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Rhamnolipids Modulate Swarming Motility Patterns of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **is capable of twitching, swimming, and swarming motility. The latter form of translocation occurs on semisolid surfaces, requires functional flagella and biosurfactant production, and results in complex motility patterns. From the point of inoculation, bacteria migrate as defined groups, referred to as tendrils, moving in a coordinated manner capable of sensing and responding to other groups of cells. We were able to show that *P. aeruginosa* produces extracellular factors capable of modulating tendril movement, and genetic analysis revealed that modulation of these movements was dependent on rhamnolipid biosynthesis. An *rhlB* mutant (deficient in mono- and dirhamnolipid production) and an *rhlC* mutant (deficient in dirhamnolipid production) exhibited altered swarming patterns characterized by irregularly shaped tendrils. In addition, agar supplemented with rhamnolipid-containing spent supernatant inhibited wild-type (WT) swarming, whereas agar supplemented with spent supernatant from mutants that do not make rhamnolipids had no effect on WT *P. aeruginosa* swarming. Addition of purified rhamnolipids to swarming medium also inhibited swarming motility of the WT strain. We also show that a *sadB* mutant does not sense and/or respond to other groups of swarming cells and this mutant was capable of swarming on media supplemented with rhamnolipid-containing spent supernatant or purified rhamnolipids. The abilities to produce and respond to rhamnolipids in the context of group behavior are discussed.**

Rhamnolipids, glycolipids comprised of L-rhamnose and 3-hydroxylalkanoic acid, were first identified in the mid 1900s in cultures of *Pseudomonas aeruginosa* (23, 29), and the structure of a rhamnolipid molecule was first reported in the mid 1960s (17). Rhamnolipids are comprised of mono- and dirhamnose groups linked to 3-hydroxy fatty acids that vary in length, the most common being L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (monorhamnolipid) and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (dirhamnolipid) (14, 34, 36).

Ketoacyl reduction, catalyzed by an NADPH-dependent β-ketoacyl reductase (RhlG), initiates fatty acid synthesis of the lipid component of rhamnolipids from the general pool of fatty acids (9). These are converted to the rhamnolipid precursor, 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), by the proposed acyltransferase activity of the RhlA protein (14). The final steps in rhamnolipid production require rhamnosyl transfer reactions catalyzed by the RhlB and RhlC rhamnosyltransferases to sequentially add dTDP-L-rhamnose to HAAs, resulting in mono- and dirhamnolipids, respectively (38, 47).

Rhamnolipids are amphipathic molecules composed of a hydrophobic lipid and a hydrophilic sugar moiety. This provides these molecules with tensioactive properties capable of reducing surface tension, forming emulsions, and causing pseudosolubilization of insoluble substrates, which allows *P. aeruginosa* to utilize diverse carbon sources such as alkanes (3, 19). They also increase binding to such substrates by enhancing cell surface hydrophobicity through displacement of lipopolysaccharides (10). Both properties make *P. aeruginosa* a candidate bioremediation species. Furthermore, addition of rhamnolipids to contaminated soils has been shown to recover pyrene (which results from the incomplete combustion of fossil fuels) and heavy metals, such as copper and zinc (6, 37).

Rhamnolipids are found in the sputa of cystic fibrosis patients and can inactivate tracheal cilia of mammalian cells, indicating that they are virulence factors (22, 31). Consistent with their role in pathogenesis, rhamnolipids induce fast cell lysis of *Dictyostelium discoideum* (11), and *Burkholderia pseudomallei* produces rhamnolipids that alter the F-actin network of macrophages that reduces phagocytosis (24).

Rhamnolipid production coincides with stationary-phase growth (23) and is under quorum-sensing control (9, 39, 40, 47). The *rhlA* and *rhlB* genes are organized in an operon induced by *N*-butyryl homoserine lactone-activated RhlR (39, 40). The *rhlC* gene is in an operon located downstream of a gene (PA1131) encoding a hypothetical protein of unknown function and is also regulated by RhlR (47). Interestingly, the gene encoding RhlG contains RhlR binding sites in the promoter region but does not require RhlR for activation (9).

During biofilm development, expression of the *rhlAB* operon is observed in microcolonies greater than 20 μm in depth (35). A previous study from our group showed that maintenance of mushroom-shaped macrocolonies and open channels in a mature biofilm requires rhamnolipids (12). A second form of group behavior that requires rhamnolipid biosynthesis is swarming motility. Rhamnolipids have been thought to be required for overcoming surface tension and allow flagellum-based propulsion of *P. aeruginosa* over semisolid surfaces (30). The result of swarming in *P. aeruginosa* is complex patterns of cells organized as radiating tendrils, the spaces between which may be analogous to biofilm channels in that they remain uncolonized.

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TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristic(s)[a] or sequence	Reference or source
P. aeruginosa strains		
PA14	Wild type	48
PA14 mutants	PA14 *flgK*::Tn*5*B30; Tc^r	42
	PA14 *rhlA*::Gm; Gm^r	46
	PA14 *rhlB*::Kn; Kn^r	This study
	PA14 Δ*rhlC*	This study
	PA14 *sadB*::Tn*5*B21; Tc^r	7
	PA14 Δ*pqsD*	D. Hogan
Plasmids		
pNC5	*sadB* in pUCP18; Cb^r Ap^r	7
pEX18Gm	Suicide vector; Gm^r Sac^+	25
pEX18KO*rhlC*	*rhlC* KO construct	This study
pBT20	Transposon plasmid; Gm^r	33
Primers		
*rhl*CKO1	5′-GGCGTCTAGACTGGCGATGCTCGGCTTC-3′	
*rhl*CKO2	5′-GGAGGAATTCCAGGTCGTCGTCGCCAG-3′	
*rhl*CKO3	5′-GGCGGAATTCCGGCGAAACGCATTC-3′	
*rhl*CKO4	5′-GGCGAAGCTTCTGGAACTTTCGGTG-3′	

[a] Abbreviations: Tc^r, tetracycline resistant; Gm^r, gentamicin resistant; Kn^r, kanamycin resistant; Cb^r, carbenicillin resistant; Ap^r, ampicillin resistant; KO, knockout.

In this study, we demonstrate that rhamnolipids are required to maintain tendril organization and prevent colonization of the area between tendrils. We also identified a genetic locus called *sadB*, previously identified because of its role in biofilm formation (7), that is required by swarming cells to respond to the presence of rhamnolipids. Our data indicate that rhamnolipids, but not HAAs, are involved in modulating swarming motility.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. All bacterial strains and plasmids used in this study are shown in Table 1. *P. aeruginosa* PA14 was routinely cultured on lysogeny broth (LB) medium (4). Swarm agar was based on M8 minimal medium (30) supplemented with $MgSO_4$ (1 mM), glucose (0.2%), and Casamino Acids (CAA) (0.5%), and solidified with agar (0.5%). Swim agar contents were identical to swarm agar contents except that it was solidified with 0.3% agar. Bacteria were spot inoculated on swarm agar plates as 2.5-μl aliquots taken directly from overnight LB cultures. Unless noted otherwise, swarm agar plates were incubated for 16 h at 37°C and then incubated an additional 32 h at room temperature for a total of 48 h. For a given experiment, all swarm agar plates were poured from the same batch of agar to minimize variation in agar concentration. All plates were poured in the morning and allowed to dry on the bench throughout the day and were inoculated, in triplicate, approximately 8 h after the plates were poured. Comparisons were made only among plates poured from the same batch of agar. To establish a zone of rhamnolipids on swarm agar plates (see Fig. 2A and B and 5C), a flagellar hook mutant (*flgK*) was inoculated on swarm agar plates and incubated at 37°C for 12 h. At this point, the rhamnolipid front was traced on the petri plate, and bacteria to be assayed were inoculated on the front or within the zone of rhamnolipids and incubated overnight at 37°C. In Fig. 2A, the perimeter of the zone was visualized by spotting a drop of 0.5% methylene blue just inside the zone edge and tilting the plate such that the drop traveled along the circumference of the zone. Spent supernatants were prepared for addition to swarm and swim agar by subculturing overnight LB cultures 1:1,000 in M8 broth supplemented with $MgSO_4$, glucose, and CAA at the concentrations mentioned above and incubating for 24 h at 37°C. Bacteria were removed by centrifugation (10 min at 6,000 × *g*), and the supernatant was filtered through a 0.22-μm membrane (Millipore, Billerica, MA) and added to swarm agar as indicated. Rhamnolipids were purified for addition to swarm agar from wild-type (WT) culture grown for 24 h in M8 broth. Cells were removed by centrifugation (10 min at 6,000 × *g*), and the supernatant was acidified to pH 2 with concentrated HCl. Then 20 ml of chloroform:methanol (2:1) was added to the acidified supernatant, and the supernatant was vortexed for 1 min. The lower organic phase was collected after centrifugation (10 min at 10,000 × *g*), evaporated to dryness, resuspended in 1 ml methanol, filtered through a 0.45-μm membrane (Millipore, Billerica, MA), evaporated to dryness, resuspended in 10 ml distilled H_2O (dH_2O), and filter sterilized. This resulted in a fivefold concentration of rhamnolipids. Cells were grown in PPGAS minimal medium at 30°C to stimulate rhamnolipid production for thin-layer chromatography (TLC) and bacterial adherence to hydrocarbon (BATH) assays. PPGAS medium consists of NH_4Cl (20 mM), KCl (20 mM), Tris-HCl (pH 7.2) (120 mM), $MgSO_4$ (1.6 mM), glucose (0.5%), and peptone (1.0%). Unless noted otherwise, antibiotics were used at the following concentration: carbenicillin, 500 μg/ml; gentamicin (Gm), 100 μg/ml; kanamycin, 500 μg/ml; tetracycline, 150 μg/ml. Unless noted otherwise, all enzymes used for DNA manipulation were purchased from Invitrogen (Carlsbad, CA). Cloning was carried out in *Escherichia coli* SM10-λ*pir* by standard methods (2), and constructs were electroporated into *P. aeruginosa* as described previously (5).

Molecular and genetic techniques. (i) *rhlC* knockout. A knockout mutation of *rhlC* was generated by amplifying 5′-flanking and 3′-flanking DNA fragments using the primers listed in Table 1. The 5′ and 3′ fragments were digested using engineered restriction sites (XbaI and EcoRI for 5′ fragment; EcoRI and HindIII for 3′ fragment) and ligated into pEX18Gm (25) cut with XbaI and HindIII. The plasmid was electroporated into *E. coli* SM10-λ*pir*. Mutations were constructed as reported previously (25), and the resolved integrants were confirmed by PCR.

(ii) *rhlB* knockout. An *rhlB*::*kanMX* construct was generated to mediate allelic replacement of the chromosomal *rhlB* gene. This construct was made using yeast homologous recombination with a novel *Saccharomyces cerevisiae-E. coli* shuttle vector, pMQ30, made for allelic replacement in gram-negative bacteria unable to support replication of the ColE1 origin. Plasmid pMQ30 is a modified version of pEX18Gm (25), into which a *URA3* selectable marker and *CEN6* and *ARSH4* sequences have been introduced from pYC2-CT (Invitrogen, Carlsbad, CA) (R. M. Q. Shanks and G. A. O'Toole, unpublished data). This modification leaves the multiple cloning site of the original vector unmodified. The resulting plasmid, pRMQS66, includes an *rhlB* allele with 276 internal base pairs replaced by the *kanMX* cassette from pUG6 (21). Additional details regarding pMQ30 and the building of pRMQS66 will be reported elsewhere. pRMQS66 was transformed into *E. coli* SM10-λ*pir*, and allelic replacement was performed as noted above for *rhlC*.

(iii) Genetic screen for swarm inhibition-deficient (*sid*) mutants. Transposon mutants were generated as described previously, with slight modifications (43, 51). The recipient (*P. aeruginosa* PA14 *flgK*::Tn*5*B30 Tc^r) and donor (*E. coli* SM10-λ*pir* pBT20 Gm^r) were grown to late stationary phase. The donor (0.25 ml) and the recipient (1.0 ml) were mixed in a 1.5-ml tube, pelleted in a microcentrifuge, resuspended in 0.1 ml of LB broth, spotted on LB agar, and incubated at 30°C for 3 h. Cells were then scraped from the LB plate, resuspended in 1.0 ml

of LB broth, pelleted in a microcentrifuge, resuspended in 0.1 ml LB broth, and plated on LB agar supplemented with tetracycline and gentamicin. Mutants were screened by spotting them (eight per swarm plate) in a circular array with the WT strain located in the center of the circle. Plates were incubated at 37°C for 16 h and then incubated at room temperature for 32 h. Mutant strains into which the WT swarmed were purified, retested, and designated swarm inhibition-defective strains (*sid* mutants). Transposon insertions were mapped using arbitrarily primed PCR (44).

Rhamnolipid detection by TLC. Rhamnolipids were extracted as previously described (26). Briefly, cells were removed from PPGAS cultures grown at 30°C for 24 h by centrifugation (10 min at 6,000 × *g*), and the supernatant was acidified to pH 2 with concentrated HCl. Equal volumes (750 μl) of acidified supernatant and chloroform:methanol (2:1) were mixed and vortexed for 1 min, and the lower organic phase was collected after centrifugation (10 min at 10,000 × *g*). The extraction was repeated, and the pooled organic phases were evaporated to dryness, resuspended in 1 ml methanol, filtered through a 0.45-μm membrane, evaporated to dryness, and resuspended in 20 μl methanol. The samples were analyzed by TLC (Kieselgel 60/Kieselgur F_{254}; Merck, Darmstadt, Germany) with a mobile phase consisting of 80% chloroform, 18% methanol, and 2% acetic acid. Anthrone reagent dissolved in concentrated H_2SO_4 at a final concentration of 2% was used to visualize rhamnolipid spots (53).

Drop collapse assay. Drop collapse assays were performed as reported previously (14). Bacteria were subcultured from overnight LB cultures into PPGAS minimal medium and grown for 16 h at 30°C. Cells were removed by centrifugation, and the supernatant was filtered through a 0.22-μm membrane and serially diluted in dH_2O. Aliquots of 30 μl were spotted in circles located on the underside of a lid to a Corning 96-well plate (Costar 3595; Corning, NY) and assayed for bead formation. Samples that did not form a bead were defined as having drop collapse activity.

BATH assays. BATH assays were performed as previously described (55). Briefly, cells were grown for 24 h at 30°C in PPGAS minimal medium, washed twice in M8 minimal medium, diluted 1:4 in wash medium, and the optical density at 600 nm was measured. Cells (1.2 ml) were mixed with hexadecane (0.2 ml), vortexed for 2 min, and allowed to stand at room temperature for 15 min. The aqueous phase was recovered, and the optical density at 600 nm was measured. The results were expressed as the percentage of cells bound to hexadecane.

Plate detection of biosurfactants. Bacteria from overnight LB cultures were spot inoculated as 2.5-μl aliquots onto M8 medium supplemented with $MgSO_4$ (1 mM), glucose (0.2%), CAA (0.5%), cetyltrimethylammonium bromide (CTAB) (0.02%), and methylene blue (0.0005%) and solidified with agar (1.5%). Plates were incubated at 37°C for 24 h, followed by incubation at room temperature for an additional 24 h for a total of 48 h. Biosurfactant production results in the precipitation of CTAB and the formation of a dark blue halo that is outlined in white surrounding the colony (50).

RESULTS

Swarm inhibition. In the course of a swarm experiment with *P. aeruginosa* PA14, tendrils migrated from the point of inoculation to form complex radial patterns (Fig. 1A). We noticed that tendrils of a given WT swarm rarely intersect, and furthermore, tendrils from different swarms change course as they approach each other (Fig. 1A). When WT tendrils of opposing swarms approach, they change direction and swarm parallel to each other instead of crossing paths (Fig. 1A). This avoidance phenotype can be observed after overnight incubation. When plates are left for 48 h, tendrils thicken in size but avoid contacting neighboring tendrils.

A flagellar hook mutant (*flgK*) was inoculated on swarm agar along with the WT strain to test whether active swarming is required for this avoidance phenotype (Fig. 1B). In this experiment WT tendrils radiated linearly in all directions except towards the *flgK* mutant. Tendrils from the WT strain most proximal to the *flgK* mutant arced around this nonmotile mutant and thus maintained a constant distance from the colony edge. The ability of the nonmotile *flgK* mutant to modulate WT tendril movement indicates active swarming is not required for this process.

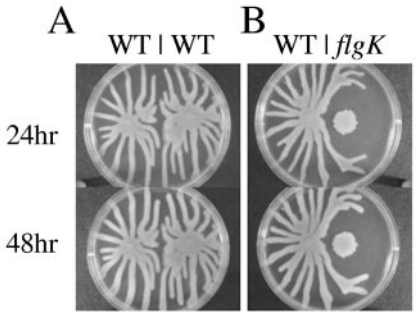

FIG. 1. Swarms of *P. aeruginosa* are able to sense and respond to neighboring swarms. (A) Two aliquots of WT cells were spotted on 0.5% swarm agar plates and monitored at 24 and 48 h. (B) Aliquots of WT and *flgK* mutant cells were spotted on 0.5% swarm agar plates and monitored at 24 and 48 h.

The swarm inhibition signal is extracellular. A zone of fluid surrounded the nonmotile *flgK* colony after ~12 h of incubation, suggesting bacterial cells secrete factors into the extracellular environment while swarming. The perimeter of this zone can be visualized using methylene blue (Fig. 2A) (see Materials and Methods for details). This zone was also present around WT tendrils (data not shown), but its irregular shape made it less amenable to the experimental manipulations outlined below. An *rhlA* mutant did not produce the zone of liquid that surrounds WT tendrils and the nonmotile *flgK* colony (data not shown). The *rhlA* gene product catalyzes the synthesis of 3-(3-hydroxyalkanoyloky)alkanoic acids, the lipid precursor of rhamnolipids (14), suggesting rhamnolipids comprise, at least in part, the zone surrounding swarming cells.

Because the *flgK* mutant is nonmotile and forms a uniform zone of rhamnolipids, this mutant was used to establish rhamnolipid-containing zones in experiments presented in Fig. 2B. The outline of this zone is marked in Fig. 2B by a white circle. To test the rhamnolipid-containing zones for swarm-modulating properties, suspensions of WT cells were inoculated within these zones (Fig. 2B, top) or on the zone edge (Fig. 2B, bottom) and incubated overnight. In both cases WT cells swarmed away from the zone, indicating it contains extracellular factors that modulate swarming.

To test whether extracellular swarm-modulating factors were specific to the *flgK* mutant, swarm agar was supplemented with WT spent supernatant prepared from stationary-phase cultures grown in M8 medium, the same medium used to prepare swarm agar. When inoculated onto the swarm agar containing 7.5% filter-sterilized WT spent supernatant, the WT strain did not swarm but formed colonies that lacked tendril radiation (compare Fig. 2C with Fig. 1A). A range of spent supernatant concentrations was tested for swarm inhibition, and it was found that plates containing 5% to 15% spent supernatant could inhibit WT swarming. The WT strain was able to swarm on plates containing less than 5% spent supernatant (data not shown), and plates containing more than 15% spent supernatant were too wet to assay for swarming. The *flgK* mutant serves as a nonswarming negative control in this experiment. Similar results were observed for spent supernatant

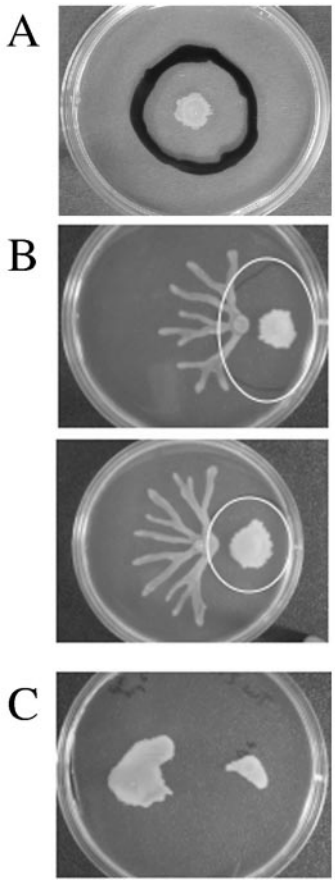

FIG. 2. An extracellular signal modulates swarming in *P. aeruginosa*. (A) A *flgK* mutant was inoculated on 0.5% swarm agar plates and incubated at 37°C for 12 h to allow the formation of a rhamnolipid zone. The perimeter of the zone was visualized using methylene blue. (B) A *flgK* mutant was inoculated on 0.5% swarm agar plates and incubated at 37°C for 12 h to allow the formation of a rhamnolipid zone. The white circle depicts the edge of the rhamnolipid zone surrounding the *flgK* mutant colony at the time of WT inoculation. WT cells were then inoculated within the zone (top) or on the zone edge (bottom) and incubated for 16 h at 37°C. (C) Aliquots of WT and *flgK* cultures were spotted on 0.5% swarm agar plates containing 7.5% WT spent supernatant and incubated at 37°C for 16 h.

prepared from the *flgK* mutant (not shown). Spent supernatant additions to swim media (see Materials and Methods for details) had no effect on swimming motility (data not shown). These observations verify that the WT strain produces an extracellular swarm-modulating factor (or factors) that does not impair swimming motility and that production of this factor is not a phenotype specific to the *flgK* mutant. In addition, these results show that production of the factor(s) is not specific to cells growing under swarm conditions.

Identification of swarm inhibition factor(s). Two genetic approaches were taken to identify genes responsible for production of the putative extracellular swarm-modulating factor. For the first approach, transposon mutagenesis was performed in the *flgK* background and mutants were screened for their ability to "repel" WT *P. aeruginosa* swarm tendrils (see Materials and Methods for details). The *flgK* mutant was a useful genetic background for this screen, because it does not swarm, but this mutant modulates swarming at a level equivalent to that of the WT strain (Fig. 1B). Furthermore, since the starting genetic background was nonswarming, it was clear that any interactions between strains were initiated by WT swarms. A mutant was considered swarm inhibition defective (*sid* mutant) if WT tendrils contacted the *flgK*-derived mutant colony. Screening ~1,000 individual transposon mutants for their ability to inhibit WT swarming identified 6 *sid* mutants, a frequency of ~0.6%. The transposon insertions carried by the *sid* mutants were mapped by arbitrary primed PCR, as reported previously (44), and are shown in Table 2.

The *sid* mutants could be classified by their ability to produce surfactants on the basis of the CTAB plate assay results (50). Interactions with surfactants result in the precipitation of CTAB and the formation of a ring surrounding the colonies plated on this medium. One group of mutants produced no surfactant activity, and the other class of mutants produced decreased activity relative to the WT strain (Table 2). Therefore, all *sid* mutants had either low levels of rhamnolipid or no production of rhamnolipids. The rhamnolipid production phenotypes of the *sid* mutants were confirmed by TLC analysis as described in Materials and Methods (data not shown). The *sid* mutants that produced low levels of surfactant activity relative to the WT on CTAB plate produced less rhamnolipids than the WT did, as determined by TLC. The *sid-5* and *sid-6* mutants produced no surfactant activity as determined by CTAB and produced no detectable rhamnolipids by TLC analysis. Interestingly, the *sid-15* (*crc*) mutant produced no surfactant activity by CTAB analysis but produced rhamnolipids at levels comparable to those produced by the WT as determined by TLC analysis. A major difference between CTAB analysis of surfactants and TLC analysis of rhamnolipids is that the former analyzes cells grown on a solid surface and the latter analyzes cells grown in liquid culture. Therefore, the gene mutated in *sid-15* (*crc*) might be required for surface-associated rhamnolipid synthesis. Consistent with this idea is that *crc* mutants are defective for biofilm formation (41). Alternatively, the difference in medium composition between the plate and liquid assays could account for the difference in rhamnolipid production in the strain mutated for the Crc regulator.

To complement the *sid* screen, a directed approach was taken by analyzing mutants known to be involved in cell-cell

TABLE 2. *sid* mutants

sid allele	Surfactant production[a]	Gene	Description[b]
sid-5	No	*cysQ*	Inositol monophosphatase family signature 1[c]
sid-6	No	*cysQ*	Inositol monophosphatase family signature 1[c]
sid-12	Low	ND[d]	
sid-15	No	*crc*	Catabolite repression control protein
sid-17	Low	*truB*	tRNA pseudouridine 55 synthase
sid-20	Low	PA4999	Hypothetical membrane protein

[a] As judged by CTAB plate assay.
[b] Description based on comparison of DNA sequence flanking insertion sequence compared to the *P. aeruginosa* PAO1 genome sequence (www.pseudomonas.com/).
[c] These *cysQ* mutations are independent mutations.
[d] ND, not determined.

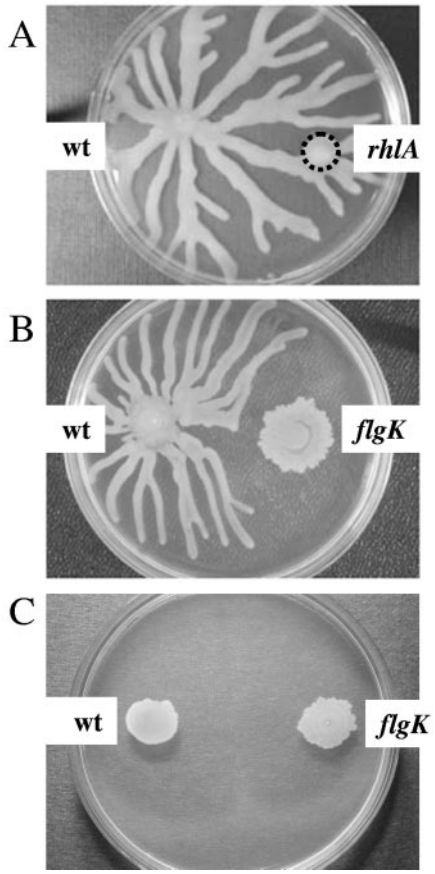

FIG. 3. Rhamnolipids are required for modulating swarming behavior. (A) Aliquots of the WT cells and *rhlA* mutant cells were spotted on 0.5% swarm agar plates and monitored over the course of 16 h. The dotted line indicates the outline of the colony formed by the nonswarming *rhlA* mutant. (B) Aliquots of the WT and *flgK* mutant cultures were spotted on 0.5% swarm agar plates containing 7.5% *rhlA* spent supernatant and incubated at 37°C for 16 h. (C) Aliquots of the WT and the *flgK* mutant cultures were spotted on 0.5% swarm agar plates containing purified rhamnolipids and incubated at 30°C for 16 h. The amount of rhamnolipids added to these plates is approximately equivalent to the level of rhamnolipids found in agar supplemented with 7.5% spent supernatant.

communication. This second strategy revealed that the Las and Rhl quorum-sensing systems were required for modulating swarming. Mutations in *lasI* or *rhlI* resulted in nonmotile colonies that WT tendrils did not avoid while swarming (data not shown). Upon testing strains carrying mutations in genes under quorum-sensing control, we found that an *rhlA* mutant did not inhibit WT swarming. When inoculated on the same plate, WT tendrils radiated linearly in all directions and did not change course when moving towards the *rhlA* colony and ultimately contacted this mutant (Fig. 3A). This is the same phenotype observed for the *sid* mutants (not shown). The *rhlA* mutant phenotype is different from the *flgK* mutant (Fig. 1B), implicating the *rhlA* gene product in production of extracellular swarm-modulating factors. RhlA is required for the synthesis of an early precursor in rhamnolipid biosynthesis. Therefore, both genetic approaches indicated that the production of rhamnolipids is necessary for modulating swarming motility and prompted the experiments described below.

Supernatant of the *rhlA* mutant does not inhibit swarming motility. The observations above could be explained in one of two ways. First, rhamnolipids and/or their precursors play two roles in swarming—as surface-wetting agents and as swarm-modulating compounds, or alternatively, the zone of rhamnolipids surrounding swarming cells carries a diffusible signaling molecule that modulates swarming motility. To distinguish between these models, swarm agar was supplemented with filter-sterilized spent supernatant prepared from the *rhlA* mutant. When inoculated onto the plates containing *rhlA* spent supernatant, the WT strain produced motile swarms with radial arrays of tendrils indistinguishable from those produced when inoculated on plates lacking spent supernatant (Fig. 3B). In contrast, spent supernatant from the WT completely inhibited swarming motility (Fig. 2C).

To demonstrate that rhamnolipids and not other components of spent supernatant modulated swarming motility, agar was supplemented with rhamnolipids extracted from WT cultures grown in M8 medium (see Materials and Methods for details). Purified rhamnolipids were added at a level approximately equivalent to that found in plates containing 7.5% spent supernatant. In these experiments, purified rhamnolipids completely inhibited WT swarming (Fig. 3C) in a fashion similar to rhamnolipid-containing spent supernatant (Fig. 2C).

Rhamnolipids have also been shown to increase the solubility and bioactivity of the *Pseudomonas* quinolone signal (PQS) (8), making PQS a candidate molecule that could be carried by rhamnolipids and that could modulate swarming. To test this idea, a *pqsD* mutant, which does not make PQS (18), was coinoculated with WT on swarm agar, and in these experiments WT tendrils avoided the *pqsD* mutant, indicating that PQS is not required for swarm modulation (data not shown). The *pqsD* mutant produces rhamnolipids at a level comparable to that of the WT strain (data not shown). These data suggest that rhamnolipids or precursors of these molecules are swarm-modulating factors and not simply vehicles for carrying such molecules.

Rhamnolipids and their biosynthetic precursors have different effects on swarming motility. The data in Fig. 3 suggest rhamnolipid biosynthesis is required both for swarming and for negatively modulating swarming behavior. The former has been previously demonstrated in *P. aeruginosa* (14, 30), but the latter has not been described and is in fact contradictory to the reported role of these molecules with respect to swarming.

An interesting characteristic of the rhamnolipid biosynthetic pathway is that the intermediates (HAAs and monorhamnolipids) and end product (dirhamnolipids) are found in the extracellular environment (14, 17, 28). We hypothesized that these factors have different roles in swarming, for example, enabling swarming through surface wetting or acting to modulate swarming movement. To test these hypotheses, we used strains with mutations in the rhamnolipid biosynthesis pathway genes that would accumulate different intermediates in the pathway. A mutation in the *rhlA* gene was available (46), and we constructed mutations in the other genes encoding the enzymes involved in rhamnolipid biosynthesis in the *P. aeruginosa* PA14 genetic background. The *rhlB* gene was replaced by a kanamycin resistance cassette, and the *rhlC* gene was deleted

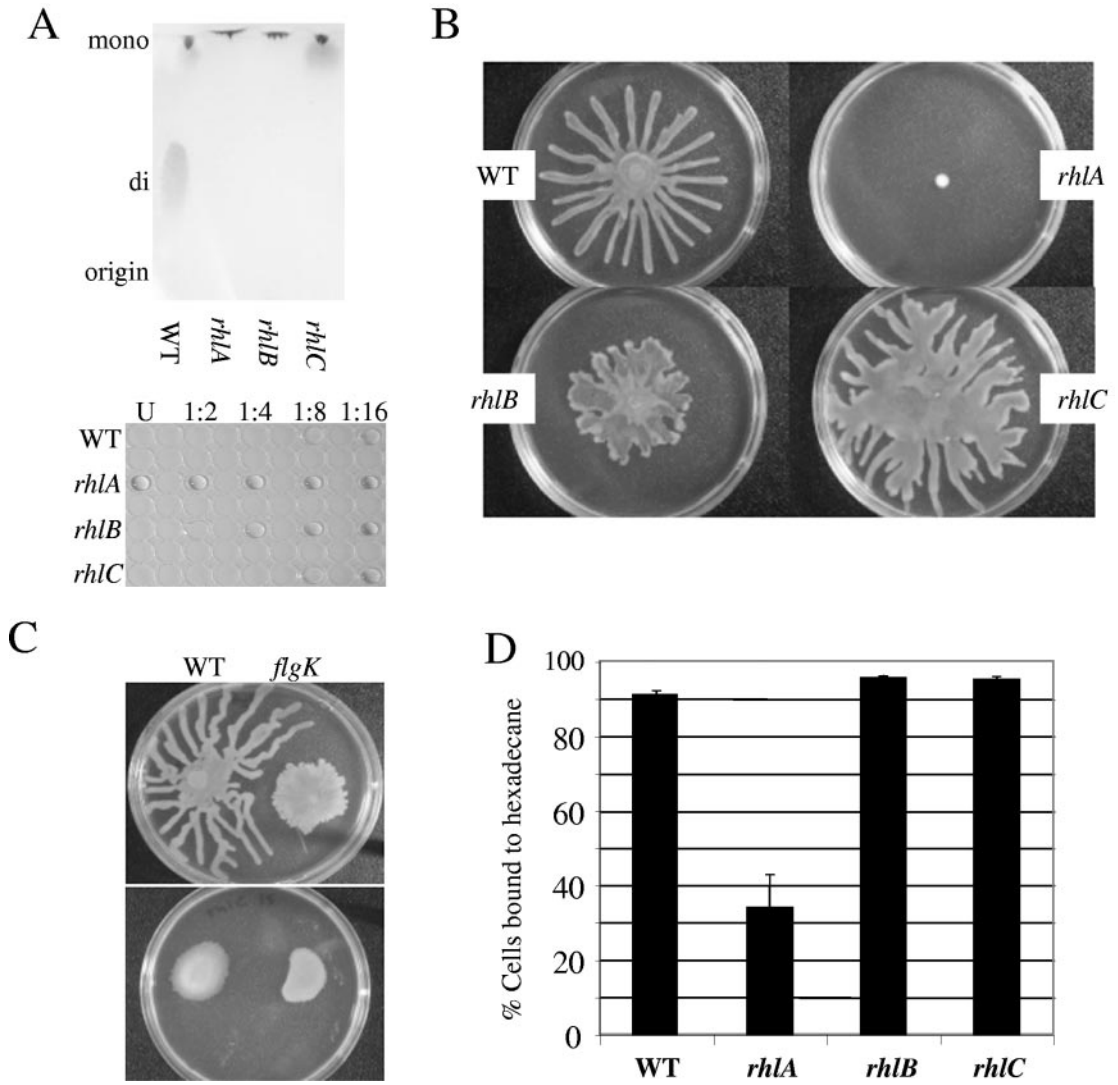

FIG. 4. Dissection of the rhamnolipid biosynthetic pathway and its role in modulating swarming behavior. (A) (Top) Thin-layer chromatographic analysis of *P. aeruginosa* rhamnolipid biosynthesis mutants grown in PPGAS medium for 24 h at 30°C. The positions of monorhamnolipids (mono) and dirhamnolipids (di) are shown to the left of the plate. (Bottom) Drop collapse analysis of *P. aeruginosa* rhamnolipid biosynthesis mutants grown in PPGAS medium for 24 h at 30°C. Samples were diluted in dH_2O, spotted on the lid of a microtiter plate, and assayed for bead formation. The dilutions are shown at the top of the gel. U, undiluted. (B) Swarming phenotypes of the WT and the *rhl* mutants grown on 0.5% agar for 16 h at 37°C. (C) Aliquots of the WT and *flgK* mutant were spotted on 0.5% swarm agar plates containing 7.5% *rhlB* (top) or *rhlC* (bottom) spent supernatant and incubated at 37°C for 24 h. (D) Rhamnolipid mutants were assayed by the BATH test to determine cell surface hydrophobicity as described in Materials and Methods. The percentage of bound cells was plotted versus the strain tested.

from the chromosome. Both the *rhlB* and *rhlC* genes encode rhamnosyltransferases catalyzing the formation of mono- and dirhamnolipids, respectively.

Biochemical analysis of rhamnolipid biosynthetic mutants by TLC is shown in Fig. 4A (top). The WT strain makes all rhamnolipid biosynthetic intermediates and produces two anthrone-positive spots—the fast-migrating spot represents monorhamnolipids, and the slow-migrating spot represents dirhamnolipids. The *rhlA* and *rhlB* mutants do not produce anthrone-positive spots, indicating that neither mutant produces rhamnolipids. The *rhlC* mutant produces a fast-migrating spot, indicating that it produces monorhamnolipids. The dark spot present in each lane that migrates ahead of monorhamnolipids corresponds to material migrating with the solvent front. These spots could be visualized without the use of the anthrone reagent, indicating that they are not rhamnolipid species. These data are consistent with the known functions for these enzymes (38, 47).

The RhlA enzyme catalyzes synthesis of HAAs, which are converted to monorhamnolipids by the RhlB enzyme (14, 38). The difference between the *rhlA* mutant and the *rhlB* mutant is that the former does not make HAAs and the latter accumulates this intermediate. HAAs cannot be detected by the TLC analysis used here because they will not react with anthrone due to an absence of sugar moieties. To phenotypically separate the *rhlA* mutant and the *rhlB* mutant, we took advantage of the surface-wetting properties of HAAs (14). The *rhlB* mutant supernatant should produce more surfactant activity than

the *rhlA* mutant supernatant, and the drop collapse assays performed using spent supernatant from WT and rhamnolipid biosynthetic mutants confirmed this prediction (Fig. 4A, bottom). The WT spent supernatant does not form a bead until diluted 1:16, indicating drop collapse capability for a dilution of up to 1:8. The same phenotype is observed for the *rhlC* mutant. The *rhlB* spent supernatant has drop collapse activity only as far as a 1:2 dilution. The *rhlA* spent supernatant forms beads at all concentrations tested, indicating no measurable drop collapse activity. The difference in drop collapse activity between the *rhlA* mutant and the *rhlB* mutant is a result of HAAs produced by the *rhlB* mutant, but not by the *rhlA* mutant (14). The *rhlB* mutant supernatant has less drop collapse activity than the *rhlC* mutant and WT, consistent with rhamnolipids also having surface-active properties.

Rhamnolipid biosynthetic mutants were tested in swarming assays (Fig. 4B). The *rhlA* mutant that makes neither HAAs nor rhamnolipids has a nonswarming phenotype. The *rhlB* and *rhlC* mutants are able to swarm. These data suggest HAAs are the minimal surfactant required for swarming in *P. aeruginosa* PA14 and are consistent with data published by Deziel et al. working with *P. aeruginosa* PG201 (14).

Although the *rhlB* and *rhlC* mutants can swarm, the patterning of swarming in these mutants differs from that of the WT. The WT produces a radial array of long, thin tendrils resulting in large cell-free areas between each tendril. In contrast, the *rhlB* and *rhlC* mutants form short, irregular tendrils, and most of the area between the tendrils is colonized. These observations suggest that while rhamnolipids are not required for swarming motility, both of these compounds modulate swarming and contribute to the maintenance of the cell-free regions between tendrils.

To test the idea that rhamnolipids modulate swarm behavior, WT cells were assayed for swarming motility on plates containing spent supernatant derived from either *rhlB* or *rhlC* strains. The *flgK* mutant was used as a nonswarming control. Wild-type cells were able to swarm on agar containing the *rhlB* spent supernatant (Fig. 4C, top) but exhibited inhibited swarming on medium containing spent supernatant from the *rhlC* mutant (Fig. 4C, bottom). The *rhlC* mutant-derived spent supernatant contains monorhamnolipids, which are absent in the *rhlB* supernatant, and indicates that monorhamnolipids are sufficient for swarm inhibition. However, the altered swarming phenotype of the *rhlC* mutant compared to the WT phenotype (Fig. 4B) indicates that dirhamnolipids also contribute to modulating swarming behavior.

The data above suggest HAAs are required for surface wetting and rhamnolipids are required for modulating swarming behavior. The functions of HAAs and rhamnolipids can also be separated by effects on cell surface hydrophobicity as measured by BATH assays (Fig. 4D). When mixed with hexadecane, ~30% of the *rhlA* population bound hexadecane, indicating a majority of the cells (~70%) are not hydrophobic enough to adhere to this substrate. The *rhlB* and *rhlC* mutants bound as well as WT did, resulting in ~95% of the culture being hydrophobic enough to interact with hexadecane. The WT level of hexadecane binding demonstrated by the *rhlB* and *rhlC* mutants indicates that rhamnolipids are not required for altering cell surface hydrophobicity and that the altered cell surface hydrophobicity of the *rhlA* mutant is due to the loss of production of HAAs.

SadB is required for normal swarm pattern formation and response to rhamnolipid-mediated modulation of swarm behavior. The data presented above suggested that rhamnolipids could modulate swarming behavior. We hypothesized that *P. aeruginosa* might have evolved a pathway for sensing and responding to the presence of rhamnolipids. To begin to address this idea, we used a candidate approach to identify mutants that did not respond to the presence of rhamnolipids. We speculated that because biofilm formation, like swarming, was a group behavior and, furthermore because some investigators have suggested a link between biofilm formation and swarming (13, 27), mutants defective in biofilm formation might also be altered in their ability to respond to rhamnolipids.

We tested biofilm mutants identified in our lab in the swarming assay shown in Fig. 1 by coinoculating the WT and these mutants on swarm agar plates. The *sadB* mutant, a biofilm-defective mutant previously reported by our group (7), had a swarming phenotype at 48 h that consisted of broader tendrils and smaller cell-free regions between tendrils in comparison to WT (Fig. 5A). Other published biofilm mutants, including *pilA*, *sadARS* (*rocARS*), and *gacA* (20, 32, 42, 45) mutants, did not have the *sadB*-like swarming phenotype (data not shown).

The swarming phenotype of the *sadB* mutant could be the result of this strain not sensing or, alternatively, not producing swarm-modulating factors. To test this idea, the *sadB* mutant was placed on swarm agar plates with the WT or the *flgK* mutant (Fig. 5B). In the former, the *sadB* tendrils radiated in all directions and did not change direction as they approached WT tendrils, eventually making contact with WT tendrils (Fig. 5B, left panel). When coinoculated with the *flgK* mutant, the *sadB* mutant radiated tendrils in all directions, many of which contacted the nonmotile *flgK* colony (Fig. 5B, center panel). In both circumstances the phenotype of the *sadB* mutant is in stark contrast to the phenotype of the WT under identical conditions (Fig. 1). Introducing the *sadB* gene in *trans* on plasmid pNC5 (*sadB*$^+$) complemented the swarming phenotype of the *sadB* mutant (Fig. 5B, right panel).

Because tendrils of the *sadB* mutant initiate contact with WT tendrils and WT tendrils bend away from the *sadB* tendrils, it is likely that the *sadB* mutant produces swarm-modulating factors and its phenotype results from an inability to respond to these factors. Indeed, the *sadB* mutant was shown to produce both mono- and dirhamnolipids by TLC analysis and displayed WT levels of drop collapse activity (data not shown).

To confirm the *sadB* mutant was unresponsive to rhamnolipids as swarm-modulating factors, the *flgK* mutant was preinoculated on swarm plates for 12 h to accumulate a zone of rhamnolipids indicated by the white circle in Fig. 5C, left panel. The WT (top) and the *sadB* mutant (bottom) were inoculated on the zone edge, and plates were incubated for an additional 24 h at 37°C. In contrast to the WT strain, which swarms only away from the rhamnolipid zone, the *sadB* mutant radiates tendrils in all directions, including into the rhamnolipid zone. In separate experiments, swarm agar was supplemented with WT supernatant (Fig. 5C, middle panel) or rhamnolipids purified from WT supernatant (Fig. 5C, right panel). In the former, unlike the WT coinoculated on the same plate, the

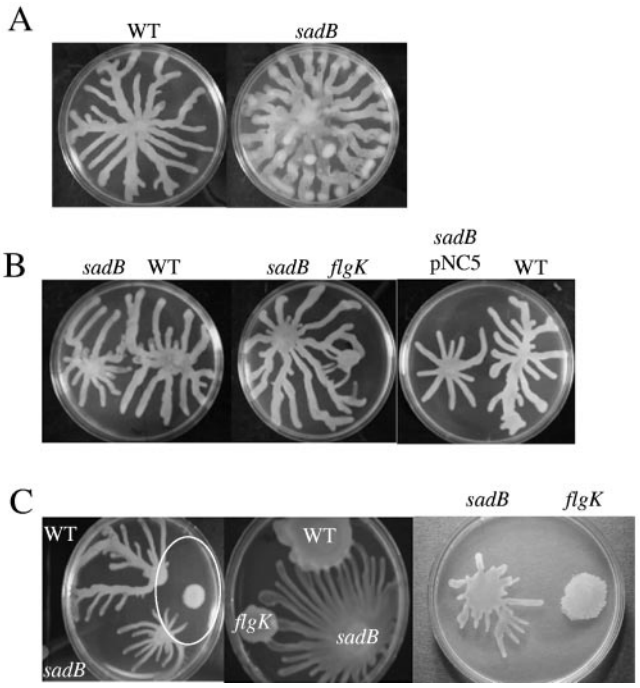

FIG. 5. The *sadB* gene has a role in rhamnolipid sensing. (A) The swarming phenotypes of WT and the *sadB* mutant after 48 h of incubation inoculated individually on a 0.5% swarm agar plate. (B) The *sadB* mutant does not avoid other groups of swarming cells. The phenotype of the *sadB* mutant 24 h after inoculation on 0.5% swarm agar plates in the presence of the WT (left panel) or the *flgK* mutant (middle panel) is shown. Complementation of the swarming phenotype of the *sadB* mutant by expressing *sadB* in *trans* on plasmid pNC5 (right panel) is depicted. (C) The *sadB* mutant does not respond to extracellular rhamnolipids. The *flgK* mutant was inoculated on 0.5% swarm agar plates and incubated at 37°C for 12 h to allow the formation of a rhamnolipid zone (left panel). The white circle depicts the edge of the rhamnolipid zone surrounding the *flgK* colony at the time of WT and *sadB* inoculation. The WT (top) and the *sadB* mutant (bottom) were then inoculated on the zone edge and incubated for 24 h at 37°C. Aliquots of the WT, the *flgK* mutant, and the *sadB* mutant were spotted on 0.5% swarm agar plates containing 7.5% WT spent supernatant and incubated at 37°C for 24 h (middle panel). Aliquots of the *sadB* mutant and the *flgK* mutant cultures were spotted on 0.5% swarm agar plates containing purified rhamnolipids and incubated at 30°C for 16 h (right panel). The amount of rhamnolipids added to these plates is approximately equivalent to the level of rhamnolipids found in agar supplemented with 7.5% spent supernatant.

sadB mutant was able to swarm and produce tendrils (Fig. 5C, middle panel). The *flgK* mutant was used as a nonswarming control. In the latter, unlike the WT inoculated on rhamnolipid-containing media (Fig. 3C), the *sadB* mutant was able to swarm and produce tendrils (Fig. 5C, right panel). These data further suggest that the *sadB* mutant is unable to respond to rhamnolipids as extracellular swarm-modulating factors.

DISCUSSION

Previous observations and data from this study show that biosurfactants (HAAs and rhamnolipids) and functional flagella are required for swarming motility (14, 16, 30, 52). However, to the best of our knowledge, previous reports have not provided a mechanism explaining how swarming cells coordinately regulate behaviors that result in complex motility patterns in *P. aeruginosa*. When *Proteus* species swarm towards each other, a line of inhibited growth results where strains meet. This was first observed in 1946 by Dienes and is referred to as the Dienes phenomenon (15). This line of inhibited growth results from the production of and sensitivity to different types of bacteriocins, namely, proticines, produced by different strains of *Proteus* species (49). If two strains were able to detect different proticines produced by a neighbor, there would be growth inhibition and the formation of a Dienes line. Alternatively, if no proticine difference were detected, the neighboring swarmer would be recognized as "self" and the swarms would interact (49).

We observed that in *P. aeruginosa*, WT tendrils move linearly unless encroaching upon another swarm at which point they change course, swarm parallel to nearby swarms, and maintain spacing similar to that of neighboring tendrils in a given WT swarm. Therefore, we hypothesized that the mechanism that allowed tendrils from different swarms to sense and respond to each other also controlled the radial array of adequately spaced tendrils in a single swarm. We predicted that this mechanism would require the production of extracellular factors capable of modulating swarming at a distance. Consistent with a role for an extracellular factor modulating swarming is the large swarm inhibition zone surrounding the *flgK* colonies (Fig. 1B). Our genetic data show that this zone is in part comprised of and results from the diffusion of rhamnolipids. Since the *flgK* strain is nonmotile, these factors will accumulate in the area surrounding the colony and likely reach higher concentrations than in the area around motile cells and thus have a more obvious effect on neighboring swarms.

The ability of the *flgK* mutant to inhibit WT swarming was a key observation and provided a useful tool for studying swarming behavior. In addition to its nonmotile phenotype, the *flgK* mutant generated a very regular and reproducible zone of liquid, likely rhamnolipids on the basis of the results of our genetic analysis, which modulated swarming. WT cells also produce an extracellular factor(s) capable of modulating swarming, as shown by the inhibitory ability of WT spent supernatant in swarming assays (Fig. 2C).

A genetic approach was taken to identify genes involved in production of factors that modulate swarming with the goal of elucidating the mechanism by which swarming cells communicate and regulate this group behavior. Transposon mutagenesis in the *flgK* background and a candidate approach screening quorum-sensing-controlled genes revealed that rhamnolipid biosynthesis was required for swarm inhibition. This finding was intriguing, since the work presented here and the work of other groups have shown that rhamnolipids are surface-wetting agents required for swarming (14, 30). This posed the obvious question of how can rhamnolipids both promote and inhibit swarming? To address this question, we genetically dissected the rhamnolipid biosynthetic pathway. A previous publication by Deziel and colleagues showed that intermediates and the end product of rhamnolipid biosynthesis are found in the extracellular milieu of swarming cells and one such intermediate (HAAs) was sufficient for swarming (14). Similar results in *P. aeruginosa* PA14 were described here—the *rhlB* mutant makes HAAs but does not make rhamnolipids and is still capable of swarming (Fig. 4B).

The findings above prompted us to hypothesize that surface motility, while dependent upon HAAs, is not modulated by HAAs but via rhamnolipids. The altered swarm patterns produced by the *rhlB* and *rhlC* mutants supported our hypothesis. While both strains make HAAs and can swarm, the *rhlB* mutant does not make rhamnolipids and the *rhlC* mutants makes only monorhamnolipids. When swarming, these mutants differ from WT by not producing long, isolated tendrils radiating from a small core of cells. Instead, the *rhlB* and *rhlC* mutants produce a dense core of cells on swarm agar, and the tendrils appear to expand concomitantly with this core, resulting in colonization of regions between tendrils (Fig. 4B). These regions between the tendrils are typically sparsely populated in WT swarms. The *rhlB* mutant has been examined in swarming motility in a previous report, but the phenotype we report here was not identified in this previous publication because their medium conditions did not promote tendril formation (14). Therefore, our data show that tendril maintenance is modulated by rhamnolipid surfactants.

Further evidence supporting rhamnolipids as swarm modulators was acquired by adding spent supernatant to swarm agar (Fig. 4C). Spent supernatant from the *rhlB* mutant had no impact on swarming, thus eliminating HAAs as a factor involved in swarm modulation. However, spent supernatant from the WT or the *rhlC* mutant effectively blocked swarming, indicating that monorhamnolipids are the minimal factor required to observe this effect. These data are in agreement with the swarm phenotypes of rhamnolipid mutants. (i) The *rhlB* mutant makes no rhamnolipids, has the shortest tendrils, and lacks supernatant-associated inhibition. (ii) The *rhlC* mutant makes monorhamnolipids, produces few defined tendrils, and has supernatant-associated inhibition. These data suggest that monorhamnolipids can inhibit swarming, but modulation of this group behavior also requires dirhamnolipids because the tendrils formed by the monorhamnolipid-producing *rhlC* mutant are less defined than the tendrils produced by the WT strain (Fig. 4B). Thus, our data are consistent with three lines of evidence indicating distinct roles for HAAs versus rhamnolipids. (i) HAAs are the primary surface-wetting agent for swarming (Fig. 4B). (ii) Rhamnolipids, but not HAAs, modulate swarming behavior (Fig. 4C). (iii) HAAs, but not rhamnolipids, alter cell surface hydrophobicity (Fig. 4D).

On the basis of the data above, we propose a model for tendril production in swarming motility that requires rhamnolipid biosynthesis to coordinate group behavior. This is a new role for these surfactants. Our model does not explain the initial radiation of tendrils from the core, which may be stochastic and dependent on surface features of the agar not visible to the naked eye. Once tendrils emerge, we propose that secreted rhamnolipids form a gradient. As these molecules diffuse from neighboring tendrils, they will accumulate and lead to increased rhamnolipid concentrations between tendrils—the lowest concentrations of these compounds should be directly in front of advancing tendrils. This model explains why tendrils travel in a linear fashion until they approach another swarm, at which point the gradient of rhamnolipids is increased in front of the tendril tip, resulting in a direction change. Consistent with a rhamnolipid gradient controlling swarming are the data presented in Fig. 2B. In this experiment, a zone of rhamnolipids is established around the *flgK* strain, and diffusion principles predict that rhamnolipid concentrations should be highest at the colony edge and decrease with increasing distance from the colony. If this were true, our model would predict that cells would swarm down the gradient (away from the *flgK* colony) if spotted in the rhamnolipid zone, and indeed this is shown in Fig. 2B. Along the same principle, we believe adding spent supernatant to swarm agar inhibits swarming because no gradient is present, since rhamnolipids are uniformly present at high concentrations.

Rhamnolipids are also required for preserving cell-free regions in another form of group behavior, namely, channel maintenance between macrocolonies of cells during biofilm development (12). Previous studies have shown that the *sadB* mutant is required for biofilm formation, and in particular, a mutation in this gene renders the cell defective for the transitioning from reversible-to-irreversible attachment during biofilm development, resulting in an unstructured biofilm lacking fluid-filled channels (7). Because biofilm formation and swarming motility are forms of group behavior with apparently analogous roles for rhamnolipids in the maintenance of cell-free regions of the bacterial community and, furthermore, because the *sadB* mutant produces mature biofilms devoid of macrocolony structures requiring rhamnolipids, we tested the *sadB* mutant in swarming assays. The *sadB* mutant still swarms, but the pattern of swarming is more similar to the pattern of the *rhlB* or *rhlC* mutant (compare Fig. 4B and 5A) than to the pattern of the WT (Fig. 5A). The *sadB* mutant is not altered in swimming or twitching motility (7).

To our surprise, the *sadB* mutant did not change direction in response to approaching tendrils and was able to swarm through rhamnolipid zones and media supplemented with rhamnolipids, indicated by data shown in Fig. 5. However, the tendrils that the *sadB* mutant extended towards the *flgK* colony are shorter than those tendrils that radiate in the opposite direction (Fig. 5C) and indicate that other factors may be involved in responding to rhamnolipids or modulating swarming. We are currently investigating how SadB, which is a cytoplasmic protein (7), is involved in sensing rhamnolipids. For example, rhamnolipids could act as classic signaling molecules and bind to a cognate receptor, these molecules could have effects on the cell envelope due to their amphipathic properties and thus initiate a signaling cascade, or alternatively, rhamnolipids could exert their effects solely through altering physical properties of the cell surface. For example, rhamnolipids have been shown to extract lipopolysaccharides from the outer membranes of gram-negative bacteria (1) and thereby alter cell surface hydrophobicity and/or exposure of outer membrane proteins (54). Alternatively, if rhamnolipids were acting as signals through their effects on the cell surface, we might expect to find membrane proteins conferring swarming phenotypes similar to the phenotype of the *sadB* mutant—presumably these membrane proteins would sense rhamnolipids directly or, alternatively, the effects of these surfactants on the cell envelope. We also speculate that the membrane perturbations caused by rhamnolipid surfactants may be analogous to membrane perturbations that occur when cells come into contact with a surface, explaining why SadB is required for both biofilm formation and swarming motility. In the long term, our goal is to determine whether other swarming mutants with a *sadB*-like phenotype also have biofilm defects and to further

explore at what levels (if any) these forms of group behavior are related.

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