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FEATURES

How *Pseudomonas aeruginosa* Regulates Surface Behaviors

At surfaces, these bacteria either form biofilms or swarm, a regulated behavior with important consequences for pathogenesis

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Should I stay or should I go now?
If I go there will be trouble
An' if I stay it will be double

–The Clash

The opportunistic pathogen *Pseudomonas aeruginosa* displays several behaviors when it encounters solid or semisold surfaces. For instance, the bacterial cells form biofilms, swarm, and engage in pili-mediated twitching. Although each of these group behaviors is extensively studied on its own, little is known about the relationships among these behaviors.

As *P. aeruginosa* cells transition from planktonic growth in liquids to life on solid surfaces, each cell first interacts with the substratum. Subsequent to contacting that surface, cells might display any of these three surface-only behaviors, leading one to suspect that *P. aeruginosa* coregulates these behaviors. Here we focus on one specific regulatory feature of group surface behaviors, namely the inverse control of biofilm formation and swarming motility. For the sake of this article, biofilms are defined as surface-associated communities of microbes that produce an extracellular matrix, which consists in part of extracellular polysaccharides. When such cells engage in swarming motility, they depend on flagella and surfactants to move across surfaces.

Summary

- *P. aeruginosa* cells coregulate several behaviors when they encounter surfaces, including whether to swarm or form biofilms.
- The transition from biofilm formation to swarming, and vice versa, likely has an important impact on interactions between the pathogen, *P. aeruginosa,* and its hosts.
- Biofilm development proves to be a more complicated process than earlier thought, forcing us to reenvision this pathway in *P. aeruginosa* as well as in other bacterial species.
- Chemotaxis, polysaccharide production, intracellular levels of c-di-GMP, and viscosity of the medium are among the factors affecting transitions between swarming behavior and biofilm formation.

Transition between Swarm and Biofilm Likely Affects Pathogenic Properties

Transitioning between a sessile biofilm on a surface to a swarm that moves across a surface, and vice versa, likely has an important impact on interactions between *P. aeruginosa* as a pathogen and its host(s). While the precise advantages to these bacteria of being "on the go" via swarming motility are uncertain, this group behavior likely plays a role in disseminating this microorganism and affecting its ability to acquire nutrients.

Meanwhile, the "troubles" that biofilms cause hosts are well documented. Forming a biofilm renders such microbes resistant to conventional antibiotic therapy, causing significant problems for patients, including individuals with cystic fibrosis or contaminated medical implants. Established biofilms may serve as a source of persistent infection, periodically shedding planktonic microbes or stimulating chronic and de-

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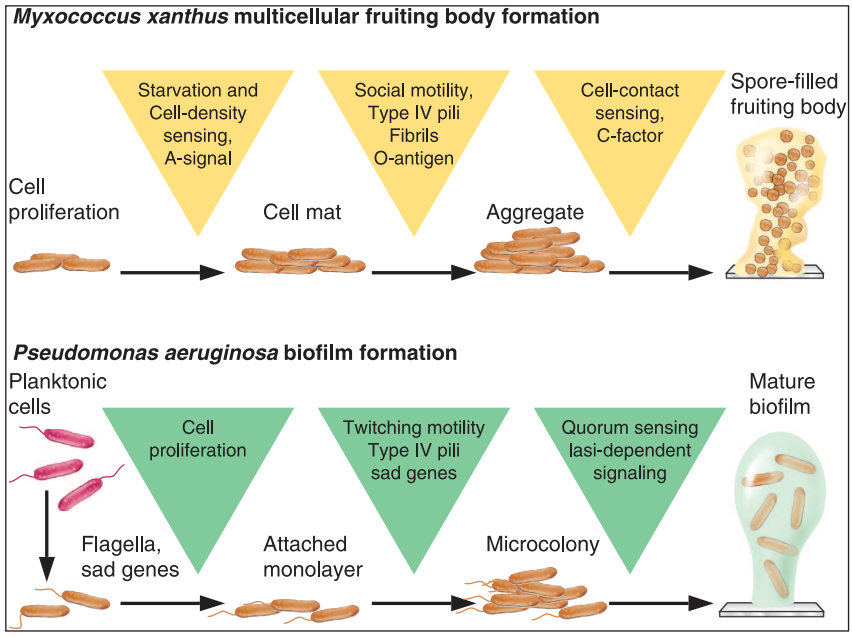
FIGURE 1



An early model of biofilm development. Adapted from figure from an article entitled "Biofilm formation as microbial development" (Annu. Rev. Microbiol. **54**:49–79, 2000) which compared the developmental pathway for fruiting body development in *Myxococcus xanthus* to the biofilm formation pathway known at the time.

structive inflammatory responses. Understanding how *P. aeruginosa* and other bacterial species regulate such surface behaviors might provide insights as to how to treat such infections.

We need to rethink how biofilm formation and swarming motility are regulated—in figurative terms, how *P. aeruginosa* determines whether to "stay or go." In 2000, Roberto Kolter of Harvard Medical School in Boston, Mass., Heidi Kaplan of the University of Texas, Houston, and I proposed that biofilms form by following a developmental pathway that is akin to other microbial developmental programs such as what occurs when *Myxococcus xanthus* cells form fruiting bodies. According to our model (Fig. 1), *P. aeruginosa* has a pathway dedicated to controlling development of a biofilm from planktonic, or free-swimming, bacterial cells.

This model has helped in developing an experimental framework for studying early events in biofilm formation. For example, investigators can introduce mutations to define discrete steps in the developmental pathway, opening new avenues of investigation and improving our understanding of those early events.

The current model of biofilm development is based in part on observations by microbiologists who, in the first few decades of the 20th century, noticed that initial interactions between a bacterial cell and a surface proceeded via discrete, reproducible steps. For example, the pole of a *P. aeruginosa* cell initially encounters a surface, reversibly attaching to it through an unstable interaction. Next, interactions with that surface are stabilized when the long axis of the cell irreversibly attaches to that surface.

We believe that this transition from reversible to irreversible attachment, first observed during the 1930s, is the first committed step to a biofilm lifestyle. During the late 1990s, investigators identified several genes that are required for irreversible attachment. They also began studying several additional steps of biofilm development that involve the flagellum, type IV pili, the Las/Rhl quorum sensing systems, and various *sad* gene products (for surface attachment defective). More recent studies during the past decade support the concept that *P. aeruginosa* has a genetic pathway dedicated to forming biofilms.

Biofilm Developmental Pathway Proves More Complex than Once Thought

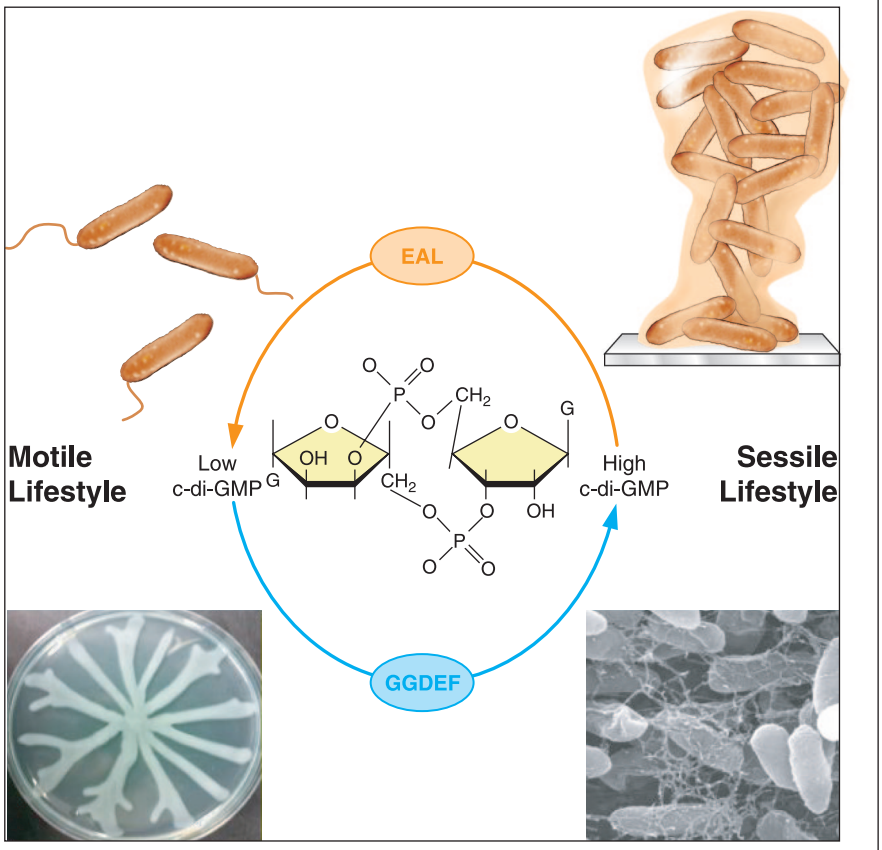
Biofilm development proves to be a more complicated process than earlier thought, forcing us to reevaluate current models. In particular, researchers studying a variety of microbes, including *Vibrio cholerae*, *Escherichia coli*, and *Salmonella*, are finding instances of inverse relationships between biofilm formation and motility.

For example, several years ago we noticed an inverse relationship between biofilm formation and swarming motility of *P. aeruginosa* strain PA14 (a close relative of the PA01 strain). Thus, we began exploring the mechanistic determinants required for inversely regulating biofilm formation and swarming motility. Our first insights came from studying a mutation in the

sadB gene, which codes for a protein of unknown function. Loss of SadB function both blocks the formation of biofilms and stimulates the ability of the microbe to swarm. Conversely, overexpressing SadB stimulates biofilm formation and reduces swarming. These data provided the first hint that *P. aeruginosa* depends on a common pathway to coregulate biofilm formation and swarming motility.

We next sought to identify other factors that coregulate surface behaviors. A search through our *sad* mutants for those altered in both swarming and biofilm behaviors led us to identify the *sadC* locus. Like *sadB* mutants, *sadC* mutants are defective in forming biofilms and are hyper-swarmers. The SadC protein contains a GGDEF domain, a common feature among proteins with diguanylate cyclase (DGC) activity, which is required for synthesizing the intracellular signaling molecule bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP). Levels of c-di-GMP in different species of bacterial cells correlate with the transition between motility and sessility. Thus, high cellular levels of this molecule promote biofilm formation while low levels correlate with motility (Fig. 2). Therefore, loss of the SadC DGC would be expected to lower c-di-GMP levels and promote motility. Those are the phenotypes that we observed.

FIGURE 2



The role of c-di-GMP in bacterial sessile and motile lifestyles. *P. aeruginosa* inversely regulates swarming motility (left) and biofilm formation (right) in response to changing pools of c-di-GMP. On the left is an image of the swarming tendrils formed by *P. aeruginosa* PA14 at ~24 h of incubation on 0.5% agar. On the right is an SEM of a *P. aeruginosa* biofilm.

We also identified a second locus, designated *bifA* (for biofilm formation), that codes for a c-di-GMP phosphodiesterase (PDE). When *bifA* is mutated, we observe increased cellular pools of c-diGMP, higher polysaccharide production and biofilm formation, and inhibition of swarming motility. Therefore, the phenotype of this *bifA* mutant is opposite to that observed for the *sadC* mutant. Epistasis analysis supports our conclusion that SadC and BifA are in the same pathway.

Other Genes and Gene Products Modulate Surface-Related Behaviors

P. aeruginosa has upwards of 60 genes involved in making, breaking, or binding c-di-GMP. Trying to understand how all these proteins modulate and respond to c-di-GMP pools is a significant challenge. We began by focusing on how changes in levels of this molecule, mediated by SadC and BifA, affect biofilm formation and swarming motility in *P. aeruginosa*.

Among motile bacteria, two factors appear to contribute universally to biofilm formation—extracellular polysaccharides and flagellar-mediated motility. *P. aeruginosa* cells also require flagellar motility for swarming motility. Further, in *P. aeruginosa*, changing c-di-GMP pools affect biofilm formation and swarming motility through two means: (i) production of polysaccharides in the biofilm matrix and (ii) control of flagellar function.

In making polysaccharides, *P. aeruginosa* PA14 relies on the *pel* locus to produce a glu-

cose-rich extracellular polysaccharide, which becomes part of the pellicle (thus the name *pel*), and to form biofilms, according to Lisa Friedman and Roberto Kolter at Harvard Medical School. When we mutated the *pel* locus, swarming increased. Put another way, Pel levels, which are affected by c-di-GMP, can help determine whether cells form a biofilm or undergo swarming. More specifically, c-di-GMP allosterically regulates Pel polysaccharide production through PelD, a protein that binds c-di-GMP, according to Stephen Lory of Harvard Medical School and his collaborators.

In addition to affecting polysaccharides, c-di-GMP also affects flagellar function. Although mutations in *sadC*, *sadB*, and *bifA* affect biofilms and swarming, these mutations do not perturb the flagellar or type IV pili systems. However, a mutation in the *sadB* gene increases flagellar reversal rates, but has only a minimal effect on linear swim speed. One means by which bacteria control flagellar function is by changing the rate of switching between the clockwise and counterclockwise rotation of this organelle, as typically occurs during chemotaxis.

Strikingly, effects of *sadB* mutations on flagellar reversal rates are observed only under conditions of high viscosity—perhaps explaining why we observe effects on swarming motility but not on swimming. Similarly, a *sadC* mutant also increases flagellar reversals in high viscosity conditions but has no impact on swimming motility at lower viscosities. In contrast, a *bifA* mutant accumulates high levels of c-di-GMP and shows a decrease in flagellar reversal rates compared to wild-type cells. Thus, we conclude that SadB, SadC and BifA affect the same pathway, a conclusion supported by epistasis studies.

Chemotaxis-Like Cluster also Regulates Flagellar Function

We next sought a link between the SadB, SadC, and BifA proteins and control of flagellar reversal rates. In *E. coli* cells, flagella response to extracellular cues requires the chemotaxis system, the flagellar switch complex, and the flagellar motor, which includes stators, the static element of the bacterial motor that provides energy to turn this appendage. The *E. coli* chemotaxis system controls the relative rates of clockwise and counterclockwise rotation of a flagellum via a signal transduction system involving both methylation and phosphorylation.

A methyl-accepting chemotaxis protein (MCP) is involved in sensing extracellular signals and transducing these signals to the cytoplasm. Other components include a MCP-specific methylase and demethylase as well as response regulator proteins, including CheY. The phosphorylation state of CheY in turn influences its ability to interact with the flagellar switch complex, and thus to control clockwise (CW) versus counterclockwise (CCW) flagellar rotation. The flagellar switch complex determines the direction of motor rotation.

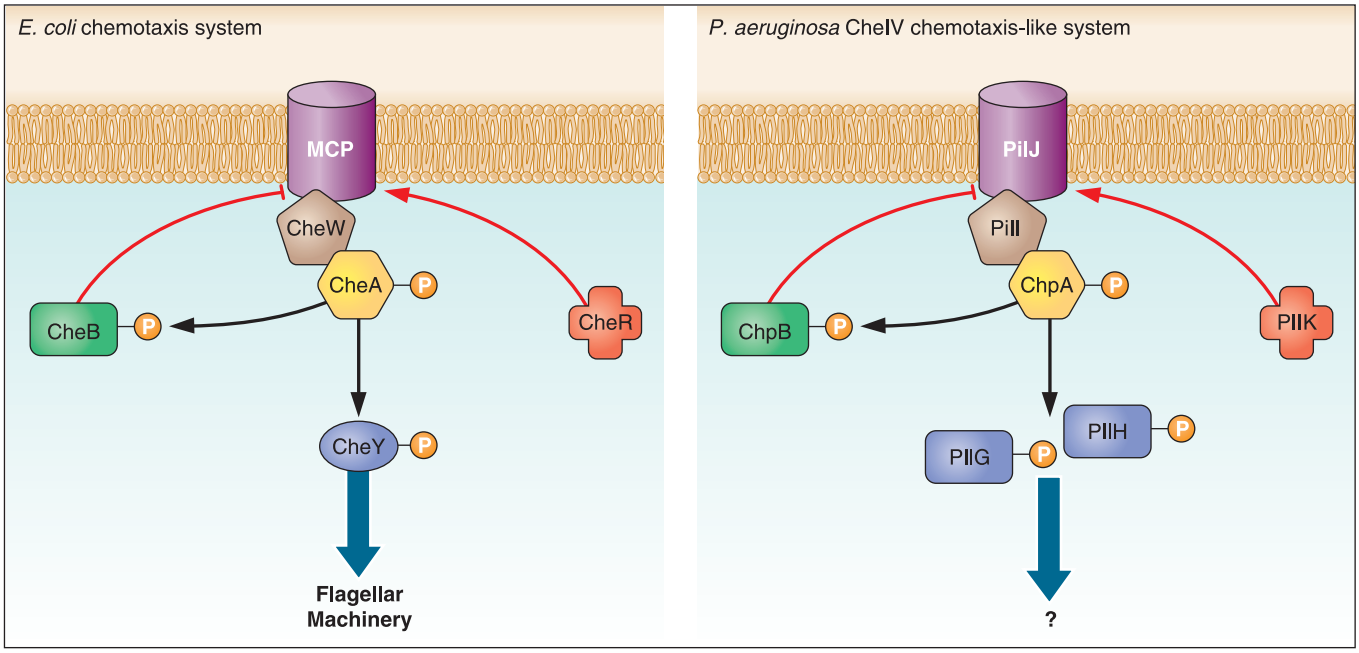
Amid the five complete or partial chemotaxis-like systems within the *P. aeruginosa* genome, we determined that the CheIV chemotaxis-like cluster works with SadB and SadC to regulate flagellar function (Fig. 3). This complex chemotaxis-like locus includes most of the components of the classic *E. coli* chemotaxis system, as well as several additional genes. This locus was originally identified because mutations in genes within the CheIV chemotaxis-like cluster rendered cells defective for type IV pili-mediated twitching motility, suggesting a role for this cluster in yet another surface-associated behavior.

We identified two mutations in the CheIV cluster that have opposite phenotypes. Mutating *pilJ*, which codes for MCP, blocks signaling through this chemotaxis system, leads to a loss in biofilm formation, and confers a hyperswarming phenotype. The increased flagellar reversal phenotype upon mutation of the PilJ MCP is similar to that observed for our *sadB* and *sadC* mutants. Furthermore, PilJ is epistatic to SadB, indicating that PilJ functions downstream of SadB and other upstream functions such as SadC and BifA.

The other mutation in ChpB, a demethylase, is expected to lead to hypermethylation of PilJ and increased basal signaling through this MCP. In contrast to the *pilJ* mutant, loss of ChpB results in increased biofilm formation and a nonswarming phenotype. Surprisingly, a *chpB* mutant shows no difference in flagellar reversal rates, but does increase production of the Pel polysaccharide, although not by affecting *pel* gene expression. Taken together, these data indicate that the Che IV cluster affects both flagellar function and polysaccharide production.

Studies of the stators of *P. aeruginosa* further

FIGURE 3



Comparison of the Che system of *E. coli* and the CheIV chemotaxis-like cluster of *P. aeruginosa*. On the left are the major components of the *E. coli* chemotaxis system. On the right are the corresponding components of the CheIV chemotaxis-like system. This chemotaxis-like system has homologs of the *E. coli* MCP, CheW adaptor protein, CheB methylase and CheR demethylase. The CheA-like protein, ChpA, is much larger with a more complex domain structure than the *E. coli* CheA protein. Furthermore, PilH and PilG both appear to be CheY-like response regulator proteins, indicating that there may be a complex set of outputs from this system. The thin black arrows indicate phosphorylation events, and the red arrows indicate methylation and demethylation reactions. The thick arrow indicates the output of the system.

support links among flagellar function, swarming motility, and biofilm formation. *P. aeruginosa* PA14 has two sets of stators, MotAB (PA4954-PA4954) and MotCD (PA1460-PA1461). Although mutating either *motAB* or *motCD* does not detectably affect swimming, losing either of these stators blocks early biofilm formation. Further, mutating either of those stators increases flagellar reversal rates, a phenotype reminiscent of the strains carrying mutations in *sadB*, *sadC*, and *pilJ*. The stators function downstream of and in the same pathway as SadB, according to epistasis analysis.

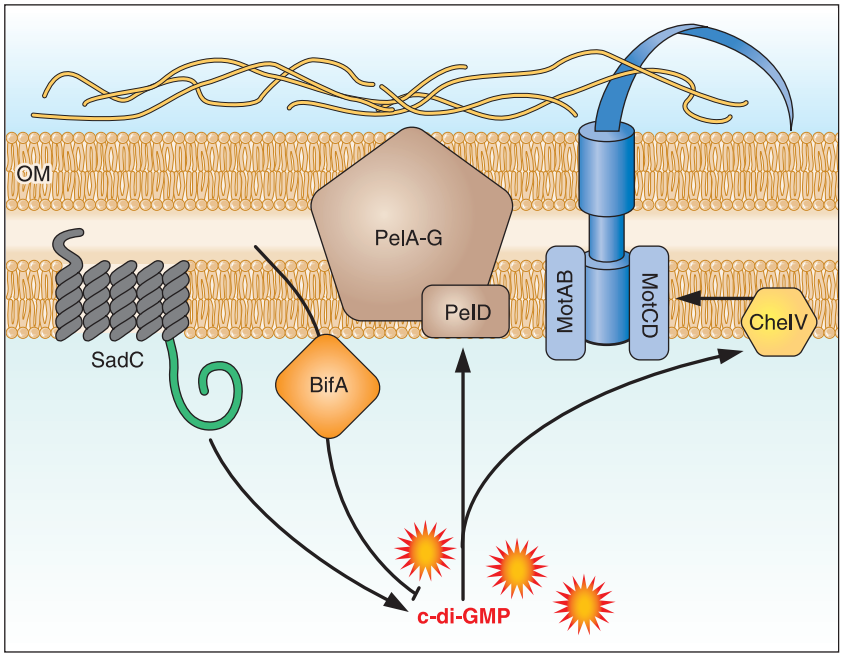
Results of the genetic analyses, phenotypic assays, and epistasis studies are consistent with a pathway that uses SadC/BifA, SadB, the CheIV chemotaxis-like cluster, and the stators to modulate flagellar reversals. Furthermore, this modulation apparently depends on viscosity, which might reflect how *P. aeruginosa* cells respond to liquids versus semisolid or solid surfaces.

How To Account for Viscosity, Tumbling, and Other Effects?

How do increased reversals in flagellar rotation promote swarming motility and interfere with biofilm formation? Although we do not understand the underlying mechanisms, viscosity appears to figure prominently in these processes. For example, those *E. coli* "cells that do not tumble tend to get trapped in agar," when swimming through 0.3% agar, according to a 1989 report from Howard Berg of Harvard University in Cambridge, Mass., and his collaborator Alan Wolfe, now of Loyola University in Chicago, Ill. We propose that an analogous phenomenon extends to swarming. Thus, strains with decreased rates of reversals under high-viscosity conditions swarm poorly, while strains with increased reversals under high-viscosity conditions show increased swarming.

With regard to biofilm formation, when *E. coli* mutants are locked in the tumbling state

FIGURE 4



A model for the components of a pathway required to coregulate surface behaviors of *P. aeruginosa*. This model shows that c-di-GMP levels, modulated by SadC and BifA, impact both Pel polysaccharide production and flagellar function. Levels of c-di-GMP exert their effects on Pel production via an allosteric mechanism requiring PelD. How c-di-GMP impacts flagellar function is not clear, but appears to require the CheIV chemotaxis-like cluster and the MotAB amd MotCD flagellar stators.

(CW), which means they reverse flagellar rotation more frequently, they attached to a glass surface less effectively than do smooth-swimming, nonreversing cells (CCW). These data might help to explain why increased flagellar reversal rates interfere with *P. aeruginosa* cells forming biofilms.

Thus, by controlling flagellar function and Pel polysaccharide production, *P. aeruginosa* cells appear to regulate whether they swarm or form a biofilm on surfaces. Further, decreasing flagellar reversal rates and increased Pel production correlate with biofilm formation, while a higher frequency of flagellar reversal rates and reduced Pel production are associated with swarming. Whether these relationships are causative or correlative is not known.

We can also begin to fit c-di-GMP signals, modulated by the SadC and BifA proteins, into these phenomena (Fig. 4). For one thing, there is evidence that c-di-GMP modulates polysaccharide production via an allosteric regulatory mechanism involving the PelD protein rather than through transcriptional mechanisms. However, we know less about how c-di-GMP levels affect component(s) of the CheIV chemotaxis-like cluster, although we speculate that c-di-GMP binding proteins, such as PilZ or other binding proteins, monitor cellular levels of c-di-GMP. Other yet-to-be identified components of the pathway could also link c-di-GMP to flagellar function.

Whether *P. aeruginosa* cells swarm or form biofilms when they encounter a host surface may have important consequences for how individuals deal with infections. For example, being in a swarming state likely renders this opportunistic pathogen more susceptible to antibiotics than if it were growing as a biofilm. Alternatively, coregulation of these behaviors may affect biofilm structure. For example, increased swarming motility in a flow cell results in flat, carpet-like biofilms, while suppressing swarming leads to large macrocolonies, according to Joshua Shrout working with Matthew Parsek at the University of Washington, Seattle, and their collaborators. Our goal is to gain insight into the mechanistic underpinnings that determine whether *P. aeruginosa* cells "stay" or "go" from a planktonic to a surface lifestyle.

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