Fifty Ways To Inhibit Motility via Cyclic Di-GMP: the Emerging Pseudomonas aeruginosa Swarming Story

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There are numerous ways by which cyclic dimeric GMP (c-di-GMP) inhibits motility. Kuchma et al. (S. L. Kuchma, N. J. Delalez, L. M. Filkins, E. A. Snively, J. P. Armitage, and G. A. O’Toole, J. Bacteriol. 197:420–430, 2015, http://dx.doi.org/10.1128/JB.02130-14) offer a new, previously unseen way of swarming motility inhibition in Pseudomonas aeruginosa PA14. This bacterium possesses a single flagellum with one rotor and two sets of stators, only one of which can provide torque for swarming. The researchers discovered that elevated levels of c-di-GMP inhibit swarming by skewing stator selection in favor of the nonfunctional, “bad” stators.

A seemingly bizarre way to inhibit motility. Since its discovery in the 1980s (1) and its very humble beginnings, cyclic dimeric GMP (c-di-GMP) has risen to the limelight of bacterial signal transduction as one of the most common bacterial second messengers (2). While c-di-GMP signaling pathways affect various aspects of bacterial physiology and metabolism, perhaps the best-known processes are the transition of motile bacteria to the nonmotile, sessile state and the regulation of biofilm formation. c-di-GMP inhibits motility via many mechanisms, most of which remain poorly understood. The study by the O’Toole and Armitage groups in this issue of the Journal of Bacteriology offers a new, previously unseen twist on c-di-GMP-dependent swarming motility control in Pseudomonas aeruginosa PA14 (3). This bacterium possesses dual flagellar motors that function under different conditions. The researchers discovered that elevated levels of c-di-GMP stop swarming by affecting stator selection. Stators are the proton-conducting membrane channels that generate torque, which powers flagellar rotation. Removing high-functioning stators and allowing the “bad” ones that do not support swarming motility to stay seems like a very strange way to stop. To put this study in perspective, let us take a closer look at the structures and functions of flagella, at the various stator arrangements, and at the ways and means by which c-di-GMP is known to affect flagellar functions.

Diversity in flagellar organization and function. Flagellar propelled motility is a very effective and widespread mode of microbial locomotion. The types of flagella and their arrangement on the cell body and the motors, the energy sources, and the chemotactic navigation systems found in microbes are amazingly diverse (4). Flagella can be single polar, multiple tufted polar, sheathed polar, peritrichous (around), lateral (near but not at a pole), and even periplasmic. The same flagella often propel bacteria in low-viscosity and relatively high-viscosity media, but this is possible only up to a point (5). Under conditions of high load, i.e., very high viscosity or on a solid surface, flagellar performance diminishes. To aid motility, some bacteria employ performance-enhancing stratagems. For example, representatives from Aeromonas, Azospirillum, Shewanella, and Vibrio use a distinct second flagellar system optimized for swarming over a surface, whereas Proteus greatly increases flagellar numbers to permit surface translocation (reviewed in references 6 and 7). Yet other bacteria elegantly engage new motor parts to accommodate changing demands (reviewed in reference 8).

Flagella are turned by a rotary motor (reviewed in reference 9), which couples ion flux through the cell membrane to motor rotation. The motor proteins designated MotA and MotB form the stator, which is the ion-conducting channel complex that generates the torque. It can be anchored to the cell wall via MotB, which possesses a peptidoglycan-binding domain. Tethering is dynamic, not permanent, and there is rapid turnover of MotB in the motor (10). The stator interacts with the rotor/switch element, which is made of the FliG, FliM, and FliP proteins. Ion flow through the MotA,B complex is tightly coupled to rotation via specific electrostatic interactions between the MotA protein of the stator and the FliG protein of the rotor. Most flagellar motors are reversible rotary machines, and switching of the direction of rotation or pausing is key to responding in changes in environmental conditions via chemotaxis and adaptation (reviewed in reference 11).

The rotor is surrounded by multiple stators, which appear like studs encircling the rotor in freeze fracture and electron cryomicroscopy micrographs (12, 13) (Fig. 1). The number of stators engaged is dynamic; a maximally functioning motor has ~11 units (14). For the Vibrio Na+-driven motor, PomAB stator localization at the rotor is dependent on Na+ concentration and ion flux (15). In Escherichia coli, a single torque generator rotates the flagellum under conditions of low load, but as load increases, the number of stators recruited to the motor increases (16, 17). Sometimes dual stators power a single rotor, and each stator set can have specific contributions. For example, the H+-type motor of B. subtilis enables fast swimming speeds, but input by its Na+-type mo-
tor contributes to motility at high pH, elevated salt, and high viscosity (18). Stator swapping occurs in *Shewanella oneidensis* MR-1: the Na\(^{+}\)-type stator is most efficiently associated with the rotor when Na\(^{+}\) concentration is high, but the H\(^{+}\)-type torque stator aids motility in low-Na\(^{+}\) conditions (19). Some torque generators, such as the Na\(^{+}\)-driven PomAB motor of *Vibrio*, are more complex and possess additional parts (MotX and MotY) that are essential for motor torque generation (reviewed in reference 20). Thus, environmental conditions can influence motor configuration and performance. The configuration of a single flagellar rotor with multiple potential stator systems (up to four) is found in the genomes of some members of the *Proteobacteria*, *Acidobacteria*, and *Firmicutes* (21).

**The dual stators of *P. aeruginosa*.** *P. aeruginosa* has a single flagellum made of one rotor and two sets of H\(^{+}\)-driven MotAB-like stators, PA1460/1461 and PA4954/4953 (Fig. 1). Genes encoding PA1460/1461 are found in an operon with the central chemotaxis phosphorelay genes, a similar genetic context for many members of the *Gammaproteobacteria*, to which *P. aeruginosa* belongs. PA1460/1461 are regulated as part of the flagellar regulon (22). In contrast, PA4954/4953 are “orphans”; i.e., they are encoded in a two-gene operon that is not in a flagellar island and not regulated by the flagellar regulators. Regrettably, these gene sets have accumulated multiple names: PA1460/1461 is called or annotated as *motAB* and *motCD*, and PA4954/4953 is named *motCD*, *motAB*, and *rpmAB* (22, 23, 24). Here, we adopt the nomenclature used by Kuchma et al. (3): *motAB* designates PA4954/4953, and *motCD* refers to PA1460/4961 (note that this is opposite to the designations used by Dasgupta et al. and Doyle et al. [22, 23]).

Early studies reported that mutants in either of the two motor sets retained nearly wild-type rates of swimming motility in liquid, whereas deletion of both gene sets completely eliminated it (23–26). With respect to swimming speed in liquid, stator performance in strains with only MotAB-type or MotCD-type stators was slightly diminished. However, the contribution of each stator changed under conditions of increasing load (agar concentration or viscosity). The Δ*motCD* mutant was severely compromised with respect to radial expansion rates in plates with 0.325% semi-solid agar and was unable to swarm over the surface of 0.5% agar; in contrast the Δ*motAB* mutant was only slightly defective in semi-solid motility medium and displayed swimming motility equal to or better than that of the wild type (23, 26). So, with respect to swimming in an aqueous environment, a single stator type was sufficient to support motility. However, the MotCD stator was critical for swimming and swimming under other conditions of increasing load (23, 26). The MotCD stator has an additional performance-enhancing feature, MotY (23), that may contribute to stator stabilization under conditions of high load.

**Fifty ways to inhibit motility via c-di-GMP.** How does c-di-GMP enter this picture? Elevated intracellular c-di-GMP levels are synonymous with motility inhibition (2, 27, 28), and *P. aeruginosa* swarming is no exception (29). How c-di-GMP inhibits motility inhibition is overall poorly characterized. Even in the most studied system of *Escherichia coli* and *Salmonella enterica*, the situation remains somewhat murky. What is known is that these bacteria employ a specific c-di-GMP receptor/effector protein, YcgR (30).

At elevated c-di-GMP levels, YcgR–c-di-GMP interferes with flagellar rotation by strongly biasing rotation in the counterclockwise direction (smooth swimming). The block imposed by YcgR–c-di-GMP on the reversal of flagellar rotation to the CW direction has been named a “backstop brake” (31). Going forward all the time without adjusting the direction is the surest way to get stuck, and that is exactly what happens—enteric bacteria quickly run into obstacles, such as the blind alleys in semisolid agar (32). Therefore, bacteria “blindfolded” by YcgR–c-di-GMP cannot spread in the semisolid agar despite having functional flagella. When YcgR–c-di-GMP is overexpressed, flagellar rotation itself is impaired, i.e., the rotation speed is lower and the fraction of non-rotating flagella is higher. What remains somewhat controversial is where, exactly, YcgR binds. Some studies, relying primarily on genetic and protein-protein interaction evidence, suggest that YcgR–c-di-GMP binds to the FlIG and FlIM subunits of the rotor (31, 33), while others, relying on the genetic and FRET-based assays, suggest interactions with the stator subunit MotA (34).

*P. aeruginosa* has a putative YcgR homolog (PA3353), but it is unclear whether the data presented by Kuchma et al. (3) fit the enteric YcgR paradigm. The *P. aeruginosa* scenario does not seem to suggest the involvement of c-di-GMP-dependent regulation of chemotaxis, as observed in *Borrelia burgdorferi* (35) and *Azospiril-
lum brasiliense (36). Another mechanism available to P. aeruginosa is a “lazy” way to inhibit swarming, i.e., by trapping flagella in the exopolysaccharides whose synthesis is induced by elevated c-di-GMP levels. This would not be unprecedented, as bacteria as diverse as Salmonella enterica (37) and Listeria monocytogenes (38) use such a mechanism when their intracellular c-di-GMP levels are high. However, under the conditions used in the study by Kuchma et al. (3), exopolysaccharide synthesis does not seem to be the major contributor. P. aeruginosa could also follow the Vibrio paradigm, where motility strongly depends on the regulation of flagellar gene expression by the c-di-GMP-dependent transcription factors (39–41). While c-di-GMP-mediated transcription inhibition does play a role in P. aeruginosa (42), a different mechanism is at play here.

To find out how c-di-GMP inhibits P. aeruginosa swarming, Kuchma et al. (3) performed transposon mutagenesis on the poorly motile, high-c-di-GMP strain that lacked a potent c-di-GMP phosphodiesterase, BfA (43). They found that some motile suppressors have transposons located in the motAB locus. Overexpression of the motAB genes was sufficient to inhibit swarming, while their deletion from the wild type somewhat enhanced swarming. This clearly established MotAB as a “bad” stator to have for swarming. The MotA-FliG interactions were found to be important for the c-di-GMP-dependent inhibition of motility; i.e., MotA must physically inhibit flagellar function under the swarming conditions. But how is it connected to c-di-GMP? The current study provides only a hint of what may be happening. The authors showed that high c-di-GMP levels interfere with proper localization of the MotD protein, which they tracked using a green fluorescent protein (GFP) fusion. The levels of MotD at the expected flagellum site were decreased in the high-c-di-GMP strain.

The ability of P. aeruginosa to control stator composition by c-di-GMP is unique in comparison to the situation in other bacteria that modulate a motile-to-sessile lifestyle switch via c-di-GMP. Since translocation on surfaces has important consequences with respect to surface colonization, this mechanism suggests a new strategy for community architecture development and subsequent biofilm formation (44). This and earlier work by O’Toole’s laboratory (45) demonstrate key roles for P. aeruginosa flagellar motors in these processes. Many interesting questions related to this story remain to be answered. What c-di-GMP receptor/effector protein affects swapping of the “good” swarming stators (MotCD) with the “bad” ones (MotAB)? How does flagellum load affect c-di-GMP levels? What are the benefits of having this kind of motor regulation compared to other mechanisms? While we await answers to these questions, it is clear that just as “there must be 50 ways to leave your lover,” according to Paul Simon’s 1975 hit, there must be 50 ways to inhibit motility via c-di-GMP. Kuchma et al. (3) have just begun unraveling one such way that seems bizarre and elegant at the same time.

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REFERENCES


