-GMP can be degraded by two families of phosphodiesterases (PDE); those with an EAL domain linearize the molecule to produce pGpG, and proteins with an HD-GYP domain generates 2 molecules of GMP from the signal. Illustration ©2014 William Scavone, Kestrel Studio, reprinted with permission.

Figure 2. A model for biofilm formation and dispersion in *Pseudomonas aeruginosa*

The steps of biofilm formation, as described in this chapter are: (1) Reversible attachment, likely via the flagellar pole; (2) Irreversible attachment via the long axis of the cell, resulting in a monolayer of cells; (3) microcolony formation; (4) macrocolony formation; and (5) dispersion. See the text for more detailed explanations of each step, which is based largely on laboratory studies. Illustration ©2014 William Scavone, Kestrel Studio, reprinted with permission.