Cross-regulation of Pseudomonas motility systems: the intimate relationship between flagella, pili and virulence
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Pseudomonas aeruginosa navigates using two distinct forms of motility, swimming and twitching. A polar flagellum and Type 4 pili power these movements, respectively, allowing P. aeruginosa to attach to and colonize surfaces. Single cell imaging and particle tracking algorithms have revealed a wide range of bacterial surface behaviors which are regulated by second messengers cyclic-di-GMP and cAMP; the production of these signals is, in turn, responsive to the engagement of motility organelles with a surface. Innate immune defense systems, long known to recognize structural components of flagella, appear to respond to motility itself. The association of motility with both upregulation of virulence and induction of host defense mechanisms underlies the complex contributions of flagella and pili to P. aeruginosa pathogenesis.

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Introduction
Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen associated with freshwater and soil reservoirs. In humans, it causes a broad range of infections associated with epithelial barrier disruption (corneal infections and keratitis, burn superinfections, ventilator-associated pneumonia), immunocompromise (neutropenic septicemia), and the genetic disease Cystic Fibrosis. Motility and attachment facilitate P. aeruginosa exploitation of and adaptation to these varied environments, and have been extensively studied. In this review, we will focus on new findings regarding the regulation of flagellar and Type 4 pili assembly and motility. Changes in motility are often associated with other phenotypic changes that alter bacterial behavior and virulence toward hosts, and these will be highlighted throughout.

The transition between planktonic and sessile growth of P. aeruginosa is often depicted as a cycle of regulated events that allow bacterial populations to sense and adapt to surface-associated growth. Swimming bacteria arrive at a surface, undergo a transition from reversible to irreversible attachment that is accompanied by changes in flagellar function [1], production of Type 4 pili (T4P) [2], intracellular accumulation of the second messenger cyclic-di-GMP (c-di-GMP) [3], and production of adhesins and exopolysaccharides that contribute to the formation of an organized biofilm community [4]. The recent application of particle-tracking algorithms to the analysis of P. aeruginosa motility has complicated our view of this process by showing the wide range of behaviors that occur at the single-cell level as bacteria come to a surface.

Coming to grips: initial encounters of P. aeruginosa with a surface
P. aeruginosa motility in low viscosity liquids is powered by a single polar flagellum. Only ca. 10% of such liquid grown bacteria assemble polar T4P [2], and so it is not surprising that initial binding of P. aeruginosa to surfaces is observed to occur via the spontaneous attachment of the polar flagellum [5,6]. Attached, vertically oriented bacteria spin (a flagellar-mediated movement), occasionally cartwheel (a T4P-mediated movement), and often detach. Bacteria that assume a horizontal orientation vis a vis the surface are more likely to remain attached, especially if they lack T4P (pilA). This somewhat paradoxical observation may indicate a role for T4P in pulling horizontal cells to a vertical orientation, where the probability of detachment is greater. Conrad et al. also followed the fates of post-division cells and found that >99% of siblings exhibit discordant motility behaviors, with one sibling remaining horizontally attached, while the second detaches, crawls or ‘walks’ away from the division site. pilA bacteria do not move after division, indicating that postdivision motility requires T4P, while fliM (aflagellate) bacteria are more likely to move away after division (60%) compared to wild-type cells (40%) [5].

The tremendous heterogeneity of motility behaviors revealed by Conrad et al. highlights the additional information provided by single cell versus bulk analyses of
motility, but also raises questions of how such variation is achieved. A recently developed FRET-based biosensor for visualizing c-di-GMP concentration within individual *P. aeruginosa* cells may provide part of the answer. Using this tool, Miller and colleagues demonstrated asymmetry of [c-di-GMP] in daughters following cell division [7**]. These differences in [c-di-GMP] result from asymmetric localization of a c-di-GMP phosphodiesterase (PDE) Pch (PA5O17) to the flagellar pole, via direct or indirect interaction with the chemotaxis protein CheA. The authors postulate that c-di-GMP heterogeneity may generate many different motility phenotypes within a population, and demonstrate that ectopic expression of a PDE, which further lowers intracellular c-di-GMP, results in a greater mean swimming velocity and a greater number of flagellar reversals than exhibited by the population of wild-type *P. aeruginosa*. Conversely, the few Δpch bacteria that were observed to swim did so with a slower mean velocity and exhibited fewer reversals [7**]. In many bacterial species, sensors that regulate flagellar rotation in response to c-di-GMP have been described. These include the PilZ-domain protein YcgR of *Escherichia coli*/*Salmonella* [8,9], which is postulated to disrupt motor-stator interactions and slow the flagellar rotor upon binding c-di-GMP. The c-di-GMP responsive regulator of flagellar rotation in *P. aeruginosa* appears not to be the YcgR homolog PA3353, however, as deletion of this gene had no effect on *P. aeruginosa*'s response to changing of c-di-GMP levels in these experiments [7**].

**Making a commitment to stay put**

It is not yet clear how surface binding is ‘sensed’ by *P. aeruginosa*, and it is likely that there are many independent mechanisms that trigger surface-associated behaviors in this organism. For some bacteria, it appears that the inhibition of flagellar rotation leads to a developmental switch that favors bacterial adhesion, such as assembly of a holdfast by *Caulobacter crescentus* [10] or production of exopolymers by *Bacillus subtilis* [11]. In each of these instances, flagellar surface binding is likely to impose a severe load on the flagellum that stops rotation: in the case of *B. subtilis*, contact by several of the peritrichous flagella may suffice, while surface contact by the polar pili of *C. crescentus* stops rotation of this organism’s single polar flagellum. Experimental conditions that artificially stop flagellar rotation, such as the addition of antiflagellin antiserum or polymers such as dextran or Ficoll, can trigger these developmental ‘switches’ to attachment, suggesting that these bacteria respond to flagellar stalling. No studies published to date suggest that *P. aeruginosa* responds to flagellar stalling, but a few older papers have linked changes in the frequency of flagellar reversals with defects in biofilm formation [12]. The mutations that decrease biofilm formation increase swimming, a form of motility powered by flagella and modulated by T4P in *P. aeruginosa* [13], and map to sadB and pilJ. The mechanism by which SadB and PilJ could modulate flagellar reversal frequency is still not known, but it is interesting to note that PilJ is the methyl-accepting chemotaxis protein (MCP)-like component of a chemotaxis cluster linked to T4P regulation, Pil/Chp (reviewed [14]).

*P. aeruginosa* upregulates expression of T4P upon surface binding [2], and uses these nanomachines for twitching motility, a form of surface locomotion in which repeated cycles of extension, attachment and retraction of pili allow bacteria to crawl along a surface. Rapid reorientation of twitching bacteria can follow the sudden release of one T4P while other pili remain under tension, and the frequency of this ‘slingshot’ like movement is affected by surface properties such as softness [15,16]. T4P genes are positively regulated by the cAMP-binding transcriptional regulator Vfr, while the function of T4P is under the control of the Pil/Chp chemotaxis cluster (recently reviewed by [17]). Mutation of Pil/Chp proteins disrupts T4P mediated motility and also alters intracellular cAMP levels, suggesting that T4P function might be linked to the synthesis of this second messenger. This relationship has been examined in recent papers, which track the behavior of surface-attached bacteria on different timescales. A Vfr-driven transcriptional reporter, P<sub>elQc::3ffp</sub>, was used to demonstrate induction of cAMP-dependent transcription shortly (within 30–45 min) after *P. aeruginosa* bound to surfaces [18]. This transcriptional response was absent in a mutant that does not produce the T4P subunit pilin (pilA), attenuated in the absence of the retraction ATPases (pilTU) and markedly diminished in the absence of the assembly ATPase (pilB). External tension on T4P, generated by flow applied to attached pilTU bacteria, had no measurable effect on P<sub>elQc</sub> reporter activity, consistent with a model in which pilus retraction is a required aspect of mechanosensing. Based on interaction between PilA and PilJ in a bacterial two-hybrid system, Gitai and colleagues postulate that PilJ — the MCP-like component of the Pil/Chp system — responds to PilA as the T4P is retracted. The authors speculate that the tension placed upon an attached pilus as it retracts may physically modify or stretch T4P and potentially expose novel epitopes on PilA [19], thereby allowing PilJ to respond specifically to attachment. A concurrent study, using a cAMP-Vfr dependent P<sub>P<sub>T-lacZ</sub></sub> reporter, likewise observed that bacteria increased cAMP levels when grown on solid agar in a T4P dependent manner [20]. PilA and PilJ were absolutely required for this upregulation, which was measured 5 hours after bacterial surface inoculation.

Tracking of bacteria at the surface of a flow cell over longer time periods (20–40 hours) revealed a role for PilY1, a surface adhesion upregulated by Pil/Chp and cAMP-Vfr, in irreversible surface attachment, experimentally defined as a horizontal orientation of bacteria on a flow cell surface [20]. Encoded within a polycistronic
operon along with minor pilins, PilY1 is secreted to the cell surface via the T4P machinery (and possibly a T4P-independent pathway) where it activates the SadC diguanylate cyclase and increases c-di-GMP production [21]. The mechanism by which PilY1 activates SadC is not known. On the time scale of these experiments, *P. aeruginosa* colonizes surfaces via T4P mediated twitching motility, laying down trails of the exopolysaccharide Psl that subsequent bacteria tend to follow [22**]. These trails guide bacterial exploration of the surface and lead to a ‘hierarchical’, rather than uniform, distribution of bacteria into aggregates and microcolonies. Interestingly, Psl also serves as a signal for c-di-GMP production by activating the diguanylate cyclases SadC and SiaD [4]. Whether PilY1 plays a role in Psl-mediated signaling to SadC remains to be addressed, but this c-di-GMP signal further upregulates production of biofilm-promoting protein and exopolysaccharide adhesins [4].

**Virulence: the nasty side of surface associated Pseudomonas**

Surface attachment is often thought of as a first step on the path to biofilm formation, but many recent papers have focused on ways in which the surface attachment of motile bacteria affects *P. aeruginosa* virulence. An ability to use T4P for twitching motility allows *P. aeruginosa* to move ‘upstream’ against flow in microfluidic devices [23]. In branched microfluidic systems, twitching motility allows *P. aeruginosa* cells to move perpendicular to the direction of flow and gain access to side branches. Competing bacteria like *Escherichia coli* and *Proteus mirabilis*, which can swim upstream but lack pilus-mediated surface motility, can be seeded into a branched system along with *P. aeruginosa* and tracked by time-lapse microscopy. These faster growing bacteria outcompete *P. aeruginosa* in the seeded branch, but cannot access side branches, allowing *P. aeruginosa* to establish a protected niche within the system (Figure 1). T4P motility allows bacteria to move ‘upstream’ through the vascular system of an inoculated plant leaf, suggesting that the surface-mediated motility may be relevant to dispersal through a host tissue. Whether the ability to twitch influences the spatial distribution of *P. aeruginosa* within a polymicrobially infected tissue, such as the chronically colonized Cystic Fibrosis lung, is an interesting point raised by this work.

Upregulation of cAMP and c-di-GMP production by surface-attached bacteria might be expected to have far-reaching consequences on the behavior of *P. aeruginosa*, given the established roles of these second messengers in regulating multiple virulence traits. In a direct test this hypothesis, *P. aeruginosa* cells grown in a shaking Petri dish were separated into planktonic and surface-attached populations and placed into contact with the amoeba *Dictyostelium discoideum* [24*]. Calcein-AM fluorescence was tracked as a marker of amoeba membrane compromise (and therefore killing) and revealed that the attached *P. aeruginosa*, but not planktonic cells, were able to rapidly (within an hour) kill amoeba. The mediators of killing were not identified by the authors, who showed that mutation of any of the systems associated with *P. aeruginosa* virulence did not abolish amoeba death (including Type 3 and Type 6 secretion, flagellar and Type 4 pilus biogenesis, exopolysaccharide production, surface-activated diguanylate cyclases, and quorum sensing systems), with the sole exception of Las quorum-sensing mutants. This finding, perhaps reflecting the activation of multiple, redundant virulence pathways in surface associated bacteria, suggests that the sensors required for this upregulation might be better targets for disrupting *P. aeruginosa* virulence than any individual virulence factor. O’Toole and colleagues demonstrate

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**Figure 1**

Twitching motility allows *P. aeruginosa* to move ‘up’ and ‘across’ fluid stream lines. *P. aeruginosa* (blue) seeded into a branched microfluidic network uses T4P-mediated motility to move ‘upstream’ and to cross into side branches of the network. Swimming bacteria that cannot twitch, like *Proteus mirabilis* (red) outcompete *P. aeruginosa* in the seeded branch, but cannot gain access to side branches. (N.D.: not detected).
that surface virulence does not develop in bacterial populations lacking pilY1, suggesting that it and Las quorum sensing provide two signals—surface attachment and cell density—that promote expression of virulence in a host-nonspecific manner [24*].

Host immune responses to *P. aeruginosa* motility

It has long been appreciated that motility organelles of *P. aeruginosa* are recognized by mammalian innate immune sensors. The extracellular Toll-like receptor (TLR) 5 and intracellular Naip5 protein both bind flagellin and activate signaling pathways upstream of NF-κB activation and NLRC4 inflammasome activation, respectively [25,26]. The additional ability to sense and respond to flagellar activity has recently been described by Berwin and colleagues [27–29]. *P. aeruginosa* strains lacking flagellar motor stator complexes (motAB motCD) assemble a non-motile flagellum and are ca. 100-fold more resistant to phagocytosis by neutrophils and macrophages than motile bacteria. Phagocytic cells respond to flagellar motility by activating the PI3K/AKT pathway via a TLR5-independent mechanism, leading to actin-dependent bacterial engulfment [30].

*P. aeruginosa* also elicit signaling responses in epithelial cells that are dependent on the flagellum [31]. Binding of bacterial aggregates to the apical surface of polarized epithelial monolayers results in the formation of a protrusion enriched in the lipid PIP3, and polarity and adherens junction proteins such as Par3, aPKC, Rac1 and Par6α. These protrusions do not form under aflagellate mutants, although the requirement for a motile flagellum was not specifically tested in this study. Protrusion formation is necessary for subsequent activation of NF-κB, which also requires bacterial expression of the Type 3 secretion system ‘injectisome’.

Infection models suggest that Type IV pili, as well as flagella, are sensed during mammalian infection. A murine model, in which gastrointestinal colonization by *P. aeruginosa* is followed by systemic dissemination when neutropenia is experimentally induced, was recently used to examine bacterial proteins required for *in vivo* ‘fitness’ [32*]. The technique of insertion sequencing (INSeq) was used to measure the frequency of individual transposon (Tn)-insertion mutants in the infecting inoculum, at the cecal mucosa, and in the spleen. Tn insertions in genes encoding components of the flagellar system were overrepresented in bacteria that colonized the cecum. This result was attributed to recognition of flagellin by multiple innate immune sensors. Tn insertions in most Type IVa pili assembly genes—but not insertions in other surface adhesins such as Cup fimbriae or Type IVb pilins—also increased fitness for cecal colonization. As disruption of the retraction ATPases PilT and PilU, which results in increased surface expression of Type IVa pili, was associated with decreased fitness, these findings suggested that Type IVa pili might also elicit pro-inflammatory host responses that lead to increased bacterial clearance. An earlier report describing activation of caspase-1 in mouse bone-marrow derived macrophages transfected with *P. aeruginosa* pilin is consistent with this hypothesis, although a host sensor for pilin was not identified in this study [33]. A human clinical study examining correlations between virulence factor expression and *P. aeruginosa* acute infection has documented a positive association between flagellar motility and symptomatic infection in patients with urinary tract infections [34]. Whether this association reflects increased inflammatory responses to flagellated bacteria, or an advantage of flagellated bacteria in colonizing or ascending the urinary tract catheters frequently found in these patients cannot be ascertained from this observational study.

Conclusion

The past few years have literally given us a new view on *P. aeruginosa* motility, as particle-tracking algorithms have allowed single bacterial cells to be followed as they arrive at, explore and colonize experimental surfaces. The heterogeneity of bacterial behaviors documented by these studies begs the question of how such variation is achieved, with one paradigm—asymmetric inheritance of enzymes that regulate c-di-GMP levels—receiving experimental support. Recent work also highlights T4P attachment and retraction as signals for cAMP production by surface-associated bacteria, a signaling circuit that would reinforce T4P driven motility and perhaps favor transitions to surface associated virulence, c-di-GMP production and ultimately biofilm formation. These ‘pro-virulence’ aspects of motility are balanced by *in vivo* work that illustrates the costs to being motile: recognition by multiple innate immune systems that respond to motility structures and functions. A future challenge will be to understand how all of these inputs sum during infection, especially if we wish to tip the balance in favor of the host.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
  - of outstanding interest


This paper uses a novel c-di-GMP biosensor to show that asymmetric distribution of a phosphodiesterase generates variation in intracellular c-di-GMP levels and heterogeneous motility behavior in postdivision cells.


This paper uses a CAMP-responsive transcriptional reporter to demonstrate that Type IV pilus retraction is required for increased CAMP levels on surface attached bacteria, and proposes that a MCP-like protein of the Pil/Che chemotaxis cluster mediates this response.


This paper uses particle-tracking algorithms to follow Type IV pilus mediated movement of bacteria along self-deposited trails of the exopolysaccharide Pal, and proposes a power law model to explain heterogeneous surface colonization and microcolony formation.


This paper demonstrates differing levels of virulence toward amoeba by genetically identical P. aeruginosa grown in liquid versus at a surface. The authors use genetic evidence to propose a two signal model (surface attachment and cell density) to explain host-nonspecific induction of virulence.


This paper uses insertion sequencing (INSeq) to show that P. aeruginosa expression of flagella and type IV pili is associated with decreased fitness in a mouse model of colonization and dissemination, and suggests that immune responses toward these motility structures impose this negative selection.
