3-2-2007

Inverse Regulation of Biofilm Formation and Swarming Motility by Pseudomonas aeruginosa PA14

Nicky C. Caiazza
Dartmouth College

Judith H. Merritt

Kimberly M. Brothers
Dartmouth College

George A. O'Toole
Dartmouth College

Journal of Bacteriology, May 2007, p. 3603–3612 Vol. 189, No. 9
0021-9193/07/$08.00+0 doi:10.1128/JB.01685-06

Inverse Regulation of Biofilm Formation and Swarming Motility by *Pseudomonas aeruginosa* PA14[▽]

Nicky C. Caiazza,† Judith H. Merritt, Kimberly M. Brothers, and George A. O'Toole*
Department of Microbiology & Immunology, Rm. 505, Vail Building, Dartmouth Medical School, Hanover, New Hampshire 03755

Received 31 October 2006/Accepted 15 February 2007

We previously reported that SadB, a protein of unknown function, is required for an early step in biofilm formation by the opportunistic pathogen *Pseudomonas aeruginosa*. Here we report that a mutation in *sadB* also results in increased swarming compared to the wild-type strain. Our data are consistent with a model in which SadB inversely regulates biofilm formation and swarming motility via its ability both to modulate flagellar reversals in a viscosity-dependent fashion and to influence the production of the Pel exopolysaccharide. We also show that SadB is required to properly modulate flagellar reversal rates via chemotaxis cluster IV (CheIV cluster). Mutational analyses of two components of the CheIV cluster, the methyl-accepting chemotaxis protein PilJ and the PilJ demethylase ChpB, support a model wherein this chemotaxis cluster participates in the inverse regulation of biofilm formation and swarming motility. Epistasis analysis indicates that SadB functions upstream of the CheIV cluster. We propose that *P. aeruginosa* utilizes a SadB-dependent, chemotaxis-like regulatory pathway to inversely regulate two key surface behaviors, biofilm formation and swarming motility.

Pseudomonas aeruginosa is an important model organism for the study of gram-negative biofilm development, yet little is known about the molecular mechanisms underlying the initial events leading to the surface interactions that characterize the early steps in bacterial biofilm formation. Microscopic observations (23, 26, 40, 51) and genetic analyses (2) revealed two sequential events that lead to stable surface interactions. First, a bacterial cell pole contacts the surface in a process referred to as reversible attachment. This is a relatively unstable interaction, as reversibly attached bacteria can readily return to a planktonic existence. The second event is a transition from the polar association to one that is mediated by the long axis of the cell body, referred to as irreversible attachment. In *P. aeruginosa*, the only mutation known to block the transition from reversible to irreversible attachment is in the *sadB* gene (2).

Another key aspect of biofilm formation by *P. aeruginosa* is the production of an extracellular matrix. In pseudomonads, this matrix is thought to be comprised of exopolysaccharides (EPS), DNA, and protein (19). The biofilm matrix has typically been credited with structuring the mature biofilm (4). Studies have identified the *pel* and *psl* loci as two sets of genes predicted to be involved in the production of the polysaccharide component of the matrix required for biofilm maturation by *P. aeruginosa* on abiotic surfaces, although only the *pel* gene cluster is found in *P. aeruginosa* strain PA14 (7, 8, 15, 27), the focus of study in this report. Interestingly, recent studies suggest that the *pel* locus also plays a role in early biofilm formation. A *pel* mutant of *P. aeruginos*a PAK shows a strong attachment defect in a strain lacking type IV pili (48) and *P. aeruginos*a PAO1 with a mutation in the *psl* locus has a block in biofilm initiation (24).

Swarming motility, another surface behavior of *P. aeruginosa*, allows this microbe to move across surfaces (11). Swarming motility requires both a functional flagellum and the production of the surface-wetting agent rhamnolipid surfactant, but the mechanism by which *P. aeruginosa* propels itself across the surface has not been explored. Swarming motility can be distinguished from swimming motility in that swarming is required to move across a hydrated, viscous semisolid surface, while swimming allows movement through a relatively low-viscosity liquid environment. We have shown that *sadB* is also involved in modulating swarming motility in response to rhamnolipid surfactants (3).

Here we show that SadB participates in the inverse regulation of biofilm formation and swarming motility and requires chemotaxis cluster IV to mediate these effects. Our data are consistent with a model in which SadB inversely regulates these behaviors via its ability both to modulate flagellar reversals in a viscosity-dependent fashion and to influence the production of the Pel polysaccharide. We propose that *P. aeruginosa* inversely regulates biofilm formation and swarming motility upon transitioning to a surface lifestyle.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, media, and chemicals. Bacterial strains, plasmids and primers used in this study are shown in Table 1. *P. aeruginosa* PA14 was cultured on lysogeny broth (LB) medium (1) solidified with 1.5% agar. M63 minimal salts (35) medium supplemented with $MgSO_4$ (1 mM), glucose (0.2%), and (where indicated) Casamino Acids (CAA, 0.5%) was used for static biofilm assays. Swarm (0.5% and 0.55% agar) and swim (0.3% agar) plates consisted of M8 minimal medium (20) supplemented with $MgSO_4$ (1 mM), glucose (0.2%), and CAA (0.5%). Twitch plates consisted of LB medium solidified with 1.0% agar. Unless noted otherwise, antibiotics were used at the following concentrations for *P. aeruginosa*: carbenicillin, 500 μg/ml; tetracycline, 150 μg/ml, genta-

* Corresponding author. Mailing address: Dept. of Microbiology & Immunology, Rm. 505, Vail Building, Dartmouth Medical School, Hanover, NH 03755. Phone: (603) 650-1248. Fax: (603) 650-1245. E-mail: georgeo@Dartmouth.edu.
† Present address: Mascoma Corporation, Dartmouth Regional Technology Center, 16 Cavendish Court, Suite 2A, Lebanon, NH 03766.
▽ Published ahead of print on 2 March 2007.

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

Strain, plasmid, or primer	Relevant characteristics or sequence	Source or reference
P. aeruginosa PA14	Wild type	37
SMC705	PA14 *sadB*::Tn*5*B21; Tc^r	2
SMC257	PA14 *flgK*::Tn*5*B30; Tc^r	32
SMC252	PA14 *pilB*::Tn*5*B30; Tc^r	32
	PA14 Δ*pelA*	7
SMC478	PA14 *pilG*::Tn*5*B21; Tc^r	This study
SMC2992	PA14 Δ*pilJ*	This study
SMC2990	PA14 Δ*chpB*	This study
SMC3257	PA14 Δ*chpB pelA*::pMQ89	This study
SMC2958	PA14 PAO178::pKO3PAO178	This study
SMC2962	PA14 *wspA*::pKO3*wspA*	This study
SMC2949	PA14 *cheR1*::pKO3*cheR1*	This study
Plasmids		
pUCP18	Cloning vector, Cb^r Ap^r	41
pNC5 (pSadB$^+$)	*sadB* in pUCP18; Cb^r Ap^r	2
pMQ90	Cloning vector; Cb^r Ap^r	42
pChpB$^+$	*chpB* gene cloned in pMQ90	This study
pPilJ$^+$	*pilJ* gene cloned in pMQ90	This study
pMQ30	Suicide vector; Gm^r SacB$^+$ *URA3 CEN6/ARSH4*	42
pMQ30Δ*pilJ*	*pilJ* KO construct	This study
pMQ30Δ*chpB*	*chpB* KO construct	This study
pKO3	Suicide vector; Tc^r	30
pKO3-PAO178	PAO178 KO construct	This study
pKO3-*wspA*	*wspA* KO construct	This study
pKO3-*cheR1*	*cheR1* KO construct	This study
Primers		
*pilJ*KO1	5′-CAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTTGCACC ATGGCTCGTATTTTGATTGTTG-3′	
*pilJ*KO2	5′-CAGTTTGAAGCCGGATACCGAGCCCGCGAAAAGATTGCCTG-3′	
*pilJ*KO4	5′-TCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCCACTCC ATTCATGTGCCTGAG-3′	
*chpB*KO1	5′-CCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTTGGT GCTCGAGTCCGGTTTCTCC-3′	
*chpB*KO2	5′-CCAGATGCTTGACCAATGCCTCCAACGAGGTGTCGGCGATCAC-3′	
*chpB*KO3	5′-GTGATCGCCGACACCTCGTTGGAGGCATTGGTCAAGCATCTGG-3′	
*chpB*KO4	5′-CATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTGAA CTCGACGGAGGAAAGATCC-3′	
pilJ5′	5′-GGCGGAATTCAAGGAGCCAAATATGAAGAAAATCAACGC-3′	
pilJ3′	5′-GGCGAAGCTTCCTCGTCCAAGGTGTCGAC-3′	
chpB5′	5′-GGCGGAATTCCGGCCAGCATGAGTGAGC-3′	
chpB3′	5′-GGCGAAGCTTGTGTTCCGGCTCATGCCATC-3′	
PAO178KO1	5′-GCGGGGATCCCTCCAGGTGTTCTTCG-3′	
PAO178KO2	5′-GCGGGAATTCGGTGATCACCAGTTCG-3′	
*wspA*KO1	5′-GCGGCTGCAGGAACTGGACTGTTCG-3′	
*wspA*KO2	5′-GGCGAAGCTTCGTGACGAATTCGG-3′	
*cheR1*KO1	5′-GCGGGGATCCGGATTTCGAGTTGTTCAGG-3′	
*cheR1*KO2	5′-GCGGGAATTCCGAAATAGCGTTGCAGAC-3′	

[a] Ap, ampicillin; Cb, carbenicillin; Gm, gentamicin; Tc, tetracycline; KO, knockout.

micin, 100 μg/ml. All enzymes used for DNA manipulation were purchased from Invitrogen (Carlsbad, CA).

Molecular techniques. The DNA flanking the transposon carried by the *pilG*::Tn*5*B21 mutant was mapped to the *pilG* locus using the published *P. aeruginosa* PAO1 genome (45) and arbitrarily primed PCR, as previously described (34). In-frame deletions of *pilJ* and *chpB* were generated using pMQ30 (42), constructed as reported elsewhere (13), and the resolved integrants were confirmed by PCR. Single-crossover insertion of plasmid pKO3 (30) by homologous recombination was used to disrupt the PAO178, *wspA*, and *cheR1* genes. The constructs were made using the primers in Table 1 and confirmed by PCR. Plasmids for *chpB* and *pilJ* complementation were constructed in pMQ90 by amplifying *chpB* and *pilJ* by PCR using the primers listed in Table 1.

Quantitative reverse transcription PCR (qRT-PCR) was performed as previously reported (21). Bacteria were harvested either from static planktonic cultures incubated for 8 h (typical biofilm assay growth conditions) or on agar plates used for Congo red assays (see below) but lacking the dyes and solidified with 1.0% agar.

Biofilm assays. Ninety-six-well microtiter plate assays and microscopic analysis were performed as previously described (29, 33). To quantify reversible versus irreversible attachment, overnight cultures were normalized by optical density at 600 nm and diluted 1:100 in M63 supplemented with glucose, CAA, and $MgSO_4$. A 500-μl aliquot of this suspension was inoculated into the wells of a 24-well plate (in duplicate) and incubated for 1 h at 37°C. The medium was removed and gently replaced, and images were captured at two frames/second for 30 s. Images were converted to QuickTime files for analysis.

Determining swim reversals. Swim reversal rate measures the frequency at which a swimming cell changes its direction. Overnight LB-grown cultures were diluted 1:100 into fresh M63 medium supplemented with glucose. Ficoll was added at 3% for low-viscosity (swimming) conditions; this added Ficoll slowed the swimming cells sufficiently to facilitate monitoring of reversal rates. High-

viscosity conditions, mimicking conditions of swarming motility based on our previous studies (47), were achieved by adding Ficoll to 15%. Subcultured bacteria were incubated at 37°C for 1 to 2 h, and then 500-μl aliquots were added to the wells of 24-well plates. Phase-contrast, time-lapse images were acquired every 0.3 s at a ×1,400 magnification using the OpenLab software package. The time-lapse images were converted to QuickTime movies for subsequent analysis. QuickTime movies were advanced frame by frame, and individual cells were monitored for the number of times they reversed swim direction while within the field of view. Approximately 25 cells were counted in each of six wells (~125 cells total) to determine reversal rates, which are expressed as reversals per cell.

Swarm assays. Plate preparation, inoculation, and incubation were performed as previously described (47). To determine the percentage of swarm plate surface coverage by a given bacterial swarm, an image of the swarm plate was captured using a Nikon 990 digital camera (Nikon, Melville, NY) and false colored using Photoshop software (Adobe, Mountain View, CA) to provide ample contrast between the bacterial swarm and the agar surface so that the pixels could be counted using Kodak 1D Image Analysis software (Kodak, Rochester, NY). The number of pixels that comprised the swarm was expressed as a percentage of the number of pixels that comprised an image of the surface of the plate. Alternatively, the captured image was imported into PowerPoint (Microsoft), where the outline of the bacterial swarm was traced in order to distinguish it from the agar surface. ImageJ software (National Institutes of Health) was used to calculate both the area within the swarm and that of the plate surface for comparison.

Polysaccharide assays. Congo red (CR) assays were performed as reported elsewhere, except that the base medium used was M63 medium supplemented with glucose, $MgSO_4$, and CAA at the concentrations used for biofilm assays (7, 8). Scanning electron microscopy (SEM) was performed as reported elsewhere (16), except that glutaraldehyde was used at 2.5% and bacteria were grown on the plastic substrate for 38 h in minimal medium plus glucose and CAA.

RESULTS

Relationship of SadB to biofilm formation and swarming. We postulated that upon encountering a surface, *P. aeruginosa* would likely coregulate its surface-associated behaviors, including biofilm formation and swarming. The fact that SadB appeared to be required for both biofilm formation (2) and swarming motility (3) suggested that this protein might be involved in coregulating these processes; therefore, we pursued *sadB* both as a genetic link between these phenomena and to gain greater insight into how *P. aeruginosa* regulates its surface behaviors.

As part of the published characterization of SadB, we demonstrated that SadB protein levels were elevated under conditions that promote robust biofilm formation. These data suggested that there might be a correlation between the levels of SadB and the extent of biofilm formation. To test this hypothesis, we took advantage of the observation that expressing *sadB* in multicopy from a plasmid ($pSadB^+$) resulted in elevated cytoplasmic levels of the SadB protein (2). We examined the effects of overexpressing SadB from the $pSadB^+$ plasmid on biofilm formation in glucose minimal medium, a medium that does not promote robust biofilm formation by *P. aeruginosa* PA14. In a 96-well biofilm assay, biofilm formation was enhanced ~3-fold at 4 h in the wild type (WT) overexpressing SadB (WT/$pSadB^+$) in comparison to the vector control strain (WT/pUCP18) (Fig. 1A). The WT strain overexpressing SadB also showed a 2.5-fold increase in the number of bacteria attached to the surface in comparison to the vector control at this early time point, as determined by phase-contrast microscopy (Fig. 1B).

The *sadB* mutant also shows a hyperswarming phenotype compared to the WT, resulting in an ~2-fold increase in surface coverage for the *sadB* mutant (Fig. 1C). In contrast, overexpression of SadB leads to suppression of swarming motility on 0.55% agar but not on 0.5% agar, suggesting a viscosity-dependent role for SadB in swarm suppression (Fig. 1D). Together, these data suggest an inverse relationship between swarming motility and biofilm formation mediated, at least in part, by SadB.

A *sadB* mutant has a viscosity-dependent defect in flagellar reversals. Because both swarming motility and biofilm formation are dependent on a functional flagellum, we assayed the *sadB* mutant for phenotypes related to motility, in particular, swim speed and flagellar reversal rate. Directly measuring the speed of swimming under low-viscosity conditions (using 3% Ficoll [47]) revealed no difference between the WT (55.3 ± 1.83 μm/s) and the *sadB* mutant (53.38 ± 1.52 μm/s; $P = 0.37$), a result consistent with our previous findings (2). Because of the effects of a *sadB* mutation on swarming motility, we also measured swimming speed under high-viscosity conditions (15% Ficoll), which we have shown previously is a condition analogous to that encountered by the cells when swarming (47). Under high-viscosity conditions, a small but significant difference in swimming speed was measured between the WT (10.03 ± 0.59 μm/s) and the *sadB* mutant (12.29 ± 0.40 μm/s; $P = 0.003$), an increase of ~23% for the *sadB* mutant. It is formally possible that this small increase in swimming motility can also contribute to the enhanced swarming of the *sadB* mutant; however, there are no other data to support this conclusion.

Another component of controlling flagellar motility is regulating the rate of flagellar reversals; therefore, we also assessed the flagellar reversal rate of the WT and the *sadB* mutant (Fig. 1E). The rate of reversals under low-viscosity conditions (3% Ficoll) for the *sadB* mutant is equal to that of WT. Consistent with a role for SadB in the control of flagellar-mediated reversals, the *sadB* mutant displayed a >2-fold increase in flagellar reversals compared to WT cells in 15% Ficoll. Also under high-viscosity conditions (15% Ficoll), overexpression of SadB from plasmid pNC5 ($pSadB^+$) in the WT reduced reversals per cell to 0.52 ± 0.26 compared to 1.36 ± 0.04 for the WT carrying the pUCP18 vector control ($P = 0.035$). This two- to threefold change in reversal rates is on par with the magnitude of change in reversal rate observed for *Escherichia coli* in the presence of attractants and/or for mutants in the Che machinery (31, 36).

SadB-mediated effects on biofilm formation and swarming require components of chemotaxis cluster IV. Based on the viscosity-dependent alteration of flagellar reversals in the *sadB* mutant and the known role of the chemotaxis system of *E. coli* in the regulation of flagellar reversals (25), we hypothesized that one of the five chemotaxis-like clusters of *P. aeruginosa* (6) might serve as a link between SadB and flagellar function. We found that a Tn*5* insertion in *pilG*, a component of chemotaxis cluster IV (*cheIV*) of *P. aeruginosa*, resulted in a SadB-like swarming phenotype, but mutations in none of the other clusters yielded similar phenotypes (Table 2).

To confirm a role for the CheIV cluster in SadB-dependent effects on biofilm formation and swarming, in-frame deletions in two genes of the *cheIV* cluster (Fig. 2A), *pilJ*, encoding a predicted methyl-accepting chemotaxis protein (MCP), and *chpB*, encoding a predicted MCP demethylase, were constructed. We chose to mutate these genes based on previous work in *E. coli*—loss of the MCP should block signaling

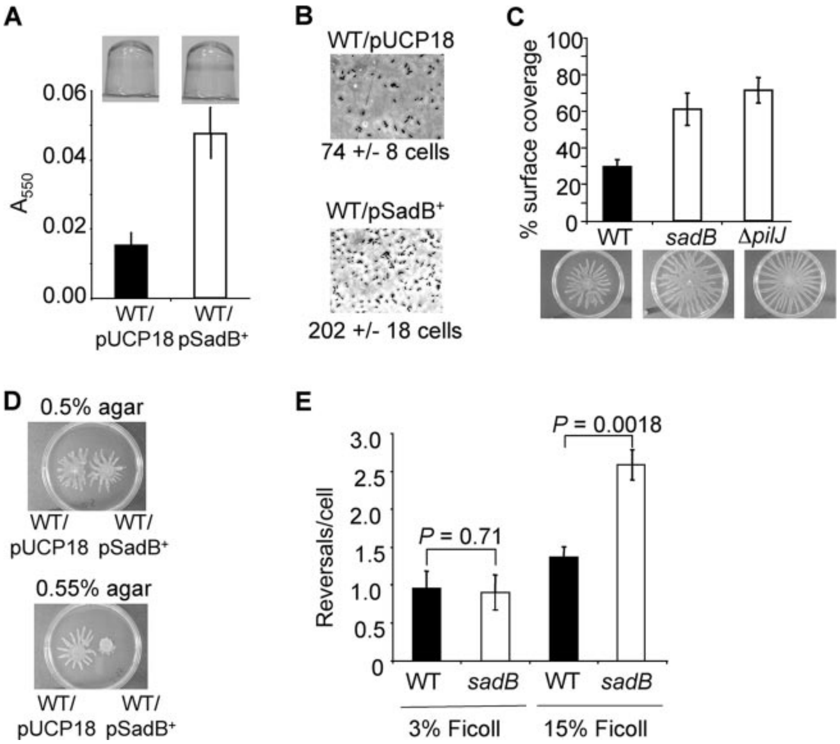

FIG. 1. Surface-associated behaviors influenced by SadB levels. (A) Biofilm formation phenotypes under static conditions. (Top) Image of crystal violet (CV)-stained biofilms formed by the wild type carrying the vector control (WT/pUCP18) and the wild type overexpressing SadB (WT/pSadB$^+$). Cells were grown in minimal medium containing (0.2%) glucose for 4 h at 37°C before staining with CV. (Bottom) Quantification of CV-stained wells. (B) Phase-contrast images of WT/pUCP18 and WT/pSadB$^+$ attached to the surface of a 24-well plate after incubation at 37°C for 1 h. For each strain, images were recorded at a magnification of ×1,400 over 10 fields of view, and the average number of surface-associated cells for each strain is indicated below the image. (C) The graph shows the average plate surface coverage that results from swarms produced by the WT, *sadB*, and Δ*pilJ* strains. Below are representative images of WT, *sadB*, and Δ*pilJ* strains after 16 h of incubation at 37°C. The percent surface coverage of the WT is significantly less than that of the *sadB* mutant ($P = 0.0029$) and the Δ*pilJ* mutant ($P = 0.000018$). (D) Aliquots of the wild type carrying the vector control (WT/pUCP18) and the wild type overexpressing SadB (WT/pSadB$^+$) were spotted on 0.5% and 0.55% swarm agar plates and incubated for 16 h at 37°C. (E) Reversal rates of WT and *sadB* mutant cells under low-viscosity (3% Ficoll) and high-viscosity (15% Ficoll) conditions.

through this chemotaxis-like system, while mutating the demethylase should result in hypermethylation of the MCP and thus presumably result in higher basal receptor activity (25). Therefore, we predicted that mutations in *pilJ* and *chpB* should have opposite effects on biofilm formation and swarming.

The Δ*pilJ* mutant is defective for biofilm formation (Fig. 2B) and displays a hyperswarming phenotype (Fig. 1C), and providing *pilJ* on a plasmid complements these phenotypes (Fig. 2B and C). The Δ*pilJ* mutant is also defective for twitching motility (Table 3). Furthermore, overexpressing SadB in the Δ*pilJ* mutant neither stimulates biofilm formation (data not shown) nor suppresses swarming motility (Fig. 2D and E), suggesting that PilJ is genetically downstream of SadB. Consistent with this hypothesis, expressing PilJ from a high-copy-

TABLE 2. SadB-dependent biofilm and swarming phenotypes of Che cluster mutants

Chemotaxis cluster	Mutation	Swarming phenotype	SadB biofilm stimulation[c,d]	SadB swarm repression[c,e]
	Δ*motAB*[a]	WT	N	N
I	Δ*motCD*	Nonswarming	N	ND
II	*PAO178*::pKO3PAO178	Decreased	Y	Y
III	*wspA*::pKO3*wspA*	WT	Y	Y
IV	*pilG*::Tn5B21	Hyperswarmer[b]	N	M
IV	Δ*pilJ*	Hyperswarmer[b]	N	N
IV	Δ*chpB*	Nonswarming	ND	ND
V	*cheR1*::pKO3*cheR1*	Decreased	Y	Y

[a] These genes are not in clusters CheI to CheV.
[b] As determined by a lack of avoidance of other bacterial swarms, as reported elsewhere (3).
[c] N, no; Y, yes; M, moderate; ND, not determined.
[d] Determined by testing whether SadB expressed from a plasmid stimulated biofilm formation (see Fig. 1A).
[e] Determined by testing whether SadB expressed from a plasmid repressed swarming motility on 0.55% agar (see Fig. 1D).

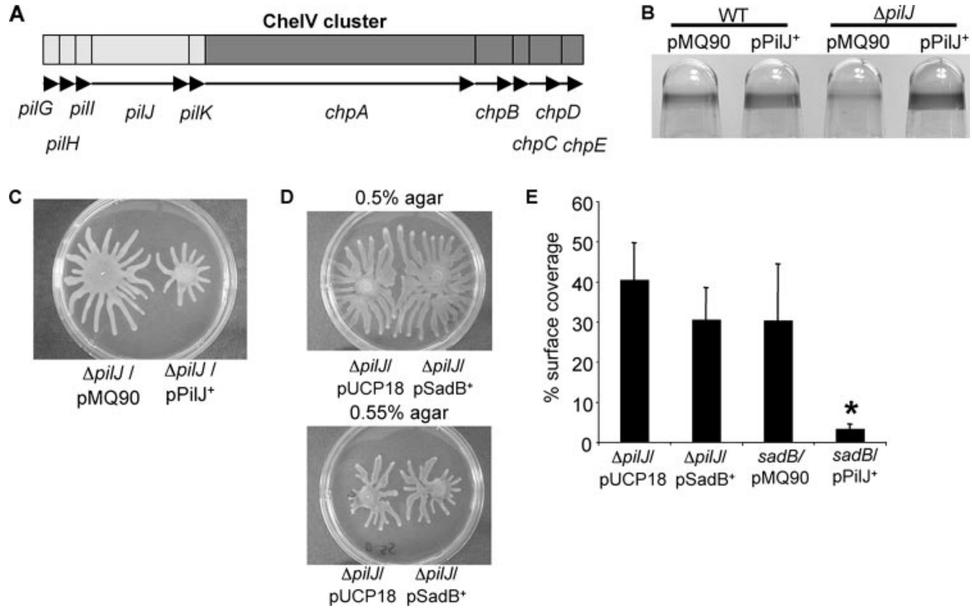

FIG. 2. The Δ*pilJ* mutant is biofilm defective and a hyperswarmer. (A) CheIV chemotaxis cluster. *pil* genes are shown in light gray and *chp* genes in dark gray. Black arrows represent open reading frames, and gene names are given below the arrows. (B) Biofilm formation by the WT, Δ*pilJ* mutant, and complemented strains at 8 h. Cells were grown at 37°C for 8 h in minimal medium containing glucose and CAA. (C) Aliquots of the Δ*pilJ* mutant carrying the vector control (Δ*pilJ*/pMQ90) or a construct expressing the *pilJ* gene in *trans* (pPilJ$^+$) were spotted on 0.5% swarm agar plates and incubated at 37°C for 16 h. (D) Aliquots of the Δ*pilJ* mutant carrying the vector control (Δ*pilJ*/pUCP18) or a construct overexpressing SadB (pSadB$^+$) were spotted on 0.5% and 0.55% swarm agar plates and incubated at 37°C for 16 h. (E) The extent of swarming, expressed as percent surface coverage, is shown on 0.5% agar for the strains indicated. *, statistically significant decrease in swarming compared to the other strains ($P < 0.001$).

number plasmid suppresses the swarming of the *sadB* mutant (Fig. 2E). In contrast to the Δ*pilJ* mutant, the Δ*chpB* mutant cannot swarm (Fig. 3A) but forms a more robust biofilm than the WT (Fig. 3B).

If the CheIV cluster is in the same genetic pathway as SadB, we predict that mutations in Δ*pilJ* and/or Δ*chpB* might cause altered flagellar reversal rates. Consistent with the observation that the Δ*pilJ* mutant and the *sadB* mutant have similar biofilm and swarming phenotypes, the Δ*pilJ* mutant also shows an ~2-fold increase in flagellar reversal rates compared to the WT under high-viscosity conditions; however, the reversal rate of the Δ*chpB* mutant is not significantly different from that of the WT (Fig. 3C).

TABLE 3. Swimming, twitching and swarming phenotypes[e]

Strain genotype	Zone (mm)		Swarming (% coverage)[c]
	Swim[a]	Twitch[b]	
WT	28.9 ± 1.9	14.3 ± 1	30
pilG::Tn5B21	29.5 ± 0.7	0	ND
Δ*pilJ*	37.0 ± 1.0	0	72
Δ*chpB*	22.0 ± 1.0	13.3 ± 0.4	<10
flgK::Tn5B30	0	ND	0
pilB::Tn5B21	ND	0	ND
sadB	ND[d]	ND[d]	63

[a] The diameter of the circular zone of motility formed on 0.3% agar plates after 24 h incubation at 37°C, as reported previously (32).
[b] The diameter of the circular zone of motility formed on 1.5% agar after 24 h incubation at 37°C, followed by 24 h at 25°C, as reported previously (32).
[c] Swarming is presented as the percentage of the agar plate covered, as reported previously (3).
[d] We reported previously that a *sadB* mutant has no swimming or twitching defect (2).
[e] ND, not determined.

Mutating *chpB* increases production of the *pel*-encoded matrix. We investigated whether the Δ*chpB* mutant is altered for other biofilm-related functions that might explain the hyperbiofilm and nonswarming phenotypes of this mutant. The Δ*chpB* mutant did not display any defects in swimming or twitching motility (Table 3). The Δ*chpB* mutant did show increased binding to CR compared to the WT (Fig. 3D, compare left and center panels). CR has been shown to bind the *pel*-encoded polysaccharide of *P. aeruginosa* PA14 (7, 8). Consistent with the conclusion that mutating *chpB* alters production of the Pel polysaccharide, introducing a *pelA* mutation into the Δ*chpB* genetic background completely eliminated CR binding (Fig. 3D).

The CR data were confirmed by SEM (Fig. 3E) using methods similar to those reported for the analysis of the Pel polysaccharide (7). The WT produced an amorphous material characteristic of extracellular polysaccharides, and consistent with the CR studies, the Δ*chpB* mutant produced more of this material (Fig. 3E, compare left and center panels).

To determine whether the Δ*chpB* mutant affected *pel* gene transcription, we measured the transcript levels of *pelA* and *pelG* using qRT-PCR. We chose to assess the expression of *pelA* and *pelG* because both of these genes are predicted to code for enzymes required to produce the glucose-rich polysaccharide component of the *P. aeruginosa* matrix (7). The WT and the Δ*chpB* mutant were grown either planktonically under static conditions (identical to conditions used for biofilm assays) or on agar plates under conditions identical to those used

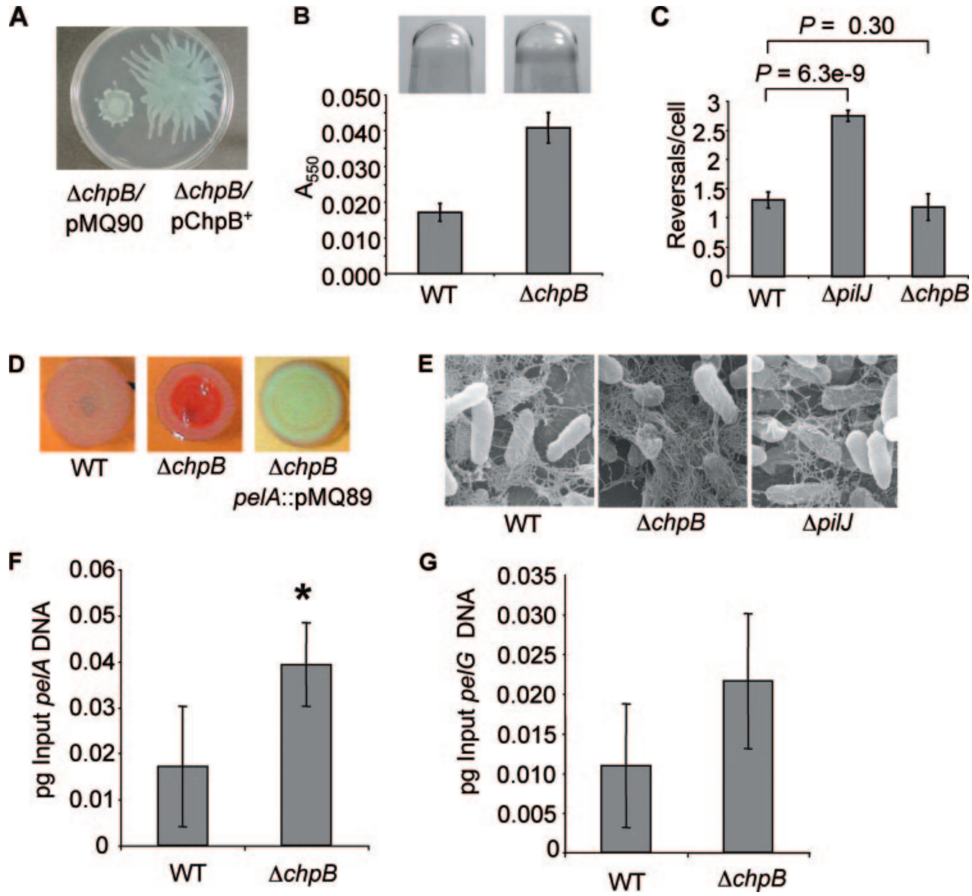

FIG. 3. The Δ*chpB* mutant is defective for swarming motility and displays increased biofilm formation and CR binding. (A) Aliquots of the Δ*chpB* mutant carrying the vector control (Δ*chpB*/pMQ90) and a construct expressing the *chpB* gene in *trans* (pChpB$^+$) were spotted on 0.5% swarm agar plates and incubated at 37°C for 16 h. (B) Biofilm formation by the WT and Δ*chpB* mutant was assessed after 4 h of growth at 37°C in minimal medium containing glucose. Shown are representative wells (top) and quantification of the biofilm assays (bottom). (C) Reversal rates of the WT and the Δ*pilJ* and Δ*chpB* mutants under high-viscosity (15% Ficoll) conditions. (D) CR assays with the WT, the Δ*chpB* mutant, and the Δ*chpB pelA* double mutant. Plates were incubated for 24 h at 37°C and an additional 24 h at room temperature. (E) SEM of WT and the Δ*chpB* and Δ*pilJ* mutants. Images were prepared with ruthenium red to highlight polysaccharides. (F and G) Quantitative RT-PCR analysis of *pelA* (F) and *pelG* (G) gene expression in agar-grown cultures of the indicated strains. *, statistically significant difference from the WT ($P < 0.05$).

for CR assays (minus the dyes). A small (~2-fold) but significant increase in *pelA* transcript, but no difference in *pelG* transcript level, was observed between the WT and Δ*chpB* mutant grown as a colony (Fig. 3F and G), and no difference was observed when the cultures were grown planktonically (data not shown). These data suggest that mutating *chpB* has little or no effect on the expression of the *pelA* and *pelG* genes.

The mutation in *pilJ* also results in a small decrease in CR staining and an altered colony morphology, but to a degree identical to that observed for a mutant lacking type IV pili (data not shown), suggesting that loss of PilJ function plays little or no role in EPS production. However, it may not be possible to observe subtle changes in EPS production using the CR assay. Consistent with the CR findings, SEM studies indicated that the WT and Δ*pilJ* mutant produced similar levels of extracellular material (Fig. 3E, right panel).

Role for *sadB* in Pel polysaccharide production. We predicted that the *sadB* mutant might also be altered for polysaccharide production. Given the lack of biofilm formation and increased swarming of the *sadB* mutant strain, phenotypes opposite those of the Δ*chpB* mutant, we predicted that the *sadB* mutant would bind less rather than more CR, and this is what we observed (Fig. 4A). Furthermore, SEM analysis revealed that the *sadB* mutant produced noticeably less extracellular matrix material than the WT (Fig. 4B). The Δ*pelA* mutant, defective in production of the Pel polysaccharide, served as a control in this study.

We determined whether the *sadB* mutant affected *pel* gene transcription. A small (~2-fold) but significant increase was observed for the *pelA* or *pelG* transcript level in the *sadB* mutant versus the WT grown under colony growth conditions (Fig. 4C and D), and no difference in transcript levels was observed under planktonic conditions (data not shown), indicating that the decrease in CR staining in the *sadB* mutant cannot be explained by decreased transcription of the *pel* locus.

Role for the *pel* locus in swarming and early biofilm formation. Given the apparent relationship between swarming and CR binding described above, we also determined whether a mutation in *pelA* might impact swarming motility. A strain mutated in the *pelA* gene showed a ~2.5-fold increase in swarming motility com-

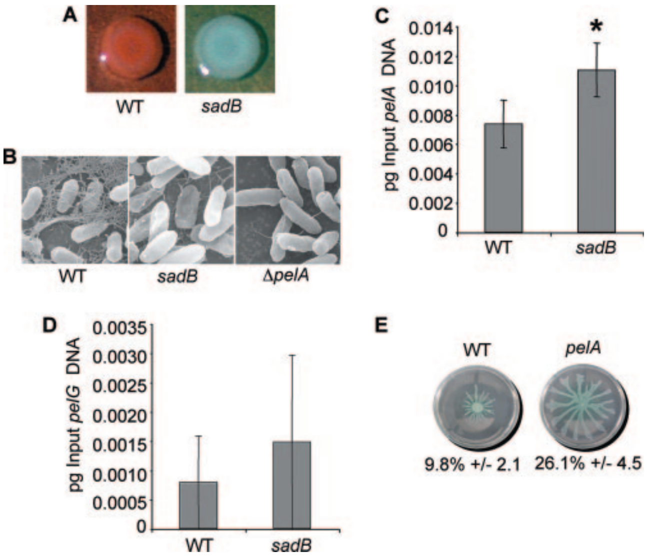

FIG. 4. A *sadB* mutant displays decreased Pel polysaccharide production but no decrease in *pel* gene expression. (A) CR binding assays with the WT and the *sadB* mutant. Plates were incubated for 24 h at 37°C and an additional 24 h at room temperature. (B) SEM of the indicated strains. Images were prepared with ruthenium red to highlight polysaccharides. (C and D) Quantitative RT-PCR analysis of *pelA* (C) and *pelG* (D) gene expression in agar-grown cultures of the indicated strains. *, statistically significant difference from the WT ($P < 0.05$). (E) Swarming phenotype of the WT and the Δ*pelA* mutant. Shown are a representative swarm plate for each strain and quantification of plate coverage for each strain (five plates).

pared to the WT strain (Fig. 4E). A mutation in the *pelA* gene does not impact swimming or twitching motility (data not shown).

Several mutations described above impact both biofilm formation and motility, and furthermore, *sadB* is known to impact biofilm formation at the transition from reversible to irreversible attachment. If the *pel*-encoded matrix also plays a role at this early step in biofilm formation, we would predict that a strain defective for matrix production would also have a defect in irreversible attachment. Consistent with this prediction, in a static assay, we observed a statistically significant decrease in irreversible attachment of the Δ*pelA* mutant (86.1% ± 3.2%) compared to the WT (94.7% ± 4.2%; $P = 0.0000583$). The decrease in irreversible attachment of the *pelA* strain is not as striking as that observed for the *sadB* mutant (67.8% ± 8.1%; $P = 0.0001417$).

DISCUSSION

Here we show that SadB, originally identified as required for early biofilm formation, is also a negative effector of swarming motility, a result consistent with our previous findings (3). We also showed previously that RpoN and FleR, known regulators of flagellum and rhamnolipid production in *P. aeruginosa* (14, 38, 46), also regulate SadB levels (2), suggesting that SadB is coregulated with other functions required for swarming and biofilm formation. How does SadB contribute to both biofilm formation and swarming behaviors? A model summarizing the findings from this study is shown in Fig. 5. While we have yet to identify the biochemical function of the SadB protein, our results implicate this protein in two pathways that impact swarming motility and biofilm formation.

First, SadB is involved in mediating flagellar reversals, but only under high-viscosity conditions likely similar to those encountered during either biofilm formation or swarming, but not swimming. A role for the chemotaxis cluster in *E. coli* in controlling flagellar reversal rates prompted us to investigate the potential involvement of the five chemosensory-like clusters of *P. aeruginosa* as a mechanism for linking SadB to flagellar function. An in-frame deletion of *pilJ*, an MCP homolog, rendered the strain biofilm defective and a hyperswarmer and resulted in increased flagellar reversals. The biofilm, swarming and flagellar reversal phenotypes of the Δ*pilJ* mutant are identical to those of a *sadB* mutant strain. Epistasis analysis indicates that SadB is genetically upstream of *pilJ*, consistent with a model wherein *sadB* exerts its effects on flagellar rotation via the CheIV chemosensory system.

Based on the *E. coli* paradigm (25), loss of the PilJ MCP should block signaling via this chemosensory system. In contrast, mutating the demethylase gene homolog, *chpB*, should result in a hypermethylated MCP with higher basal receptor activity. Consistent with these hypotheses, the Δ*chpB* mutant is defective for swarming but forms a more robust biofilm than

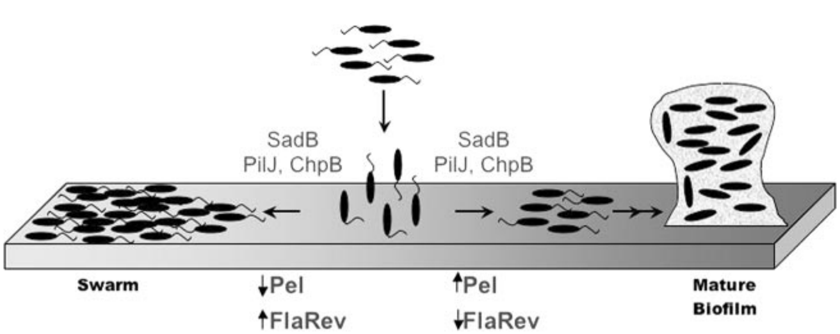

FIG. 5. Model for inverse control of biofilm formation and swarming motility. Planktonic bacteria (top) initially interact with the surface, likely via reversible polar attachment, although which end of the cell interacts with the abiotic surface is unclear. A pathway that includes SadB and components of the CheIV chemotaxis cluster (PilJ and ChpB) controls the decision to initiate biofilm formation or move via swarming. Biofilm formation is associated with an increase in the production of Pel-dependent polysaccharide (Pel) and a decrease in flagellar reversal rate (FlaRev). Swarming is associated with decreased production of polysaccharide and an increased rate of flagellar reversals. The substratum is shown as a gradient of gray to represent the fact that variations in surface properties (viscosity, wetness, etc.) might also impact the biofilm-versus-swarming decision.

the WT, phenotypes opposite those observed for the Δ*pilJ* mutant.

While mutations in *sadB* and *pilJ* resulted in increased flagellar reversals, to our surprise the Δ*chpB* mutant did not have the predicted decrease in reversals and in fact showed no discernible effect on this behavior. Perhaps the repression of flagellar reversals is accomplished via another pathway. Alternatively, while the Δ*chpB* mutation alters the basal activity of the MCP, perhaps a second input signal is still required to observe decreased reversals in this mutant background. We do show here that mutations in *chpB* impact another known biofilm-related factor, namely, the production of the putative *pel*-encoded matrix. A Δ*chpB* mutant has a CR-hyperbinding phenotype that is *pelA* dependent and results in increased matrix production as judged by SEM, but the Δ*chpB* mutation does not alter *pel* gene expression, suggesting that this mutation increased production of the Pel polysaccharide by a nontranscriptional mechanism. In contrast to the Δ*chpB* mutant, the *sadB* mutant showed decreased CR binding and matrix production, suggesting that SadB positively impacts production of the Pel polysaccharide. Despite the decrease in apparent production of the Pel polysaccharide by the *sadB* mutant, expression of the *pelA* and *pelG* mRNAs is slightly up-regulated in the *sadB* mutant versus the WT. These data indicate that the reduction of Pel polysaccharide production in a *sadB* mutant occurs via a nontranscriptional mechanism.

At this point, we do not understand how SadB or components of the CheIV cluster impact EPS production. Given the lack of change in *pel* gene expression in the *sadB* and *chpB* mutants, one obvious explanation for the changes in matrix production in strains mutated in these functions is that Pel production is controlled by a mechanism other than regulation of *pel* operon gene expression. To date, the only known means of nontranscriptional regulation of EPS production in pseudomonads is thought to be via the nucleotide signaling molecule c-di-GMP (10, 12, 17, 22, 44). However, there are no proteins with known c-di-GMP-related motifs in the CheIV cluster or in the CheI, CheII, or CheV cluster. The WspR protein, a component of the *wsp* chemosensory system (CheIII cluster) of *P. aeruginosa*, which plays a role in biofilm formation and EPS production, contains a GGDEF domain, an amino acid motif associated with the synthesis of c-di-GMP from GTP, and has been shown in vitro to catalyze c-di-GMP synthesis (12), but mutations in *wspR* do not yield SadB-like phenotypes. Furthermore, SadB lacks the EAL, GGDEF, and HD-GYP domains (39, 44) associated with c-di-GMP metabolism, and we have no biochemical evidence that SadB is involved in c-di-GMP metabolism (data not shown), nor does it appear to alter cellular pools of c-di-GMP (J. Hickman, J. Merritt, C. Harwood, and G. O'Toole, unpublished data). Therefore, the mechanism by which SadB and ChpB modulate Pel polysaccharide production remains to be elucidated.

Components of the CheIV cluster, including *pilJ* and the previously described *chpA* (49), also play a role in twitching motility, indicating that this putative chemosensory system participates in coordinating all three known surface behaviors of this microbe. We also showed that mutations in *pilJ* and *chpB* had no effect on swimming motility, further reinforcing a role for the CheIV cluster specifically in surface-associated behaviors of this microbe.

Our data suggest that SadB and PilJ modulate flagellar reversals under high-viscosity conditions but do not contribute to the control of flagellar reversals under the low-viscosity conditions that favor swimming. We hypothesize that SadB-dependent control of flagellar reversals upon polar, reversible attachment to a solid surface might decrease rotation about the cell pole and thus increase the time of interaction between the bacterium and its substratum, thereby promoting biofilm formation. In contrast, increased reversal rates appear to favor swarming motility. Wolfe and Berg postulated that for *E. coli*, increasing flagellar reversals might in some circumstances facilitate the ability to move through a semisolid matrix (50). Based on their microscopic observations, they concluded that "cells that do not tumble tend to get trapped in agar" and move less efficiently through this matrix (50). While that study was performed in the context of swimming through 0.3% agar, our data suggest that this phenomenon might also be extended to swarming conditions. Also consistent with our data is the finding that *E. coli* strains locked in the "tumbling" chemotaxis mode by mutation had a reduced ability to attach to an abiotic surface compared to the WT or mutants locked in the "running" mode (28). In addition to controlling flagellar function, by coordinating the production of the Pel polysaccharide, SadB can modulate another facet of biofilm initiation and swarming. Work presented here and recent published studies (24, 48) show that a functional *pel* locus contributes to early biofilm formation, and we show here that mutating the *pel* locus promotes swarming motility (Fig. 4D). This inverse relationship between polysaccharide production and motility has been noted in several other studies of *P. aeruginosa* (5, 9, 18, 43).

Our studies may provide a mechanistic basis for a recent exciting report by Shrout and colleagues (43). They proposed that early in biofilm formation, the extent of swarming motility helps dictate the final structure of the biofilm. That is, under conditions that promote swarming early in biofilm formation, the resulting mature biofilm is flat, while under conditions inhibitory to swarming motility, a biofilm with aggregates (distinct macrocolonies) will result. Based on their experimental work and accompanying mathematical simulations, they also postulated a role for a polysaccharide-containing matrix in the formation of the aggregates during biofilm development (43). One interpretation of the study of Shrout et al. is that *P. aeruginosa* must be able to integrate several important cell functions early in biofilm formation, namely, swarming motility and matrix production. The data presented in our report suggest that SadB and the CheIV cluster provide a molecular means for coregulating these functions.

We propose that *P. aeruginosa* inversely regulates the surface-associated behaviors of biofilm formation and swarming by controlling both flagellar reversals and the production of the Pel polysaccharide. Flagellar reversal rates in *E. coli* are largely regulated nontranscriptionally via the Che signal transduction pathway (25), and given the high sequence similarity of the *cheIV* cluster components to their *E. coli* counterparts, this is likely also the case in *P. aeruginosa*. CR binding and SEM data, together with the *pelA* and *pelG* transcriptional analysis presented here, indicate that production of the *pel*-encoded EPS may also be controlled by SadB and the CheIV cluster via a mechanism other than transcriptional control. Given that PilJ is also involved in twitching motility, the CheIV cluster may

coordinate three different surface behaviors: swarming motility, twitching motility and biofilm formation. An appealing aspect of this regulatory strategy for coregulating surface behaviors is that in adapting to a surface-associated lifestyle, *P. aeruginosa* may be able to seamlessly and rapidly transition among its surface behaviors as it encounters ever-changing substratum properties and environmental conditions.

ACKNOWLEDGMENTS

We thank C. Daghlian and the Ripple Electron Microscopy facility at Dartmouth College for assistance with the SEM studies.

This work was supported by NIH training grant T32 AI007519 (predoctoral fellowship) to N.C.C., predoctoral fellowship T32 GM08704 to J.H.M., and grant AI51360-01 to G.A.O.

REFERENCES

1. **Bertani, G.** 2004. Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. J. Bacteriol. **186:**595–600.
2. **Caiazza, N. C., and G. A. O'Toole.** 2004. SadB is required for the transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas aeruginosa* PA14. J. Bacteriol. **186:**4476–4485.
3. **Caiazza, N. C., R. M. Shanks, and G. A. O'Toole.** 2005. Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. J. Bacteriol. **187:**7351–7361.
4. **Davey, M. E., and G. A. O'Toole.** 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. **64:**847–867.
5. **Deziel, E., Y. Comeau, and R. Villemur.** 2001. Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. J. Bacteriol. **183:**1195–1204.
6. **Ferrandez, A., A. C. Hawkins, D. T. Summerfield, and C. S. Harwood.** 2002. Cluster II *che* genes from *Pseudomonas aeruginosa* are required for an optimal chemotactic response. J. Bacteriol. **184:**4374–4383.
7. **Friedman, L., and R. Kolter.** 2004. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. Mol. Microbiol. **51:**675–690.
8. **Friedman, L., and R. Kolter.** 2004. Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. J. Bacteriol. **186:**4457–4465.
9. **Garrett, E. S., D. Perlegas, and D. J. Wozniak.** 1999. Negative control of flagellum synthesis in *Pseudomonas aeruginosa* is modulated by the alternative sigma factor AlgT (AlgU). J. Bacteriol. **181:**7401–7404.
10. **Gjermansen, M., P. Ragas, C. Sternberg, S. Molin, and T. Tolker-Nielsen.** 2005. Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms. Environ. Microbiol. **7:**894–906.
11. **Harshey, R. M.** 1994. Bees aren't the only ones: swarming in gram-negative bacteria. Mol. Microbiol. **13:**389–394.
12. **Hickman, J. W., D. F. Tifrea, and C. S. Harwood.** 2005. A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proc. Natl. Acad. Sci. USA **102:**14422–14427.
13. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-*FRT* recombination system for site specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86.
14. **Ishimoto, K. S., and S. Lory.** 1989. Formation of pilin in *Pseudomonas aeruginosa* requires the alternative sigma factor (RpoN) of RNA polymerase. Proc. Natl. Acad. Sci. USA **86:**1954–1957.
15. **Jackson, K. D., M. Starkey, S. Kremer, M. R. Parsek, and D. J. Wozniak.** 2004. Identification of *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. J. Bacteriol. **186:**4466–4475.
16. **Kadouri, D., and G. A. O'Toole.** 2005. Susceptibility of biofilms to *Bdellovibrio bacteriovorus* attack. Appl. Environ. Microbiol. **71:**4044–4051.
17. **Kazmierczak, B. I., M. B. Lebron, and T. S. Murray.** 2006. Analysis of FimX, a phosphodiesterase that governs twitching motility in *Pseudomonas aeruginosa*. Mol. Microbiol. **60:**1026–1043.
18. **Kirisits, M. J., L. Prost, M. Starkey, and M. R. Parsek.** 2005. Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. Appl. Environ. Microbiol. **71:**4809–4821.
19. **Klausen, M., M. Gjermansen, J. U. Kreft, and T. Tolker-Nielsen.** 2006. Dynamics of development and dispersal in sessile microbial communities: examples from *Pseudomonas aeruginosa* and *Pseudomonas putida* model biofilms. FEMS Microbiol. Lett. **261:**1–11.
20. **Kohler, T., L. K. Curty, F. Barja, C. van Delden, and J. C. Pechere.** 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol. **182:**5990–5996.
21. **Kuchma, S. L., J. P. Connolly, and G. A. O'Toole.** 2005. A three-component regulatory system regulates biofilm maturation and type III secretion in *Pseudomonas aeruginosa*. J. Bacteriol. **187:**1441–1454.
22. **Kulasakara, H., V. Lee, A. Brencic, N. Liberati, J. Urbach, S. Miyata, D. G. Lee, A. N. Neely, M. Hyodo, Y. Hayakawa, F. M. Ausubel, and S. Lory.** 2006. Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3′–5′)-cyclic-GMP in virulence. Proc. Natl. Acad. Sci. USA **103:**2839–2844.
23. **Lawrence, J. R., P. J. Delaquis, D. R. Korber, and D. E. Caldwell.** 1987. Behavior of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. **14:**1–14.
24. **Ma, L., K. D. Jackson, R. M. Landry, M. R. Parsek, and D. J. Wozniak.** 2006. Analysis of *Pseudomonas aeruginosa* conditional Psl variants reveals roles for the Psl polysaccharide in adhesion and maintaining biofilm structure postattachment. J. Bacteriol. **188:**8213–8221.
25. **Macnab, R. M.** 1996. Flagella and motility, p. 123–145. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, DC.
26. **Marshall, K. C., R. Stout, and R. Mitchell.** 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. J. Gen. Microbiol. **68:**337–348.
27. **Matsukawa, M., and E. P. Greenberg.** 2004. Putative exopolysaccharide synthesis genes influence *Pseudomonas aeruginosa* biofilm development. J. Bacteriol. **186:**4449–4456.
28. **McClaine, J. W., and R. M. Ford.** 2002. Reversal of flagellar rotation is important in initial attachment of *Escherichia coli* to glass in a dynamic system with high- and low-ionic-strength buffers. Appl. Environ. Microbiol. **68:**1280–1289.
29. **Merritt, J. H., D. E. Kadouri, and G. A. O'Toole.** 2005. Growing and analyzing static biofilms, p. 1B.1.1–1B.1.17. *In* R. Coico, T. Kowalik, J. Quarles, B. Stevenson, and R. Taylor (ed.), Current protocols in microbiology. J. Wiley & Sons, Hoboken, NJ.
30. **Monds, R. D., P. D. Newell, J. A. Schwartzman, and G. A. O'Toole.** 2006. Conservation of the Pho regulon in *Pseudomonas fluorescens* Pf0-1. Appl. Environ. Microbiol. **72:**1910–1924.
31. **Montrone, M., M. Eisenbach, D. Oesterhelt, and W. Marwan.** 1998. Regulation of switching frequency and bias of the bacterial flagellar motor by CheY and fumarate. J. Bacteriol. **180:**3375–3380.
32. **O'Toole, G. A., and R. Kolter.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. **30:**295–304.
33. **O'Toole, G. A., and R. Kolter.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. **28:**449–461.
34. **O'Toole, G. A., L. A. Pratt, P. I. Watnick, D. K. Newman, V. B. Weaver, and R. Kolter.** 1999. Genetic approaches to the study of biofilms. Methods Enzymol. **310:**91–109.
35. **Pardee, A. B., F. Jacob, and J. Monod.** 1959. The genetic control and cytoplasmic expression of "inducibility" in the synthesis of β-galactosidase in *E. coli*. J. Mol. Biol. **1:**165–178.
36. **Prasad, K., S. R. Caplan, and M. Eisenbach.** 1998. Fumarate modulates bacterial flagellar rotation by lowering the free energy difference between the clockwise and counterclockwise states of the motor. J. Mol. Biol. **280:**821–828.
37. **Rahme, L. G., E. J. Stevens, S. F. Wolfort, J. Shao, R. G. Tompkins, and F. M. Ausubel.** 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science **268:**1899–1902.
38. **Ritchings, B. W., E. C. Almira, S. Lory, and R. Ramphal.** 1995. Cloning and characterization of *fleS* and *fleR*, new response regulators of *Pseudomonas aeruginosa* which regulate motility and adhesion to mucin. Infect. Immun. **63:**4868–4876.
39. **Ryan, R. P., Y. Fouhy, J. F. Lucey, L. C. Crossman, S. Spiro, Y. W. He, L. H. Zhang, S. Heeb, M. Camara, P. Williams, and J. M. Dow.** 2006. Cell-cell signaling in *Xanthomonas campestris* involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. Proc. Natl. Acad. Sci. USA **103:**6712–6717.
40. **Sauer, K., A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies.** 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J. Bacteriol. **184:**1140–1154.
41. **Schweizer, H. P.** 1991. *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. Gene **103:**109–112.
42. **Shanks, R. M., N. C. Caiazza, S. M. Hinsa, C. M. Toutain, and G. A. O'Toole.** 2006. *Saccharomyces cerevisiae*-based molecular tool kit for manipulation of genes from gram-negative bacteria. Appl. Environ. Microbiol. **72:**5027–5036.
43. **Shrout, J. D., D. L. Chopp, C. L. Just, M. Hentzer, M. Givskov, and M. R. Parsek.** 2006. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. Mol. Microbiol. **62:**1264–1277.
44. **Simm, R., M. Morr, A. Kader, M. Nimtz, and U. Romling.** 2004. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. Mol. Microbiol. **53:**1123–1134.
45. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L.**

Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, and I. T. Paulsen. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406:**959–964.

46. **Totten, P. A., J. C. Lara, and S. Lory.** 1990. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J. Bacteriol. **172:**389–396.
47. **Toutain, C. M., M. E. Zegans, and G. A. O'Toole.** 2005. Evidence for two flagellar stators and their role in the motility of *Pseudomonas aeruginosa*. J. Bacteriol. **187:**771–777.
48. **Vasseur, P., I. Vallet-Gely, C. Soscia, S. Genin, and A. Filloux.** 2005. The pel genes of the *Pseudomonas aeruginosa* PAK strain are involved at early and late stages of biofilm formation. Microbiology **151:**985–997.
49. **Whitchurch, C. B., A. J. Leech, M. D. Young, D. Kennedy, J. L. Sargent, J. J. Bertrand, A. B. Semmler, A. S. Mellick, P. R. Martin, R. A. Alm, M. Hobbs, S. A. Beatson, B. Huang, L. Nguyen, J. C. Commolli, J. N. Engel, A. Darzins, and J. S. Mattick.** 2004. Characterization of a complex chemosensory signal transduction system which controls twitching motility in *Pseudomonas aeruginosa*. Mol. Microbiol. **52:**873–893.
50. **Wolfe, A. J., and H. C. Berg.** 1989. Migration of bacteria in semisolid agar. Proc. Natl. Acad. Sci. USA **86:**6973–6977.
51. **Zobell, C. E.** 1943. The effects of solid surfaces upon bacterial activity. J. Bacteriol. **46:**39–56.