A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels

Jason W. Hickman*†, Delia F. Tifrea†, and Caroline S. Harwood*‡

*Department of Microbiology, Box 357242, University of Washington, 1959 Northeast Pacific Street, Seattle, WA 98195; and †Department of Microbiology, University of Iowa, 51 Newton Road, Iowa City, IA 52240

Communicated by E. Peter Greenberg, University of Washington School of Medicine, Seattle, WA, August 17, 2005 (received for review May 21, 2005)

Pseudomonas aeruginosa **causes chronic biofilm infections, and its ability to attach to surfaces and other cells is important for biofilm formation and maintenance. Mutations in a gene called *wspF*, part of a putative chemosensory signal–transduction operon, have been shown to result in cell aggregation and altered colony morphology. The WspF phenotypes depend on the presence of WspR, which is a member of a family of signal transduction proteins known as response regulators. It is likely that the effect of the *wspF* mutation is to cause constitutive activation of WspR by phosphorylation. WspR contains a GGDEF domain known to catalyze formation of a cytoplasmic signaling molecule cyclic diguanylate (c-diGMP). We determined that purified WspR catalyzed the formation of c-diGMP *in vitro* and phosphorylation stimulated this activity. We observed increased cellular levels of c-diGMP and increased biofilm formation in a *wspF* mutant. Expression of a protein predicted to catalyze degradation of c-diGMP reversed the phenotypes of a *wspF* mutant and inhibited biofilm initiation by wild-type cells, indicating that the presence of c-diGMP is necessary for biofilm formation. A transcriptome analysis showed that expression levels of at least 560 genes were affected by a *wspF* deletion. The *psl* and *pel* operons, which are involved in exopolysaccharide production and biofilm formation, were expressed at high levels in a *wspF* mutant. Together, the data suggest that the *wsp* signal transduction pathway regulates biofilm formation through modulation of cyclic diguanylate levels.**

Pseudomonas aeruginosa | GGDEF domain | exopolysaccharide | EAL domain

Biofilms are surface-associated multicellular communities encased in a self-produced extracellular matrix. Existence in a biofilm provides many advantages over a planktonic (single-cell) existence, including increased resistance to predation and antimicrobial agents (1–4). Biofilms of *P. aeruginosa* cause chronic infections of humans with underlying predispositions (5).

A number of genes involved in adherence and aggregation of *P. aeruginosa*, properties that are thought to be important during biofilm formation, have been identified (6–10). A mutation in one such gene, *wspF*, has been shown to result in increased cell aggregation and a wrinkled colony morphology (7). *wspF* is part of a gene cluster predicted to encode a signal transduction system similar to that which regulates swimming-mediated chemotaxis in bacteria (7). The WspF protein is homologous to CheB, a methylesterase involved in adaptation to chemotactic stimuli. Chemotaxis signal transduction systems also include response regulators called CheY. The response regulator of the Wsp chemosensory system, WspR, is a CheY homolog that contains the conserved GGDEF domain (7). These domains have been implicated in the formation of the intracellular signaling molecule cyclic diguanylate (c-diGMP) (11–15).

c-diGMP was first identified in *Gluconacetobacter xylinus*, where it regulates production of cellulose through modulation of cellulose synthase activity (16). More recent work has indicated that increased levels of c-diGMP in a variety of bacteria, including *P. aeruginosa*, are correlated with increased cell aggregation and surface attachment (17, 18). However, the factors that regulate c-diGMP levels and the modes of action of c-diGMP in *P. aeruginosa* are unknown. Here we describe a role for the Wsp chemosensory system in regulating biofilm formation through modulation of c-diGMP levels. We demonstrate that loss of *wspF* results in increased cellular c-diGMP, possibly by stimulating WspR-P catalyzed c-diGMP synthesis. We also show that increased levels of c-diGMP result in enhanced biofilm formation and that cells with decreased levels of c-diGMP are defective in biofim initiation. We present evidence that intracellular levels of c-diGMP control expression of genes involved in production of exopolysaccharide (EPS) in *P. aeruginosa*.

Materials and Methods

Strains, Plasmids, and Growth Conditions. The bacterial strains and plasmids used in this study are listed in Table 2, which is published as supporting information on the PNAS web site. The sequences of primers used are available upon request. All *Escherichia coli* and *P. aeruginosa* strains were grown in LB broth at 37°C unless otherwise noted. Colony morphology was visualized after growth on tryptone agar containing 40 mg of Congo red and 10 mg of Coomassie brilliant blue per liter of medium (19). Antibiotics were added at the following concentrations where appropriate: 300 μg/ml carbenicillin and 100 μg/ml gentamycin for *P. aeruginosa*, and 100 μg/ml ampicillin and 10 μg/ml gentamycin for *E. coli*.

Construction of In-Frame Deletion Mutants and Plasmids. In-frame deletions of *wspA*, *wspE*, *wspF*, *wspR*, and the double deletion *wspFR* (Fig. 1) were constructed by using overlap extension PCR as described (20–22). The resulting PCR products were cloned into the *P. aeruginosa* suicide vector pEX19Gm (23). The resulting plasmids were mated into *P. aeruginosa* from *E. coli* S17–1 and recombinants were obtained as described (20). Mutant strains were confirmed by PCR analysis of chromosomal DNA.

The cloning vector pJH1Gm was constructed from the broad-host range vector pCM62 (24). All regions of pCM62 except those necessary for tetracycline resistance were PCR amplified. The resulting DNA fragment was blunt-end ligated to a cassette encoding gentamicin resistance obtained from pUCGM (25). The cloning junctions of the resulting vector were sequenced to confirm the plasmid construction. To generate a construct for complementation of the *wspF* deletion, a PCR-amplified *wspF* gene was cloned into pJH1Gm to create pWspF1, which was transferred into *P. aeruginosa* strains from *E. coli* S17–1 by conjugation. To generate a construct for expression of PA2133

Freely available online through the PNAS open access option.

Abbreviations: c-diGMP, cyclic diguanylate; EPS, exopolysaccharide.

‡To whom correspondence should be addressed. E-mail: csh5@u.washington.edu.

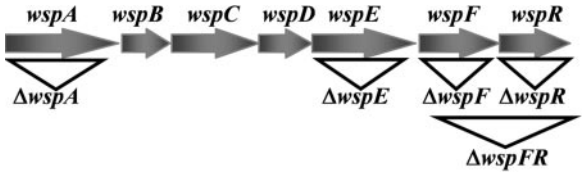

Fig. 1. Organization of genes encoding the Wsp chemosensory system in *P. aeruginosa*. Gene names are shown above, and triangles indicating in-frame deletion mutations that were constructed are shown below. The genes are predicted to encode: a membrane-bound methyl-accepting chemotaxis protein (MCP) (*wspA*, PA3708), a *cheR*-like methyltransferase (*wspC*, PA3706), two *cheW* homologs (*wspB*, PA3707; *wspD*, PA3705), a hybrid histidine kinase-response regulator (*wspE*, PA3704), a *cheB*-like methylesterase (*wspF*, PA3703), and a response regulator with a GGDEF domain (*wspR*, PA3702).

a PCR-amplified PA2133 gene was cloned into the multiple cloning site of vector pJN105, generating plasmid pJN2133.

Attachment Assays and Biofilm Formation. Attachment assays were performed as described (26, 27). Plates were incubated at 37°C with shaking for 6–7 h. Biofilms were grown in flow chambers as described by using 1% tryptic soy broth (28, 29). Chambers were inoculated with $\approx 1 \times 10^7$ cells and incubated at room temperature for 1 h to allow surface attachment, after which flow was started at a rate of 2 ml/min. Strains constitutively expressing GFP were constructed by introduction of plasmid pMR9–1 (29). Biofilm images were obtained as described by using a Radiance 2100 confocal laser scanning microscope system (Bio-Rad) (28). Images were further processed with VOLOCITY software (Improvision, Lexington, MA).

Two-Dimensional Thin Layer Chromatography (TLC) Experiments. Procedures were adapted from those previously published (17, 30). Bacteria were grown overnight in Mops minimal medium with 30 mM succinate and 0.15 mM KH_2PO_4, and then subcultured in the same medium to an OD_{600} of 0.05. After one to two doublings, 0.1 mCi of ^{32}P orthophosphate (PerkinElmer; 1 Ci = 37 GBq) was added to 1 ml of culture, and growth was continued for 4–5 h, after which 10 μl of 11 M cold formic acid was added to 100 μl of the labeled cell culture.

PEI-cellulose plates (Selecto Scientific) were washed in 0.5 M LiCl followed by dH_2O and allowed to air dry. Formic acid cell extract (5 μl) was spotted on the TLC plates. Plates were soaked in methanol for 5 min, air dried, and developed in 0.2 M NH_4HCO_3, pH 7.8, in the first dimension. The plates were then soaked in methanol for 15 min, dried, and developed in the second dimension with 1.5 M KH_2PO_4, pH 3.65. Plates were soaked again in methanol for 15 min, air dried, and exposed to a PhosphorImager screen overnight. The data were collected on a Typhoon 8600 scanner and analyzed by using IMAGEQUANT software (Molecular Dynamics).

WspR Purification and Activity Assays. WspR was purified by using an intein–chitin binding domain fusion system (New England Biolabs, Beverly, MA). To generate an N-terminal intein fusion to WspR, the *wspR* gene was PCR amplified from chromosomal DNA and purified, digested, and ligated into pTYB12 to give pWspR12.

To purify WspR, *E. coli* ER2566 containing pWspR12 (1 liter of culture) was grown to an OD_{600} of 0.5 at 37°C, 0.3 mM IPTG (final concentration) was added to induce expression of WspR, and the culture was shifted to 18°C for overnight growth. Cells were harvested by centrifugation, resuspended in 5 ml of column buffer (20 mM Tris, pH 8.0/250 mM KCl/0.1 mM EDTA/0.1% Triton X-100) and lysed by sonication. The extract was centrifuged at 15,000 $\times$ *g* for 30 min, and the supernatant was loaded on a 10-ml chitin column equilibrated with 10 volumes of column buffer. The column was washed with 10 volumes of column buffer, flushed with column buffer containing 60 mM DTT, and stored for 48 h at 4°C to allow intein cleavage. Released protein was eluted in column buffer and analyzed by SDS/PAGE. Fractions containing WspR were pooled and dialyzed against storage buffer (20 mM Tris, pH 8.0/250 mM KCl/0.1 mM EDTA/50% glycerol). WspR was estimated to be >95% pure by SDS/PAGE analysis.

The products of WspR activity were assayed by using 1D and 2D TLC. Purified WspR (final concentration 10 μM), or WspR pretreated with 25 mM acetyl phosphate for 1 h at 37°C, were incubated with [^{32}P]GTP for time intervals in reaction buffer (75 mM Tris, pH 7.8/250 mM NaCl/25 mM KCl/10 mM $MgCl_2$) at 37°C in 50-μl reactions. Reactions were stopped by addition of 50 mM EDTA, spotted on PEI-cellulose TLC plates, and developed in one dimension by using 1.5 M KH_2PO_4, pH 3.65. TLC plates were processed and analyzed as described above.

Transcriptome Analysis. Cells were grown with shaking at 37°C to an OD_{600} of 0.3. RNA isolation, cDNA synthesis, fragmentation, and end-labeling were performed as described (31). Hybridization and processing of *P. aeruginosa* GeneChips was carried out at the University of Iowa DNA Facility by using previously published protocols (31). Experiments were carried out in triplicate with independently prepared samples.

The Affymetrix Microarray Suite was used for initial data acquisition and processing. Transcript data were further analyzed by using Cyber-T (http://visitor.ics.uci.edu/genex/cybert/index.shtml) as previously described with a *P* value threshold of 0.001, resulting in a corresponding posterior probability of differential expression no lower than 0.996 (32).

Results

Loss of *wspF* Increases Surface Attachment and Enhances Biofilm Formation by *P. aeruginosa* PAO1. To investigate the function of the *wsp* gene cluster in *P. aeruginosa*, in-frame deletions of *wspA*, *wspE*, *wspF*, and *wspR* were constructed (Fig. 1, see *Materials and Methods*). As previously reported, the *wspF* mutant formed small, wrinkled colonies and cells clumped in shaken liquid cultures (Fig. 2*A*) (7). The *wspF* mutant also formed robust pellicles in standing liquid cultures. The *wspF* mutant and wild-type cells grew at similar rates. Mutations of *wspA*, *wspE*, or *wspR* had no obvious effect on colony morphology, cell aggregation, or pellicle formation. The phenotypes of the *wspF* mutant were reversed by a deletion of *wspR*, consistent with a previous report (7).

We noticed that *wspF* mutant cells growing in shaken cultures attached to the sides of the culture tubes. This observation suggested that loss of *wspF* may also result in increased adherence. To test this we performed microtiter dish binding assays. The *wspF* mutant displayed increased adherence when compared to the parent PAO1 strain (Fig. 2*B*). Again, the *wspF* phenotype depended on the presence of *wspR* (Fig. 2*B*). We complemented the increased adherence and other phenotypes of the *wspF* mutant by introducing a copy of *wspF* on a plasmid. Loss of *wspA*, *wspE*, or *wspR* alone did not result in any observed phenotypes under the conditions tested.

The increased cell aggregation and adherence of a *wspF* mutant suggested that this strain might also display enhanced biofilm formation under flow compared to PAO1. To investigate this possibility, we grew PAO1 and *wspF* mutant strains expressing GFP in continuous flow chambers. After 24 h of growth in flow chambers, the mutant lacking WspF had formed a thick biofilm, whereas the PAO1 biofilm was immature and cells were apparently still in the process of forming microcolonies (Fig. 3 *Upper*). At this time, the *wspF* mutant

MICROBIOLOGY

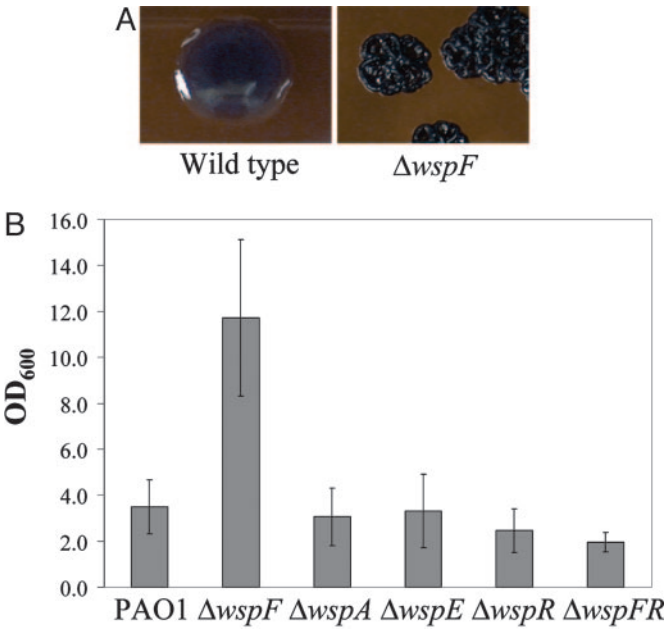

Fig. 2. Phenotypes associated with loss of *wspF*. (*A*) Colony morphologies of PAO1 and a mutant lacking *wspF*. Photographs were taken after 5 days of growth on tryptone agar containing 40 mg of Congo red and 10 mg of Coomassie brilliant blue per liter of medium. (*B*) Attachment of *P. aeruginosa* PAO1 and *wsp* deletion mutants to microtiter dish wells. Results shown are the mean of at least three independent experiments. Error bars represent the standard deviations.

had formed a biofilm ≈20–25 μm thick. After 48 h, the *wspF* mutant biofilm had doubled in thickness to 40–50 μm. At 48 h, the PAO1 biofilm was as thick as the WspF mutant biofilm, but the architecture was significantly different between the two strains (Fig. 3 *Lower*). Whereas PAO1 generated a biofilm with large mushroom structures and deep valleys, the biofilm generated by the *wspF* mutant was relatively flat without large mushroom structures. These results indicate that not only do mutants lacking *wspF* form thicker biofilms than wild-type

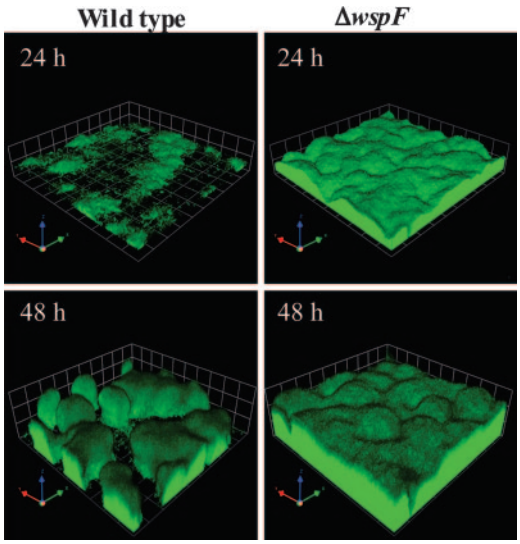

Fig. 3. Loss of *wspF* results in enhanced biofilm formation. Biofilm formation by PAO1 and a *wspF* mutant. Images were obtained by using a ×60 objective after 24- and 48-h growth in continuous flow chambers. Each square on the grid is 20 μm per side.

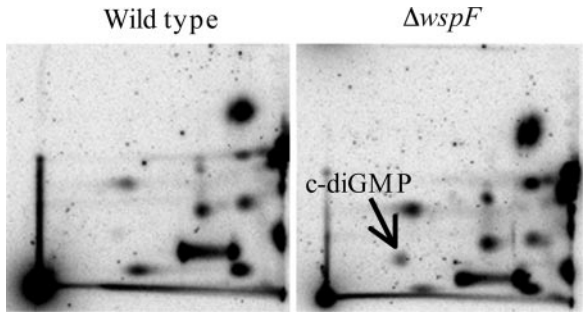

Fig. 4. Loss of *wspF* results in increased c-diGMP levels. Two-dimensional TLC analysis of acid extracts from ^{32}P-labeled cells. (*Left*) PAO1. (*Right*) *wspF* mutant. The spot corresponding to published R_f values for c-diGMP is indicated by the arrow in *Right*.

PAO1 at early stages in development, but there are significant architectural differences in mature biofilms generated by a *wspF* mutant and PAO1.

A *wspF* Mutant Has Increased Intracellular Levels of Cyclic Diguanylate. Because the phenotypes of a *wspF* mutant depended on the presence of *wspR*, we turned our attention to this response regulator. WspR contains a conserved C-terminal GGDEF domain. These domains have been implicated in the production of c-diGMP, a small intracellular signaling molecule that influences the production of cellulose and other EPS (17, 18, 33–36). It has been shown that increased intracellular levels of c-diGMP result in enhanced adherence and biofilm formation in various bacteria including *Salmonella*, *Pseudomonas*, and *Vibrio* species (17, 18, 33). Based on this and the known roles of homologous chemotaxis proteins, we hypothesized that loss of WspF would result in a WspR-P-dependent increase in intracellular c-diGMP levels. By analogy to the Che system of *E. coli*, loss of WspF, a CheB homolog, would be expected to lock the predicted Wsp signaling complex into a conformation where it constantly phosphorylates the WspR response regulator. WspR-P would in turn catalyze the synthesis of c-diGMP, and result in relatively high intracellular levels of this signaling molecule. This hypothesis predicts that we should observe elevated levels of c-diGMP in a *wspF* mutant. To test this hypothesis, we labeled cells with ^{32}P-orthophosphate, extracted nucleotide pools, and separated them by 2D TLC. When we compared extracts from PAO1 and a *wspF* mutant, we saw that the *wspF* mutant formed a metabolite with migration ratios (R_f values of 0.15 in the NH_4CO_3 dimension and 0.31 in the KH_2PO_4 dimension) that matched those previously reported for c-diGMP in other organisms (Fig. 4) (17, 37). The wild type formed barely detectable amounts of c-diGMP (Fig. 4). This finding indicates that loss of *wspF* results in increased intracellular levels of c-diGMP.

WspR Generates c-diGMP *in Vitro*. We wanted to provide further evidence that WspR was responsible for the increased c-diGMP we observed in a *wspF* mutant. To do this, we measured c-diGMP production by purified WspR that was either untreated or pretreated with acetyl phosphate for 1 h. Acetyl phosphate can phosphorylate a wide variety of response regulators *in vitro* (38). Untreated WspR generated c-diGMP from GTP and pretreatment of WspR with acetyl phosphate increased the rate of c-diGMP synthesis (Fig. 5). Stimulation of WspR activity by acetyl phosphate supports the model that this protein is a c-diGMP synthase and a response regulator. A 2D TLC analysis of the WspR reaction products confirmed the synthesis of c-diGMP (data not shown).

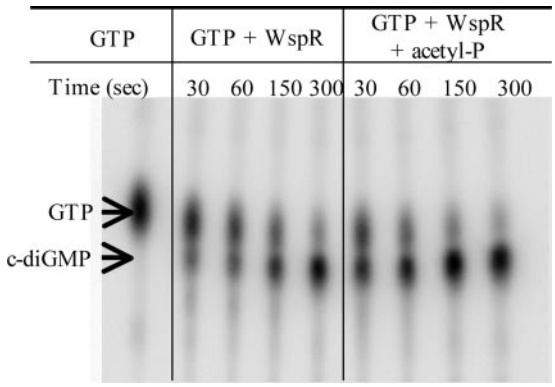

Fig. 5. WspR generates c-diGMP from GTP. Time course of formation of c-diGMP by WspR. Arrows on left indicate the relative migration of GTP and c-diGMP. The GTP-only control is shown in the left lane. Reactions with WspR are shown at left, and reactions with acetyl-phosphate-treated WspR are shown at right.

Loss of *wspF* Results in Increased Expression of Genes Involved in EPS Production. Our results suggested that elevated intracellular levels of c-diGMP resulted in increased adherence and biofilm formation. We wanted to determine whether these phenotypes were correlated with changes in the transcriptome of the *wspF* mutant. When we compared the transcript profile of the *wspF* mutant to PAO1 using the web-based program CYBER-T, 560 genes displayed a statistically significant difference in transcript levels (Table 3, which is published as supporting information on the PNAS web site). Of these, 358 genes showed higher levels, and 202 genes showed lower levels of transcripts in the *wspF* mutant compared to PAO1. A selection of these genes is presented in Table 1. Two different operons involved in EPS production and biofilm formation were expressed at higher levels in the *wspF* mutant (19, 39–41). The *psl* transcripts were 2- to 3-fold higher in the *wspF* mutant, whereas the *pel* transcripts were 3- to 13-fold higher in the mutant as compared to wild type (Table 1). These results could explain the enhanced adherence and biofilm formation we observed in the *wspF* mutant.

Table 1. Select genes expressed at higher levels in a Δ*wspF* mutant

PA number	Gene name	Fold change*	Protein description†
PA0169		2.9	GGDEF
PA0170		2.5	Hypothetical protein
PA0171		2.6	Hypothetical protein
PA0172		2.9	Hypothetical protein
PA0610	*prtN*	2.3	Pyocin regulator
PA0612–0648		3.7–22.2	R and F-type pyocins‡
PA0985		7.3	Pyocin S5
PA1150	*pys2*	5.0	Pyocin S2
PA1431	*rsaL*	2.6	Regulatory protein
PA1432	*lasI*	2.2	Quorum sensing
PA2231–2242	*pslA-L*	1.8–2.7§	EPS production
PA2440		4.3	Hypothetical protein
PA2441		12.8§	Hypothetical protein
PA3058–3064	*pelA-G*	2.6–13.3§	EPS production
PA3866		5.1	Pyocin
PA4624		6.7	Hypothetical protein
PA4625		9.3§	Putative adhesin¶

*Fold change between *wspF* and PAO1 was determined by using the web-based program CYBER-T. *P* value for all expression differences reported was <0.001.

†Protein function taken from the *Pseudomonas* genome web site www.pseudomonas.com.

‡These genes were reannotated based on published work (50).

§Expression changes confirmed by using quantitative RT-PCR.

¶This gene was reannotated based on analysis with the web-based program INTERPROSCAN, www.ebi.ac.uk/InterProScan/.

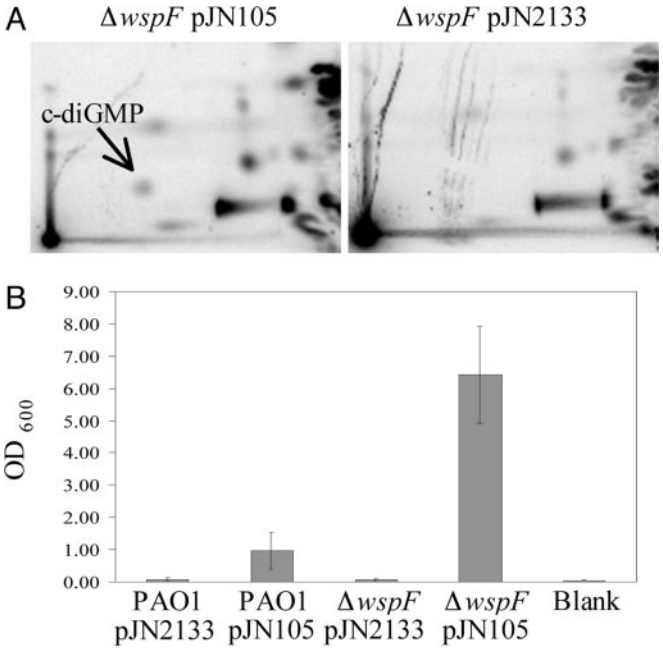

Fig. 6. Degradation of c-diGMP reverses the phenotypes of a *wspF* mutant. (*A*) Two-dimensional TLC analysis of a *wspF* mutant expressing PA2133 from a plasmid. The location of c-diGMP is indicated with an arrow. (*B*) Attachment of strains expressing PA2133 from a plasmid (pJN2133) to microtiter dish wells. The background absorbance obtained from uninnoculated control wells is shown at right. pJN105 is the control vector. Absorbance obtained is an average of at least three independent trials. The error bars represent the standard deviation.

We also saw an increase in levels of transcripts for several pyocins, including the phage-related gene clusters spanning PA0612–0648 in the *wspF* mutant. The transcript of an ORF predicted to encode a GGDEF-containing protein (PA0169) was 3-fold higher in the *wspF* mutant, and a gene encoding a possible adhesion protein (PA4625) was expressed at 9-fold higher levels (Table 1). The expression changes of *pelA*, *pslA*, PA2441, and PA4625 were confirmed by RT-PCR using RNA samples prepared from independently grown cultures of the *wspF* mutant and PAO1.

Degradation of c-diGMP in a *wspF* Mutant Reverses the Phenotypes of this Strain. Our data show that the phenotypes of the *wspF* mutant correlate with an increase in intracellular levels of c-diGMP. If increased intracellular concentrations of c-diGMP were responsible for the *wspF* mutant phenotypes, then decreasing the amount of c-diGMP should reverse these phenotypes. Some bacterial proteins containing a conserved motif, called DUF2 or an EAL domain, have been shown to catalyze the degradation of c-diGMP to two molecules of GMP (42). There are many proteins in *P. aeruginosa* predicted to contain an EAL domain, one of which is encoded by PA2133, an uncharacterized ORF. To test the hypothesis that c-diGMP is the signal responsible for enhanced biofilm formation, we introduced a plasmid expressing PA2133 into PAO1 and the *wspF* mutant. The expression of PA2133 in the *wspF* mutant restored wild-type colony morphology to this strain. *wspF* mutant cells carrying the PA2133 expression vector had undetectable levels of c-diGMP (Fig. 6*A*). Also, the surface attachment of *wspF* mutant cells expressing PA2133 was completely abolished. Interestingly, the ability of

MICROBIOLOGY

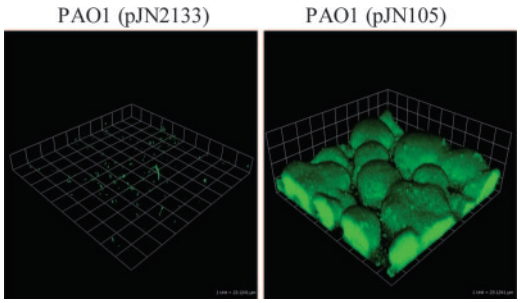

Fig. 7. Degradation of c-diGMP inhibits biofilm formation by PAO1. Biofilm formation by strain PAO1 expressing PA2133 from a plasmid (pJN2133) compared to PAO1 with a control vector. Biofilms were visualized by staining with propidium iodide (4 μM) after 72-h growth.

wild-type cells to attach to surfaces was also completely abolished when PA2133 was expressed (Fig. 6*B*). This result suggested that degradation of c-diGMP in wild-type PAO1 might inhibit biofilm formation. To test this possibility, we examined the ability of PAO1 expressing PA2133 to establish a biofilm in flow chambers. This strain failed to form a biofilm even after 72 h of incubation (Fig. 7). WspF mutant cells expressing PA2133 in trans had lower levels of *pslA* and *pelA* transcripts as compared with *wspF* cells carrying vector only, as assessed by RT-PCR (data not shown). A PA2133 mutant resembled the wild type in attachment assays.

Discussion

Here we have provided evidence that the Wsp chemosensory system regulates biofilm formation in *P. aeruginosa* through modulation of intracellular c-diGMP levels. A homologous Wsp chemosensory system in *P. fluorescens* regulates cellulose synthesis by an as yet unstudied mechanism (43, 44). We have also demonstrated that there are global gene expression changes associated with an increase in cellular levels of c-diGMP in *P. aeruginosa*. The increased expression of the *pel* and *psl* gene clusters involved in EPS production in the *wspF* mutant may explain adherence and enhanced biofilm formation observed in this strain. It has been shown that these two gene clusters are involved in biofilm formation by *P. aeruginosa* (19, 39–41). It has also been shown that increased expression of the *psl* and *pel* gene clusters in *P. aeruginosa* PAK results in increased surface attachment and wrinkled colonies, similar to the phenotypes of a *wspF* mutant (45). A recent report has noted that spontaneous hyperattaching variants isolated from biofilms have colony morphologies reminiscent of the *wspF* mutant. These variants display increased expression of the *psl* and *pel* loci, and loss of *psl* genes in these strains eliminates the hyperattachment phenotype (46). The effects of expression changes of other genes in the *wspF* mutant on biofilm formation remain to be determined. Our results add important information to the growing understanding that c-diGMP influences biofilm formation in a variety of bacterial species (16–18, 33, 34, 43, 47, 48).

We found that the degradation of c-diGMP in wild type PAO1 (through expression of PA2133) resulted in the blockage of biofilm formation (Figs. 6 and 7). This finding suggests that, not only do increased c-diGMP levels enhance biofilm formation, but decreased levels of c-diGMP prevent initiation of biofilm development. The defect in attachment and biofilm initiation that we observed in *P. aeruginosa* strains expressing PA2133 is more severe than that reported for *Salmonella* and *Vibrio* strains overexpressing proteins containing a c-diGMP phosphodiesterase domain (17, 18). This finding suggests that c-diGMP activity might represent a target for development of anti-*Pseudomonas* biofilm agents.

Although our results suggest that phenotypes associated with increased c-diGMP (i.e., increased EPS production) are, at least in part, mediated through changes in gene expression, other work has indicated that c-diGMP may exert its effects on enzyme activities. One example is the allosteric regulation of cellulose synthase activity in *Gluconacetobacter xylinus* by c-diGMP (37). Another possible example is the GGDEF-containing response regulator PleD in *Caulobacter cresentus*. PleD is involved in stalked cell development, and localizes to the stalked pole in this organism. It has been hypothesized that PleD may exert its effects on stalked cell development by increasing c-diGMP concentrations at a subcellular location (49, 50). It is possible that, in the case of *P. aeruginosa*, there are uncharacterized effects of c-diGMP on protein activities, as well as global effects on gene expression.

The *P. aeruginosa* genome contains at least 31 genes coding for proteins predicted to have diguanylate cyclase activity, 14 of which also have phosphodiesterase EAL domains predicted to catalyze c-diGMP degradation. An additional six proteins are predicted to have phosphodiesterase activity only, for a total of 37 proteins potentially involved in the formation and degradation of c-diGMP. Many of these proteins also contain a variety of predicted sensing or regulatory domains, such as PAS/PAC, response regulator, or transmembrane regions. From this, it is clear that the potential environmental or physiological signals affecting c-diGMP levels in *P. aeruginosa* are numerous and diverse.

The Wsp proteins are predicted to form a signal transduction complex similar to those responsible for bacterial chemotaxis. In chemotaxis, when membrane-bound signal transducer proteins, also called methyl-accepting chemotaxis proteins (MCPs), bind a chemical stimulus, a conformational change is propagated through the MCP that modulates the autophosphorylation activity of a physically associated histidine kinase (CheA). CheA-P phosphorylates the response regulator CheY, and CheY-P binds to the flagellar motor, causing cells to tumble and switch directions. At the same time, with a slower kinetics, CheA also modulates the phosphorylation state of a second response regulator, CheB, through phosphotransfer. CheB-P is active as a methylesterase and acts in conjunction with constitutively active methyltransferase (CheR) to adjust the methylation state of glutamate residues on MCPs. A shift in the methylation state resets the signaling activity of the MCPs to the prestimulus level, resulting in adaptation. This system of rapid response and slower adaptation allows cells to continue to swim up or down concentration gradients of chemical attractants or repellents. If this model correctly describes the main elements of Wsp chemosensory signaling, then it implies that *P. aeruginosa* uses this system to respond and adapt to an as yet unknown, but presumably constantly varying signal to fine tune intracellular levels of c-diGMP, and transition in and out of the biofilm state.

This work was supported by National Institutes of Health Grant GM056665. J.W.H. was supported by National Science Foundation Training Grant DBI9602247 and a Pulmonary Research Training Grant at the University of Iowa, and Cystic Fibrosis Foundation Postdoctoral Fellowship at the University of Washington.

1. Stewart, P. S. & Costerton, J. W. (2001) *Lancet* **358,** 135–138.
2. Mah, T. F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S. & O'Toole, G. A. (2003) *Nature* **426,** 306–310.
3. Stewart, P. S. (1994) *Antimicrob. Agents Chemother.* **38,** 1052–1058.
4. Mah, T. F. & O'Toole, G. A. (2001) *Trends Microbiol.* **9,** 34–39.
5. Lyczak, J. B., Cannon, C. L. & Pier, G. B. (2000) *Microbes Infect.* **2,** 1051–1060.
6. Drenkard, E. & Ausubel, F. M. (2002) *Nature* **416,** 740–743.
7. D'Argenio, D. A., Calfee, M. W., Rainey, P. B. & Pesci, E. C. (2002) *J. Bacteriol.* **184,** 6481–6489.
8. Häussler, S., Ziegler, I., Löttel, A., von Götz, F., Rohde, M., Wehmhöhner, D., Saravanamuthu, S., Tümmler, B. & Steinmetz, I. (2003) *J. Med. Microbiol.* **52,** 295–301.

9. Häussler, S. (2004) *Environ. Microbiol.* **6,** 546–551.
10. Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W. & Davies, D. G. (2002) *J. Bacteriol.* **184,** 1140–1154.
11. Ryjenkov, D. A., Tarutina, M., Moskvin, O. V. & Gomelsky, M. (2005) *J. Bacteriol.* **187,** 1792–1798.
12. Chan, C., Paul, R., Samoray, D., Amiot, N. C., Giese, B., Jenal, U. & Schirmer, T. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 17084–17089.
13. Pei, J. & Grishin, N. V. (2001) *Proteins* **42,** 210–216.
14. Ausmees, N., Mayer, R., Weinhouse, H., Volman, G., Amikam, D., Benziman, M. & Lindberg, M. (2001) *FEMS Microbiol. Lett.* **204,** 163–167.
15. D'Argenio, D. A. & Miller, S. I. (2004) *Microbiology* **150,** 2497–2502.
16. Ross, P., Mayer, R. & Benziman, M. (1991) *Microbiol. Rev.* **55,** 35–58.
17. Tischler, A. D. & Camilli, A. (2004) *Mol. Microbiol.* **53,** 857–869.
18. Simm, R., Morr, M., Kader, A., Nimtz, M. & Römling, U. (2004) *Mol. Microbiol.* **53,** 1123–1134.
19. Friedman, L. & Kolter, R. (2004) *Mol. Microbiol.* **51,** 675–690.
20. Ferrández, A., Hawkins, A. C., Summerfield, D. T. & Harwood, C. S. (2002) *J. Bacteriol.* **184,** 4374–4383.
21. Horton, R. M., Ho, S. N., Pullen, J. K., Hunt, H. D., Cai, Z. & Pease, L. R. (1993) *Methods Enzymol.* **217,** 270–279.
22. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989) *Gene* **77,** 51–59.
23. Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. & Schweizer, H. P. (1998) *Gene* **212,** 77–86.
24. Marx, C. J. & Lidstrom, M. E. (2001) *Microbiology* **147,** 2065–2075.
25. Schweizer, H. D. (1993) *BioTechniques* **15,** 831–834.
26. O'Toole, G. A. & Kolter, R. (1998) *Mol. Microbiol.* **28,** 449–461.
27. O'Toole, G. A. & Kolter, R. (1998) *Mol. Microbiol.* **30,** 295–304.
28. Singh, P. K., Parsek, M. R., Greenberg, E. P. & Welsh, M. J. (2002) *Nature* **417,** 552–555.
29. Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. & Greenberg, E. P. (1998) *Science* **280,** 295–298.
30. Bochner, B. R. & Ames, B. N. (1982) *J. Biol. Chem.* **257,** 9759–9769.
31. Schuster, M., Lostroh, C. P., Ogi, T. & Greenberg, E. P. (2003) *J. Bacteriol.* **185,** 2066–2079.
32. Schuster, M., Hawkins, A. C., Harwood, C. S. & Greenberg, E. P. (2004) *Mol. Microbiol.* **51,** 973–985.
33. Garcia, B., Latasa, C., Solano, C., Garcia-del Portillo, F., Gamazo, C. & Lasa, I. (2004) *Mol. Microbiol.* **54,** 264–277.
34. Boles, B. R. & McCarter, L. L. (2002) *J. Bacteriol.* **184,** 5946–5954.
35. Amikam, D. & Benziman, M. (1989) *J. Bacteriol.* **171,** 6649–6655.
36. Weinhouse, H., Sapir, S., Amikam, D., Shilo, Y., Volman, G., Ohana, P. & Benziman, M. (1997) *FEBS Lett.* **416,** 207–211.
37. Ross, P., Aloni, Y., Weinhouse, H., Michaeli, D., Weinberger-Ohana, P., Mayer, R. & Benziman, M. (1986) *Carbohydr. Res.* **149,** 101–117.
38. McCleary, W. R. & Stock, J. B. (1994) *J. Biol. Chem.* **269,** 31567–31572.
39. Matsukawa, M. & Greenberg, E. P. (2004) *J. Bacteriol.* **186,** 4449–4456.
40. Friedman, L. & Kolter, R. (2004) *J. Bacteriol.* **186,** 4457–4465.
41. Jackson, K. D., Starkey, M., Kremer, S., Parsek, M. R. & Wozniak, D. J. (2004) *J. Bacteriol.* **186,** 4466–4475.
42. Christen, M., Christen, B., Folcher, M., Schauerte, A. & Jenal, U. (2005) *J. Biol. Chem.* **280,** 30829–30837.
43. Spiers, A. J., Bohannon, J., Gehrig, S. M. & Rainey, P. B. (2003) *Mol. Microbiol.* **50,** 15–27.
44. Spiers, A. J., Kahn, S. G., Bohannon, J., Travisano, M. & Rainey, P. B. (2002) *Genetics* **161,** 33–46.
45. Vasseur, P., Vallet-Gely, I., Soscia, C., Genin, S. & Filloux, A. (2005) *Microbiology* **151,** 985–997.
46. Kirisits, M. J., Prost, L., Starkey, M. & Parsek, M. R. (2005) *Appl. Environ. Microbiol.* **71,** 4809–4821.
47. Bomchil, N., Watnick, P. & Kolter, R. (2003) *J. Bacteriol.* **185,** 1384–1390.
48. Güvener, Z. T. & McCarter, L. L. (2003) *J. Bacteriol.* **185,** 5431–5441.
49. Paul, R., Weiser, S., Amiot, N. C., Chan, C., Schirmer, T., Giese, B. & Jenal, U. (2004) *Genes Dev.* **18,** 715–727.
50. Aldridge, P., Paul, R., Goymer, P., Rainey, P. & Jenal, U. (2003) *Mol. Microbiol.* **47,** 1695–1708.