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Pseudomonas aeruginosa Exhibits Sliding Motility in the Absence of Type IV Pili and Flagella▽†

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Pseudomonas aeruginosa **exhibits swarming motility on 0.5 to 1% agar plates in the presence of specific carbon and nitrogen sources. We have found that PAO1 double mutants expressing neither flagella nor type IV pili (*fliC pilA*) display sliding motility under the same conditions. Sliding motility was inhibited when type IV pilus expression was restored; like swarming motility, it also decreased in the absence of rhamnolipid surfactant production. Transposon insertions in *gacA* and *gacS* increased sliding motility and restored tendril formation to spreading colonies, while transposon insertions in *retS* abolished motility. These changes in motility were not accompanied by detectable changes in rhamnolipid surfactant production or by the appearance of bacterial surface structures that might power sliding motility. We propose that *P. aeruginosa* requires flagella during swarming to overcome adhesive interactions mediated by type IV pili. The apparent dependence of sliding motility on environmental cues and regulatory pathways that also affect swarming motility suggests that both forms of motility are influenced by similar cohesive factors that restrict translocation, as well as by dispersive factors that facilitate spreading. Studies of sliding motility may be particularly well-suited for identifying factors other than pili and flagella that affect community behaviors of *P. aeruginosa*.**

Pseudomonas aeruginosa is an opportunistic pathogen that can cause either acute or chronic infection in susceptible hosts. *P. aeruginosa* has two surface organelles responsible for motility: a single polar flagellum that promotes swimming motility in liquid environments, and polar type IV pili (TFP) responsible for twitching motility across solid surfaces (5, 25). *P. aeruginosa* also exhibits swarming on semisolid surfaces (0.5 to 1.0% agar) in the presence of specific nitrogen and carbon sources, such as glutamate and glucose (17, 32). Swarming motility occurs in many organisms and is often associated with changes in flagellar number and/or placement (10). Although most authors report that flagella are required for *P. aeruginosa* swarming motility, one of the first descriptions of *P. aeruginosa* swarming noted that a PAO1 *fliC* mutant (Flagellin$^-$) had decreased but not abolished motility under swarming conditions (17). The role of TFP in swarming remains unclear. A recent transposon screen to identify genes required for swarming in the common laboratory strain PAO1 identified multiple *pil* genes involved in TFP regulation (30). This is consistent with reports that TFP are required for *P. aeruginosa* swarming (17). However, other authors have reported that *pilA* mutations in PAO1 and PA14 either have no phenotype or result in increased spreading on swarming plates (32, 38).

Swarming motility generally requires the production of surface wetting material (10). In *P. aeruginosa*, mono- and dirhamnolipids, as well as their precursor 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA), are secreted during swarming (3, 7, 43). HAA and rhamnolipids are synthesized by three enzymes (RhlA, RhlB, and RhlC) that are under control of the *N*-butyryl homoserine lactone-activated quorum-sensing regulator RhlR. Although HAA is required for *P. aeruginosa* swarming under many conditions (3, 43), a recent report showed that a *rhlAB* mutant, which cannot synthesize HAA, still swarms on FAB plates in the presence of succinate or glutamate (38).

The identification of regulatory proteins that control swarming motility in response to environmental cues remains an area of active research. One regulator that has been implicated in swarming motility is the GacS/GacA two-component system. Mutation of the response regulator GacA results in increased swarming motility in *P. aeruginosa* (31). Spontaneous loss-of-function mutations that map to *gacA* and *gacS* have also been described in *Pseudomonas fluorescens*, where they result in hypermotile bacteria that outcompete the wild type during competitive root colonization assays (24).

GacS and GacA are also implicated in the regulation of biofilm formation, along with two hybrid sensor kinase-response regulators named RetS and LadS (9, 45). Δ*retS* bacteria exhibit a hyperbiofilm phenotype, while Δ*ladS*, Δ*gacS*, or Δ*gacA* mutants are defective in biofilm formation (9, 31). It is not known how signals from these three sensor kinases are integrated during bacterial signaling; however, the current model suggests that activity of GacS/GacA and LadS leads to increased levels of the small RNAs RsmZ and RsmY, while activity of RetS results in decreased levels of these small RNAs. RsmZ and RsmY, in turn, are hypothesized to sequester the posttranscriptional regulator RsmA (12). Low levels of free RsmA lead to the expression of genes associated with biofilm formation, including the *pel* and *psl* exopolysaccharide synthesis operons.

P. aeruginosa swarming motility and biofilm formation are also regulated by pathways that use cyclic-di-GMP (c-di-GMP) as a second messenger. Two recent papers described a cyclic-

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype or characteristics[a]	Source or reference
E. coli strains		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*(Tc^r)]	Stratagene
S17.1	*thi pro hsdR recA RP4-2* (Tc::Mu) (Km::Tn*7*)	39
P. aeruginosa strains		
PAO1	Wild type	42
PA103	Wild type	22
PA103 *pilA*	PA103 Δ*pilA*::Gm^r; Gm^r	47
pilA	PAO1 Δ*pilA*	6
fliC	PAO1 Δ*fliC*::Tc^r; Tc^r	6
fliC pilA	PAO1 Δ*fliC*::Tc^r Δ*pilA*; Tc^r	6
fliC pilA	PAO1 Δ*fliC*Δ*pilA*	This work
fliC pilA gacA	PAO1 Δ*fliC*::Tc^r Δ*pilA gacA*::Tn<Gm^r>; Tc^r Gm^r	This work
fliC pilA gacA	PAO1 Δ*fliC*Δ*pilA gacA*::Tn<Gm^r>; Gm^r	This work
fliC pilA gacS	PAO1 Δ*fliC*::Tc^r Δ*pilA gacS*::Tn<Gm^r>; Tc^r Gm^r	This work
fliC pilA retS	PAO1 Δ*fliC*::Tc^r Δ*pilA retS*::Tn<Gm^r>; Tc^r Gm^r	This work
fliC pilA gacA attB::*pilA*	PAO1 Δ*fliC* Δ*pilA gacA*::Tn<Gm^r> attB::*pilA*; Gm^r	This work
fliC pilA rhlA	PAO1 Δ*fliC*::Tc^r Δ*pilA* Δ*rhlA*; Tc^r	This work
Plasmids		
pX1918G	Cloning/expression vector; Gm^r	36
pMOD2<MCS>		Epicentre
pMOD<Gm^r>	*aacC1-xylE* cassette cloned at MCS of pMOD2<MCS>	This work
mini-CTX2	Contains attP site for integration at *P. aeruginosa* chromosomal attB site; Tc^r	13
mini-CTX-*pilA*	*pilA* under control of its own promoter in mini-CTX2	This work
pUCP-KS	Shuttle vector; Ap^r Cb^r	46
pRhlA	*rhlA* under control of the P_{lac} promoter in pUCP-SK; Ap^r Cb^r	This work
pEX18 Gm	Suicide vector; Gm^r; *sacB*	13
pEX18-Δ*pilA*	*pilA* knockout construct in pEX18 Gm; Gm^r; *sacB*	6
pEX18-Δ*fliC*	*fliC* knockout construct in pEX18 Gm; Gm^r; *sacB*	This work
pMQ30	Suicide vector; *sacB*; Gm^r URA3 CEN6/ARSH4	37
pKO-*rhlA*	*rhlA* knockout construct in pMQ30; Gm^r; *sacB*	This work
pMQ80	Cloning vector; Gm^r URA3 P_{BAD}; *araC*	37
pBifA	His-tagged *bifA* under control of the P_{BAD} promoter in pMQ80; Gm^r URA3	18
pSadC	C-terminal HA-tagged *sadC* under control of the P_{BAD} promoter in pMQ80; Gm^r URA3	26

[a] Abbreviations: Ap, ampicillin; Cb, carbenicillin; Gm, gentamicin; Tc, tetracycline; Km, kanamycin.

di-GMP phosphodiesterase, BifA, and a diguanylate cyclase, SadC, that inversely regulate biofilm formation and swarming motility of *P. aeruginosa* PA14 by modulating c-di-GMP levels (18, 26). BifA and SadC likely exert some of their effects by altering exopolysaccharide synthesis; indeed, c-di-GMP binding to one of the *pel*-encoded enzymes, PelD, is required for exopolysaccharide synthesis (19). However, as O'Toole and colleagues demonstrated, additional c-di-GMP-regulated targets also influence swarming motility.

We have observed a novel surface behavior of *P. aeruginosa* PAO1 that allows bacteria to spread on semisolid surfaces in the absence of both flagella and TFP. This behavior is consistent with the description of "sliding motility" presented by Jorgen Henrichsen in his seminal 1972 review of bacterial surface translocation (11). In this work we demonstrate that sliding motility responds to many of the same regulatory proteins and environmental cues as swarming motility. These include the GacA/GacS and RetS two-component system proteins and the c-di-GMP modulators BifA and SadC.

MATERIALS AND METHODS

Strain and plasmid construction. All bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were propagated in Luria-Broth (LB) or Vogel-Bonner minimal (VBM) medium unless otherwise noted. PPGAS medium was prepared as described elsewhere (3). Antibiotics were used as required for *Escherichia coli* (ampicillin, 100 μg ml^{-1}; gentamicin, 15 μg ml^{-1}; tetracycline, 20 μg ml^{-1}) and *P. aeruginosa* (carbenicillin, 200 μg ml^{-1}; gentamicin, 50 μg ml^{-1}; tetracycline, 100 μg ml^{-1}).

Standard molecular biology techniques were employed for restriction digests, ligations, transformations, and selections (35). PCR primers were designed based on sequence information in the PAO1 genome database (http://v2.pseudomonas.com) and are listed in Table 2 (42). PCR amplifications were carried out using recombinant *Pfu* Turbo polymerase (Stratagene, La Jolla, CA) or *Taq* polymerase (New England BioLabs, Beverly, MA). *E. coli* strain XL-1 Blue was used for cloning and plasmid propagation; vectors were transformed into *E. coli* S17.1 for mobilization by mating into *P. aeruginosa*. All *P. aeruginosa* mutants were constructed in the PAO1 background. The Δ*pilA* strain was constructed by allelic exchange. Upstream (PCR amplified with pilA N1 and pilA N2) and downstream (PCR amplified with pilA C1 and pilA C2) (Table 2) regions flanking the *pilA* gene were cloned in tandem in pEX18 Gm^r to make pEX18-Δ*pilA*; this plasmid was then mobilized into PAO1 by mating. Exconjugants were initially selected on VBM-gentamicin and then plated to VBM-sucrose (5%) to select for loss of vector backbone sequences, as previously described (6, 36). Candidates were screened by PCR. The Δ*fliC*::Tc^r and Δ*fliC*::Tc^r Δ*pilA* mutants were made as previously described (6). An unmarked Δ*fliC* Δ*pilA* strain was constructed by mating Δ*fliC*::Tc^r Δ*pilA* with *E. coli* S17.1 carrying pEX18-Δ*fliC* and selecting for the loss of Tc^r. Again, all strains were screened for gene deletion by PCR; in each case, the correct genotype was confirmed by Southern blot analysis. For complementation of Δ*pilA*, PCR primers *pilA* N1 and *pilA* C2 were used to amplify the full-length *pilA* gene from PAO1 along with its promoter. This product was cloned into the mini-CTX2 plasmid and integrated into the chromosomal *attB* site (1).

The *rhlA* deletion strains were constructed with pMQ30, which allows for

TABLE 2. Primers

Primer	Sequence[a]
pilA N1	CTCGAGCTCGGTGCTGAACTGGACATC ATTG
pilA N2	GAATTCCATGAATCTCTCCGTT
pilA C1	GAATTCGATAACTAAGGTGATCGAA GGTG
pilA C2	CTGCAGCCGCGAGTGCTGGTG
rhlA expN	CGAGCTCAAGAGCACCTACGCGCGTTG
rhlA exp C	CGGTACCGGTCTTCGCAGGTCAAGG
rhlA N1	CAGACCGCTTCTGCGTTCTGATTTAAT CTGTATCAGGCGAACTCCTTCGCCC TGGC
rhlA N2	CCGGCCAGGCCGGGTCTTCGCAGGTCA AGGGTTTCAGGCGTAGCCGCGCCGC ATTTCACAC
rhlA C1	GCCTGTTCGAAAATTTTTGGAGGTGTG AAATGCGGCGCGGCTACGCCTGAAC CCTTGACCTGC
rhlA C2	GTATGTTGTGTGGAATTGTGAGCGGAT AACAATTTCAGCGTTGCAGTTCGT CGTC
gacA expN	CCGAATTCAATGCGCGACGAGGT GCAG
gacA expC	GGGCGGCCGCGATTGCTACAGGTAGC GAGG
Tn for 1	ATGCCTGCAGGTCGACTCTAGAGGATC
Tn for 2	CAAGCTTGCCAACGACTACGCAC
Tn rev 1	CGAGAACACCCGAGAAAATTCATC
Tn rev 2	CATATTGGCTCGAATTCCGAT

[a] Underlined portions of sequences represent restriction sites incorporated into primers to facilitate cloning.

plasmid construction by gap repair in yeast (37). PCR primers *rhlA* N1 and *rhlA* N2 and *rhlA* C1 and *rhlA* C2 were used to amplify DNA flanking *rhlA* (Table 2). These PCR products as well as SmaI-linearized pMQ30 were transformed into *Saccharomyces cerevisiae* EGY40 (*MAT*α *trp1 his3 ura3 leu2*:0LexAop-LEU2), and transformants were selected on Ura- dropout medium (Q-Biogene, Irvine, CA) as previously described (37). The pMQ30-Δ*rlhA* plasmid was purified from yeast, transformed into electrocompetent *E. coli* XL-1 Blue, and verified by analytic restriction digests. The pMQ30-Δ*rlhA* vector was subsequently mobilized into PAO1 by mating, and candidate Δ*rhlA* mutants were selected, screened, and confirmed as described above.

Full-length *rhlA* and *gacA* genes were amplified with a 1:1 ratio of *Pfu* Turbo and *Taq* polymerases using primer pairs rhlA expN with rhlA expC and gacA expN with gacA expC, respectively (Table 2). The PCR products were cloned into pUCP-KS, placing them under control of the constitutively active *lac* promoter in *P. aeruginosa*. All constructs were verified by sequencing to confirm that no errors were introduced during PCR amplification and then transformed into electrocompetent PAO1 (4).

Motility assays. Swarming motility was assayed as previously described on 0.5% M8 plates supplemented with 0.2% glucose and 0.05% glutamate (28) or 0.5% plates with FAB medium supplemented with 12 mM of either glutamate, succinate, or glucose (38). Motility was also measured on 0.5% agar PPGAS plates prepared as described above. Single colonies were plated overnight at 30°C and then placed at room temperature for an additional 24 h. Twitching motility was determined by subsurface stab assays through 1.5% LB agar plates. The twitching zone size at the plastic-agar interface was visualized by Coomassie blue staining after overnight incubation at 37°C. Swimming motility was assayed by point inoculation of 0.3% LB agar plates; zone sizes were measured after overnight incubation at 30°C. Motility assays were carried out on three to five replicates and repeated at least three times. The images presented are representative with regard to both the extent of spreading and morphology of motile colonies.

Construction of pMOD-2-<Gm^r> and transposon mutagenesis. A 2.3-kb fragment containing the *xylE aacC1* cassette was generated by EcoRI digestion of pX1918G and subcloned into the unique EcoRI site of pMOD-2-<MCS> (Epicentre Biotechnologies, Madison, WI) to generate pMOD-2-<Gm^r>. The EZ-Tn*5*<Gm^r> transposon was prepared by digesting pMOD-2-<Gm^r> with PshA. A 200-ng aliquot of transposon DNA was incubated with EZ-Tn*5* transposase (Epicentre Biotechnologies) in the presence of glycerol to form transposon complexes according to the manufacturer's protocols. Electrocompetent Δ*fliC*::Tc^r Δ*pilA* was prepared and electroporated with one-eighth of the transposon mixture and then incubated for 2 h at 37°C in LB and plated onto LB-gentamicin plates overnight (4). The next day single colonies were picked to 150- by 15-mm 0.5% M8 swarming plates. The parent strain was included on each plate as a positive control. Colonies that displayed altered dispersion were then plated to LB-gentamicin plates for further evaluation.

Inverse PCR was done to map the location of the transposons found to alter bacterial spreading. Chromosomal DNA was prepared using the Wizard kit (Promega, Madison, WI). A 1.5-μg aliquot of chromosomal DNA was digested with either MspI or HinpI for 4 h at 37°C. The enzymes were heat inactivated at 65°C for 25 min. Ten μl of digested chromosomal DNA was ligated with 1 μl T4 ligase (New England BioLabs, Beverly, MA) overnight in a total volume of 400 μl at 4°C. The DNA was precipitated with sodium acetate and ethanol, washed with 70% ethanol, and air dried, and the pellet was resuspended in 100 μl Tris-EDTA buffer. Five μl of this material was used as template in PCRs (total volume, 25 μl). Sequences flanking the transposon ends were amplified with *Taq* polymerase (94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and then 72°C for 10 min), and PCR products were sequenced using primers Tn for1, Tn for2, Tn rev1, and Tn rev 2 (Table 2).

Rhamnolipid and drop collapse assays. Rhamnolipid was measured via the orcinol method as previously described (20). Filtered supernatants prepared from M8 plus casein or PPGAS cultures grown with aeration at 30°C for 36 to 48 h were used for this assay ($n = 3$). To confirm that the supernatants contained biologically active surfactant, drop collapse assays were also performed on PPGAS filtered supernatants (above) or on filtered supernatants prepared from LB overnight cultures for *fliC pilA rhlA* and *fliC pilA rhlA* pRhlA. Filtered supernatant was serially diluted with sterile water, and 30-μl drops were placed on the lid of a Corning 96-well dish, allowing drop collapse activity to be visualized as described elsewhere (3). Each assay was repeated a minimum of three times.

RESULTS

***P. aeruginosa* exhibits sliding motility in the absence of flagella and TFP.** We are interested in understanding the factors that influence *P. aeruginosa* motility. The standard laboratory strain, PAO1, reproducibly displays swarming motility on 0.5% M8 plates supplemented with 0.2% glucose and 0.05% glutamate. We constructed a double mutant lacking both pili and flagella, *fliC pilA*, to serve as a negative control in motility assays. Like its isogenic parent *fliC*, the double mutant did not swim in liquid broth (data not shown) or on 0.3% agar swimming plates (Fig. 1A) (6). Subsurface stab assays demonstrated that the double mutant was also defective for twitching motility (Fig. 1B). We were surprised, however, to observe that the *fliC pilA* mutant still showed surface spreading on swarming motility plates (Fig. 1C). *fliC* showed no swarming motility, as expected; however, an isogenic *pilA* mutant showed increased spreading compared to wild-type PAO1 (Fig. 1C). To confirm that the double mutant lacked both TFP and flagella, we harvested bacteria from swarming motility plates and examined them by transmission electron microscopy (TEM). As seen in Fig. 2A, both flagella and TFP were absent from the surface of the double mutant, though these could clearly be visualized on the surfaces of wild-type PAO1 harvested from swarming plates (Fig. 2B) (6).

The surface spreading of *fliC pilA* on semisolid agar is consistent with "sliding motility," i.e., "a kind of surface translocation produced by the expansive forces in a growing culture in combination with special surface properties of the cells resulting in reduced friction between cell and substrate" (11). Unlike swarming, sliding motility does not require flagella (8, 11, 23). Complementation of *fliC pilA* and *pilA* with a copy of the wild-type *pilA* gene integrated into the chromosomal *attB* site

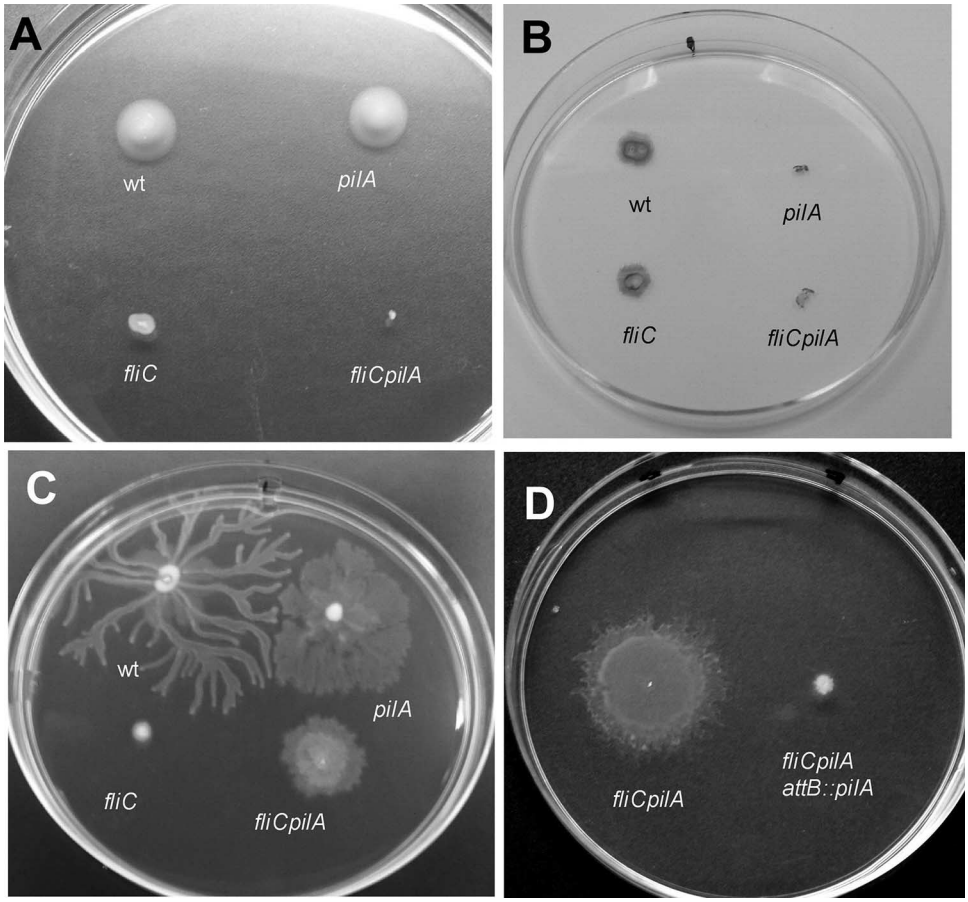

FIG. 1. PAO1 motility in strains lacking type IV pili and/or flagella. A) Flagellum-dependent swimming motility on 0.3% LB agar plates. B) TFP-dependent twitching motility at the plastic-1.5% LB agar interface. C) Motility on 0.5% M8 agar plates supplemented with 0.05% glutamate and 0.2% glucose. D) Restoration of pilin expression inhibits motility of *fliC pilA* bacteria. wt, wild type.

restored twitching motility in both strains and abolished surface spreading in the double mutant (Fig. 1D and data not shown), confirming that the observed differences in motility on semisolid agar between these two strains were indeed due to the loss of pilin expression. This suggests that type IV pili inhibit sliding motility, possibly by increasing interactions between the cell and substrate.

Rhamnolipids facilitate motility in the absence of TFP and flagella. Motility on swarming plates usually appears to be dependent on the production of rhamnolipid surface wetting agents. We tested whether motility of *fliC pilA* on swarming plates also required rhamnolipid production. An unmarked in-frame deletion of *rhlA*, which encodes the protein required for synthesis of the rhamnolipid precursor HAA, was constructed in the *fliC pilA* background and tested for motility and surfactant production. As seen in Fig. 3B, the triple mutant showed no drop collapse activity; this could be complemented by expressing RhlA from a plasmid. The *fliC pilA rhlA* strain showed decreased surface spreading compared to its isogenic parent, *fliC pilA*; motility could be restored, however, by plasmid-encoded RhlA (Fig. 3A and Table 3). These data demonstrate that motility of *fliC pilA* bacteria on swarming plates is enhanced by HAA and/or rhamnolipid production. This enhancement of flagellum-independent spreading by increased expression of surface wetting agents (i.e., rhamnolipids) is characteristic of sliding motility (11).

Sliding motility is regulated by *gacS*/*gacA* and *retS*. To identify other gene products that influence *P. aeruginosa* sliding motility, we performed random transposon mutagenesis of *fliC pilA* using miniTn*5*<Gm^r>. A group of ca. 1,000 Gm^r insertion mutants were screened for increased or decreased spreading on swarming motility plates. Figure 4A shows examples of mutants that demonstrated either decreased (type a) or increased (type b) motility compared to the *fliC pilA* parent (type c). We were particularly interested in b type mutants, which showed tendril formation reminiscent of that observed during wild-type PAO1 swarming. Two transposon insertions that resulted in this phenotype were mapped by inverse PCR to *gacA* and *gacS* (Fig. 4B and data not shown). Of interest, one of the transposon insertions that abolished motility of *fliC pilA* mapped to the *retS* gene (Fig. 4B). These sensor kinase response regulator proteins have been shown to inversely affect swarming behavior of wild-type (i.e., Fla$^+$ Pil$^+$) *P. aeruginosa* and *P. fluorescens* (9, 12).

fliC pilA gacA and *fliC pilA retS* bacteria exhibit no swimming or twitching motility (data not shown). As sliding motility is affected by bacterial growth rate (11), we confirmed that growth curves of *fliC pilA*, *fliC pilA gacA*, and *fliC pilA retS*

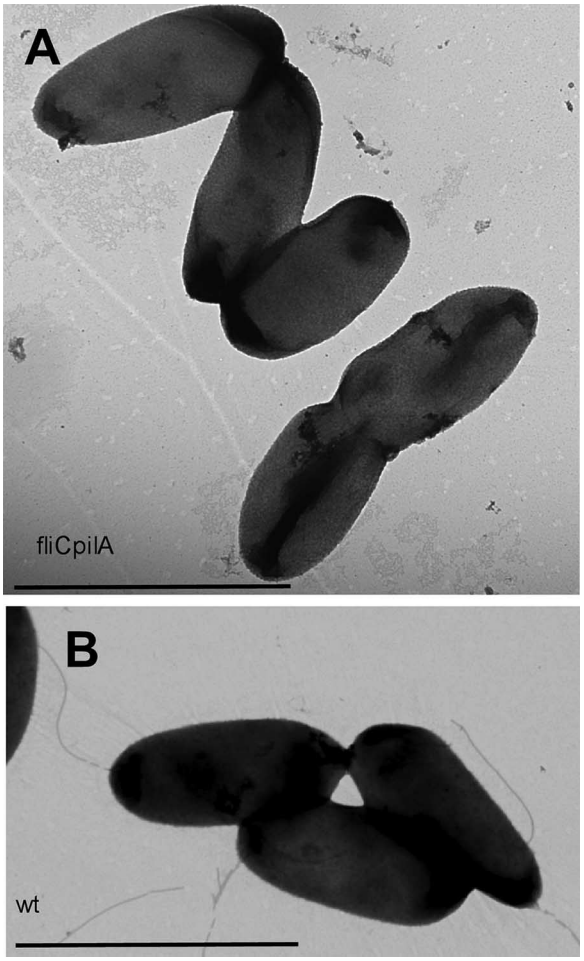

FIG. 2. Motility organelles are not present on sliding *fliC pilA* bacteria. TEM of *fliC pilA* bacteria (A) and isogenic wild-type (wt) bacteria (B) harvested directly from swarming plates and stained with 1.5% uranyl acetate. Bar, 2 μm.

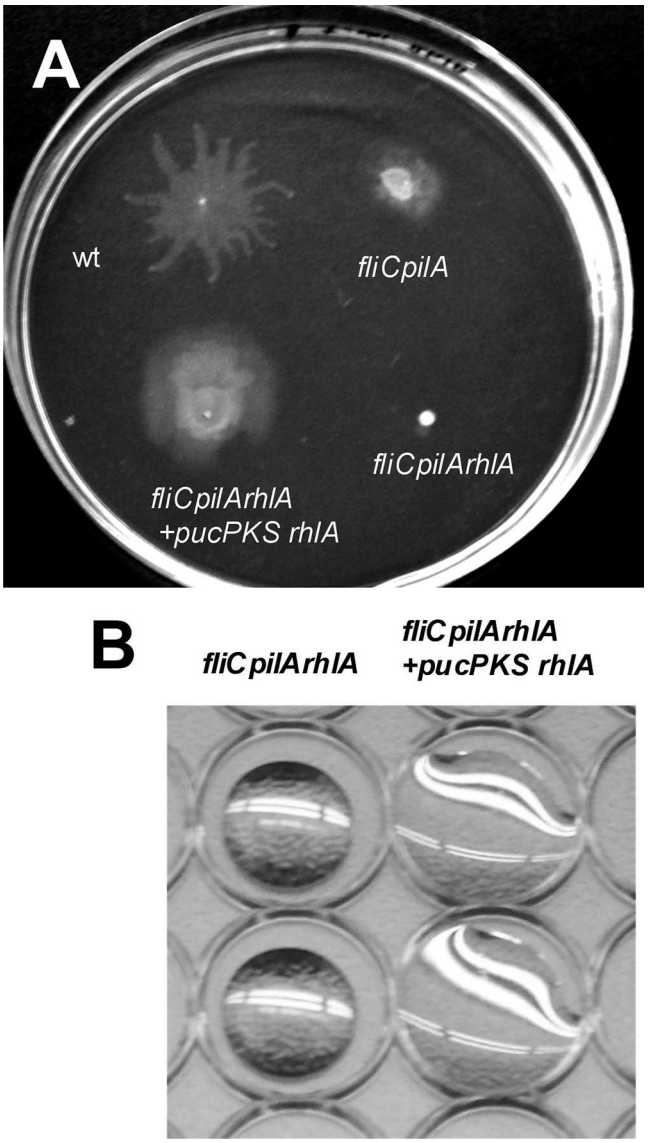

FIG. 3. *rhlA* positively influences sliding motility. (A) Dispersion of *fliC pilA* and *fliC pilA rhlA* bacteria was compared on 0.5% agar M8 plates after overnight incubation at 30°C. The extent of spread is dependent upon RhlA expression. (B) Undiluted supernatants prepared from overnight LB cultures of *fliC pilA rhlA* and the complemented strain *fliC pilA rhlA* pRhlA were assayed for drop collapse activity.

bacteria in M8–glucose–glutamate–0.5% Casamino Acids liquid medium were identical (data not shown). We also harvested *fliC pilA gacA* mutants from swarming plates and examined them by TEM to confirm the absence of pili, flagella, cup fimbriae (44), or other surface structures that might be associated with increased motility; no such structures could be visualized (Fig. 4C). Complementation of *fliC pilA gacA* with a plasmid expressing wild-type GacA (pGacA) resulted in decreased motility relative to the parental *fliC pilA* strain (Fig. 4D). We also restored pilin expression to the *fliC pilA gacA* triple mutant by integrating a wild-type copy of the *pilA* gene under its own promoter at the chromosomal *attB* site. The resulting strain, *fliC pilA gacA* attB::*pilA*, showed restored twitching motility (data not shown) but failed to spread on swarming motility plates (see Fig. S1 in the supplemental material). This underscores the ability of pili to inhibit sliding motility in nonflagellated bacteria and is consistent with the hypothesis that the *gacA* mutation alters expression of genes and/or proteins other than those that target assembly of pili and flagella to result in a hypermotility phenotype.

***fliC pilA retS* PAO1 produces rhamnolipid and exhibits drop-collapse activity.** As demonstrated above, HAA and/or rhamnolipid production enhances sliding motility of *fliC pilA*. GacA and GacS activities positively regulate rhamnolipid production (12); however, the effect of *retS* mutations on rhamnolipid production has not been described. We tested whether *gacA* or *retS* mutants showed differences in rhamnolipid production in any of several media, including PPGAS and M8 media supplemented with casein. Extraction and quantification of rhamnolipids using the orincol method demonstrated that all strains showed equivalent rhamnolipid production (data not shown).

The orcinol assay measures rhamnose and does not detect

TABLE 3. Colony sizes on swarming plates

Strain	Colony diam[a] (mm)
PAO1	42.5 ± 5.2
fliC pilA	19.5 ± 2.6
fliC pilA gacA	34.3 ± 5.0
fliC pilA retS	2.25 ± 0.50
fliC pilA rhlA	9.25 ± 1.30
fliC pilA rhlA pRhlA	19.5 ± 4.0

[a] The largest diameter of the colony was measured after overnight incubation at 30°C on 0.5% M8 swarming plates supplemented with 0.2% glucose and 0.05% glutamate. Data are means ± standard deviations ($n = 5$).

the presence of the rhamnolipid precursor HAA, which also affects swarming motility. HAA was therefore measured indirectly by carrying out drop collapse assays on the same bacterial culture supernatants. Equal amounts of surfactant were present in the supernatants of all strains tested (data not shown), arguing that changes in total HAA and/or rhamnolipid production are unlikely to account for differences in sliding motility observed between *fliC pilA*, *fliC pilA gacA*, and *fliC pilA retS*.

Sliding motility of *fliC pilA gacA* responds to changes in available carbon and nitrogen sources. *P. aeruginosa* swarming motility varies with the carbon and glucose sources provided in the swarming plate (17, 38). To determine whether sliding motilities of *fliC pilA* and *fliC pilA gacA* showed a similar nutritional dependence, we examined the extent and pattern of dispersion on 0.5% PPGAS plates and on 0.5% FAB plates supplemented with either 12 mM succinate or glutamate. The plates were incubated overnight at 30°C and then placed at room temperature and examined 48 to 72 h later. As seen in Fig. S2 of the supplemental material, both wild-type PAO1 and *fliC pilA* showed different extents of swarming and sliding, respectively, depending on available carbon and nitrogen

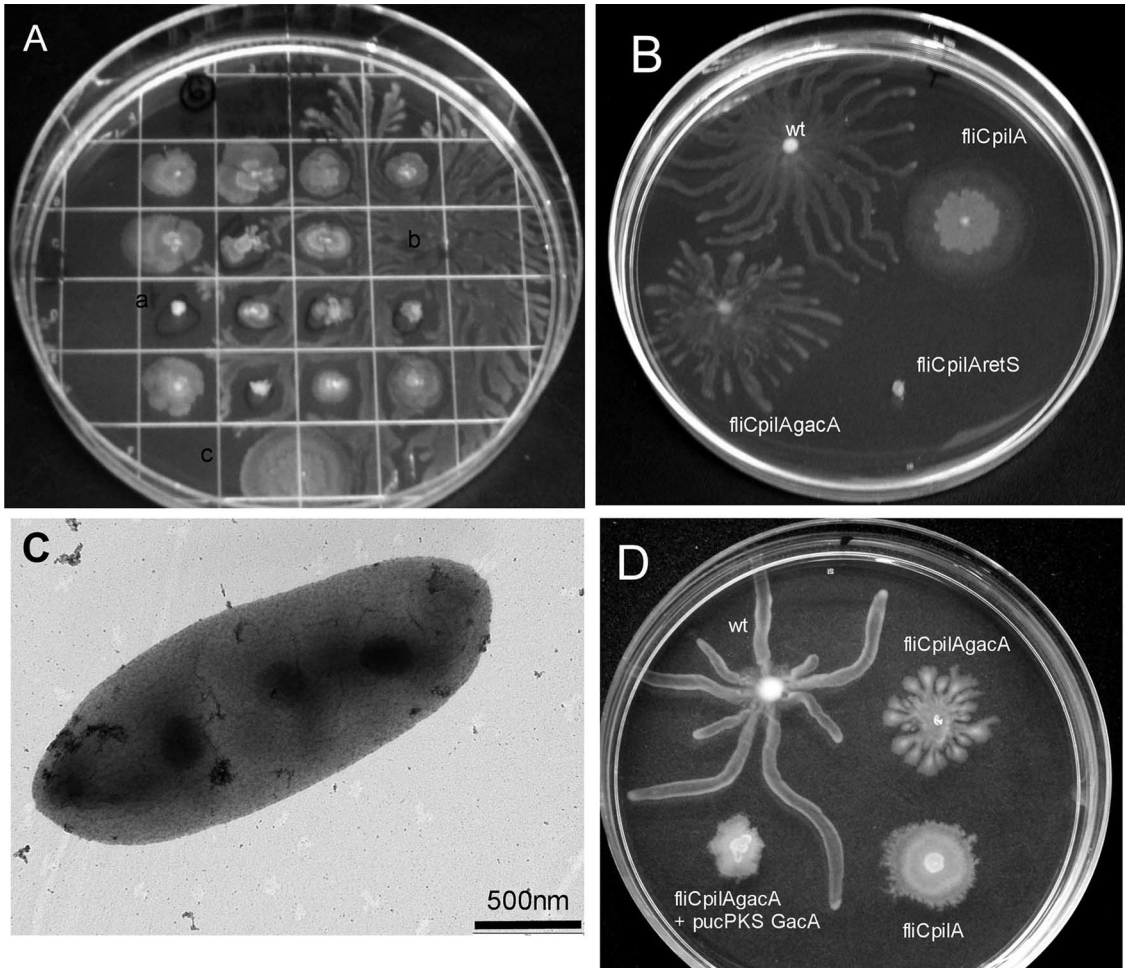

FIG. 4. GacA and RetS influence sliding motility. (A) Transposon insertion mutants of *fliC pilA* were screened on 0.5% M8 swarming plates for changes in motility. Mutants exhibiting both diminished (a) and increased (b) spreading were obtained; the parental *fliC pilA* strain was included on each plate as a control (c). Note the appearance of tendril formation in the strain marked b. (B) Sliding motility of *fliC pilA gacA* and *fliC pilA retS* mutants was assayed on 0.5% M8 plates after overnight incubation at 30°C. (C) *fliC pilA gacA* bacteria grown on swarming plates were harvested from the end of a tendril and visualized by TEM. No bacteria had recognizable surface structures; a representative image is shown. (D) Expression of GacA from a plasmid complements the *fliC pilA gacA* phenotype on 0.5% M8 plates supplemented with carbenicillin (200 μg/ml). Note that tendril formation is eliminated when GacA is reintroduced. The isogenic control strains all carry the empty vector pUCP-KS. wt, wild type.

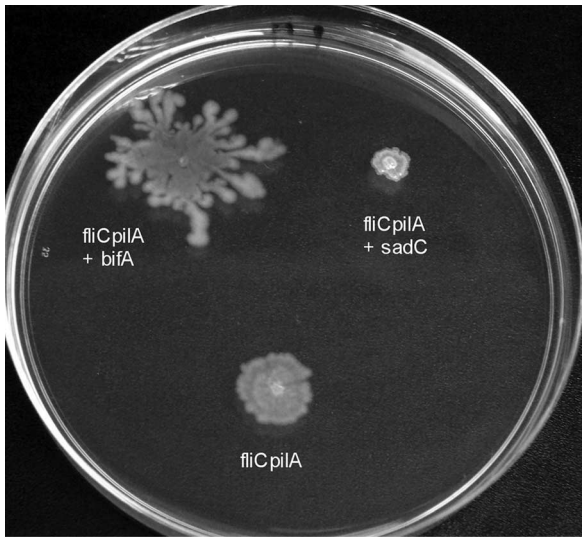

FIG. 5. BifA and SadC reciprocally regulate sliding motility. Dispersion of *fliC pilA* bacteria expressing BifA or SadC under control of the inducible P_{BAD} promoter was assayed on 0.5% M8 agar plates supplemented with 0.2% glucose and 0.05% glutamate plates that contained gentamicin and arabinose (0.5%). Plates were incubated overnight at 30°C and then left at room temperature for an additional 48 h. The control strain carries the empty vector pMQ80.

sources. *fliC pilA gacA* spread to a similar extent under all conditions but exhibited distinct morphological patterns on each medium (see Fig. S2 in the supplemental material). We could not induce motility of *fliC pilA retS* on any swarming medium that we tested; likewise, *fliC pilA rhlA* bacteria failed to spread on 0.5% FAB plates with glutamate or succinate (see Fig. S2 in the supplemental material).

Overexpression of SadC and BifA alters dispersal of *fliC pilA* on swarming plates. The diguanylate cyclase SadC and the c-di-GMP phosphodiesterase BifA have recently been shown to regulate swarming motility and biofilm formation in PA14. Overexpression of SadC represses swarming motility (26), while overexpression of BifA increases swarming zone size (18). We tested whether overexpression of these proteins in the *fliC pilA* background would similarly affect sliding motility. As seen in Fig. 5, SadC expression inhibited sliding, while overexpression of BifA resulted in both increased sliding and the appearance of tendril formation. These results argue that sliding motility, like swarming, is regulated by c-di-GMP levels.

DISCUSSION

Bacteria can move through their environment in many different ways. One relatively complex form of motility exhibited on semisolid surfaces by several genera of bacteria, including pseudomonads, is swarming motility. This form of motility appears to be "powered" by flagella but is also influenced by elaboration of surface structures (such as TFP), production of surfactants, and the presence of specific environmental signals. In this work we present evidence that *P. aeruginosa* can spread on semisolid surfaces in the absence of flagella or pili, a behavior that matches the description of sliding motility originally presented by Henrichsen (11). We also demonstrate that sliding motility is regulated by many of the factors that are known to influence classic swarming motility. Rhamnolipid production promoted sliding motility, specific carbon/nitrogen sources altered its extent, and known regulators of swarming, such as GacA/GacS, RetS, BifA, and SadC, inhibited or promoted sliding as well as swarming motility. Therefore, sliding motility may allow *P. aeruginosa* to colonize surfaces under conditions where flagellar expression is downregulated, e.g., within the human airway (14, 40).

Although we were initially quite surprised to observe surface spread by *fliC pilA* bacteria on swarming plates, the behavior of the single and double mutants is consistent with the notion that bacterial translocation of any sort is restricted by cohesive forces and facilitated by both propulsive forces and those that reduce friction between bacterium and substrate. In wild-type PAO1, TFP mediate interactions between bacteria that appear to antagonize the flagellum-dependent movement of bacteria away from each other. Thus, the disappearance in a *pilA* mutant of the tight tendrils that characterize a wild-type swarming colony is a manifestation of a net decrease in cell-cell interactions. Likewise, the increased motility of a double *fliC pilA* mutant compared to its *fliC* counterpart is facilitated by a decrease in the cell-cell interactions that restrict spreading of the piliated *fliC* strain. The importance of pilin-mediated cell-cell interactions is underscored by comparing the phenotypes of *fliC pilA gacA* with the same strain now complemented for pilin expression: tendril formation and spreading are strongly repressed in the piliated strain.

The absence of TFP and flagella is not sufficient for sliding motility. A *pilA* mutant constructed in the aflagellate PA103 background does not spread on swarming plates (data not shown), suggesting that sliding motility requires more than the absence of surface pili. This work provides evidence that rhamnolipids, which are not produced by PA103 (data not shown), are one of the factors that positively regulate sliding motility of *fliC pilA* bacteria. Nonetheless, a quantitative defect in total rhamnolipid production does not account for the failure of the *fliC pilA retS* cells to spread. Our assays, however, cannot rule out that changes in the relative amounts of HAA and mono- and di-rhamnolipids produced by the *retS* and *gacA* transposon insertion mutants underlie the different patterns and degrees of motility that they exhibit.

Many of the regulators that influence swarming motility also appear to affect production of exopolysaccharide (EPS), at either the transcriptional or posttranscriptional level (9, 18, 26). In agreement with published results, mutation of *retS* and overexpression of SadC in the *fliC pilA* background increased Congo Red staining of colonies, consistent with increased EPS production (data not shown). Thus, the increased cohesive forces provided by increased EPS production may inhibit both swarming and sliding motility. However, it is not clear that diminished or absent EPS production alone is sufficient to promote either swarming or sliding motility. Thus, while mutations in the EPS synthetic locus, *pel*, result in increased swarming of wild-type PA14, the same mutation introduced into a nonswarming Δ*bifA* mutant does not rescue swarming motility (18). These observations are consistent with the hypothesis that additional factors that promote swarming or sliding are also under the control of these regulators.

The surface translocation of *P. aeruginosa* in the absence of

recognized motility organelles likely reflects the spread of dividing organisms in an environment where dispersive factors, such as surfactant production, outweigh the cohesive force usually provided by pili. This "spreading by expansion," termed sliding motility by Henrichsen (11), has been recently described in mycobacteria (23) and nonflagellated *Bacillus subtilis* (8). Acetylated glycopeptidolipids function as surface-active compounds required for spreading of mycobacteria (33, 34), while mutations in a surfactin synthetic locus abolish sliding motility of *B. subtilis* (15, 16). Nonetheless, we cannot rule out the existence of an as-yet-unrecognized form of motility independent of pili and flagella, as has been described for other organisms, such as *Myxococcus xanthus* (27, 41), *Flavobacterium johnsoniae* (2, 21), and *Mycoplasma mobile* (29). Our ongoing screen of transposon mutants that abolish sliding motility has not yet identified genes encoding novel surface structures or homologs of motility genes found in these other organisms. Continuing identification of transposon insertions that either abolish or enhance sliding motility will provide additional information regarding regulators not only of this unusual surface behavior but also of swarming motility.

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