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Roles for flagellar stators in biofilm formation by *Pseudomonas aeruginosa*

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Abstract

While *Pseudomonas aeruginosa* has only a single flagellum, its genome encodes two flagellar stators, called MotAB and MotCD. Here we report that despite no apparent alterations in swimming motility, mutations in either the MotAB or the MotCD stator render the strains defective for biofilm formation in both static and flow cell systems. Our data suggest distinct roles for the stators in early biofilm formation, with both the MotAB and MotCD stators playing a role in initial polar attachment of the bacterial cell to the surface (reversible attachment) and the MotAB stator also participating in the downstream adherence event of irreversible attachment. We also show that the initial polar attachment of *P. aeruginosa* to two different abiotic surfaces occurs largely at the flagellated end of the cell, a finding that should help develop models for early attachment events. Interestingly, in flowing conditions, a mutation in either stator alone revealed a more severe biofilm defect than mutating both stators or mutating the flagellum. Our data suggest that defects in biofilm formation observed for the stator mutants may be in part due to impacting flagellar reversal rates.

Keywords: *P. aeruginosa*; Stators; Flagellum; Biofilm; Polar attachment

1. Introduction

Pseudomonas aeruginosa is able to attach to many abiotic and biotic surfaces, including medical devices such as contact lenses [28] and intubation tubes [18]. *P. aeruginosa* is also thought to colonize and form biofilms on biotic surfaces, such as in the lungs of cystic fibrosis patients or on the mucosal surface of patients with chronic sinusitis, where it triggers infections that are extremely difficult to eradicate [3,9].

Previous studies showed that despite the fact that this organism has only one flagellum and one motor, the genome of the *P. aeruginosa* strain codes for two flagellar stators [10,27]. The flagellar stator is the static element of the bacterial motor providing energy to turn this appendage and therefore propel the cell through its environment. The two stators of *P. aeruginosa* PA14, MotAB (PA4953-4954) and MotCD (PA1460-1461) [27], were named based on their respective level of sequence similarity to the well-characterized *Escherichia coli* stator [13].

The stators are involved in swimming and swarming motility, but play no role in the third form of motility utilized by *P. aeruginosa*, type-IV-pili-dependent twitching motility [10,27]. Swimming motility in *P. aeruginosa* requires a functional flagellum, while swarming requires both a functional flagellum and production of rhamnolipid surfactants, which act as both surface wetting agents and signals [6,16,27]. In *P. aeruginosa* PA14, the MotAB and MotCD stators are redundant for swimming motility; however, when cells are swarming, the MotAB stator of *P. aeruginosa* PA14 is dispensable, but the MotCD

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stator is required for this behavior [27]. In this report, we investigate the role of the MotAB and MotCD stators in biofilm formation.

2. Materials and methods

2.1. Bacterial strains and growth medium

All strains used in this study are derived from the *P. aeruginosa* PA14 genetic background [26]. Construction and initial analysis of strains Δ*motAB*, Δ*motCD* and Δ*motABCD* has been reported previously [27]. The *flgK* strain contains a Tn*5* transposon inserted in the *flgK* gene [22]. The medium used for all experiments (unless specified otherwise) was M63 minimal salts medium [24] supplemented with arginine (0.4%) and $MgSO_4$ (1 mM). Gentamicin (Gm) was used at 50–100 μg/ml and ampicillin (Ap) at 500 μg/ml.

2.2. Biofilm assays

Biofilm formation in microtiter dishes was measured as described previously [20,23]. For biofilms grown under flow conditions, bacteria were cultivated in flow chambers with channel dimensions of $5 \times 1 \times 30$ mm, assembled as described previously [8,17]. At day 3 of incubation, the cells were stained with Syto 9 (diluted 1:1000) (Molecular Probes, Carlsbad, CA) before obtaining phase-contrast microscopy or fluorescent Z series images. The Z series images were subjected to COMSTAT analysis to quantify biofilm structure [14].

2.3. Attachment assays

Attachment to plastic was measured using an air–liquid interface (ALI) assay [20]. The medium was supplemented with Ficoll (Type 400, Sigma, St. Louis, MO) at the final concentration of either 3 or 30%. After 4 h of incubation without shaking at 37 °C, the wells were vigorously washed with PBS several times. Phase-contrast micrographs were acquired to enumerate the attached bacteria. The ratios between reversible and irreversible attachment were determined from flow cell assays after 24 h of incubation, as reported [5].

2.4. Assessment of polar attachment

Polar attachment of *P. aeruginosa* PA14 was assessed in two ways. A PilU-YFP fusion protein (expressed from plasmid pUCP20Gm-*yfp-pilU*) is localized to the same pole as the flagellum [7] and was used to mark the flagellar pole. Overnight cultures of this strain grown in LB + Gm were diluted 1:10 into minimal M63 medium plus glucose and casamino acids (caa). Ten microliters of 0.75 μm non-fluorescent plastic beads (Polysciences, Inc., Warrington, PA) were added to the bacterial cells and allowed to incubate standing for 2–3 h at 25 °C. The mixture of bacteria and beads was examined by phase-contrast microscopy under a wet-mount and bacteria attached to the bead by a pole were imaged by fluorescent microscopy. The location of the fluorescent spot conferred by the PilU-YFP protein (marking the flagellar pole) was noted relative to the non-fluorescent bead.

A second approach used unlabeled bacterial cells diluted 1:50 into M63 plus glucose/caa medium from overnight LB-grown cultures and aliquoted (500 μl) into 24-well plates. After incubating for 5–10 min, the surface of the plates was scanned for reversibly attached bacteria in the process of undergoing cell division, as indicated by the presence of an obvious division septum. Flagella are found at the non-dividing end of the cell. Reversibly attached dividing cells rotating about a pole indicated that the bacteria were attached via their flagellated pole.

2.5. Swim reversals

Swim reversal rates were measured as described [4].

2.6. Statistical analysis

All *P* values were calculated in Excel using the Student's *t*-test with a two-tailed distribution and a two-sample unequal variance.

3. Results

3.1. Both the MotAB and MotCD stators of P. aeruginosa *PA14 are involved in biofilm formation*

We assessed biofilm formation under static conditions using a 96-well microtiter dish. Both the Δ*motAB* and the Δ*motCD* strains showed a reduction in biofilm formation compared to the WT at time points ≥6 h with the most striking phenotype at 24 h (Fig. 1A). When both stators were deleted the phenotype was similar to a *flgK* mutant (Fig. 1A), which lacks the flagellum and is non-motile [22]. None of the mutants has detectable growth defect under these conditions [22,27]. We observed similar results when biofilm formation was assessed using glass tubes as the substratum and in minimal M63 medium supplemented with glucose and casamino acids (data not shown), suggesting that these phenotypes are not surface- or medium-specific.

3.2. The ΔmotAB *and* ΔmotCD *mutants show different attachment behaviors in low and in high viscosity medium in static biofilm formation conditions*

We hypothesized that the differential ability to initially attach in these different conditions might reveal the relative contributions of each stator to the earliest steps in biofilm formation. We measured the ability of the WT and stator mutants to initiate attachment to a plastic surface in static conditions in medium containing Ficoll and then assaying for their ability to attach at 4 h. The addition of 3% Ficoll is considered a low viscosity medium wherein the cells move by swimming motility, and we showed previously that the swimming