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Genetic Analysis of the AdnA Regulon in *Pseudomonas fluorescens*: Nonessential Role of Flagella in Adhesion to Sand and Biofilm Formation

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AdnA is a transcription factor in *Pseudomonas fluorescens* that affects flagellar synthesis, biofilm formation, and sand adhesion. To identify the AdnA regulon, we used a promoterless Tn*5-lacZ* element to study the phenotypes of insertion mutants in the presence and absence of AdnA. Of 12,000 insertions, we identified seven different putative open reading frames (ORFs) activated by AdnA (named *aba* for activated by AdnA). *aba120* and *aba177* showed homology to *flgC* and *flgI*, components of the basal body of the flagella in *Pseudomonas aeruginosa*. Two other insertions, *aba18* and *aba51*, disrupted genes affecting chemotaxis. The mutant loci *aba160* (possibly affecting lipopolysaccharide synthesis) and *aba175* (unknown function) led to loss of flagella. The mutant bearing *aba203* became motile when complemented with *adnA*, but the mutated gene showed no similarity to known genes. Curiously, *aba18*, *aba51*, *aba160*, and *aba203* mutants formed biofilms even in the absence of AdnA, suppressing the phenotype of the *adnA* deletion mutant. The combined findings suggest that flagella are nonessential for sand attachment or biofilm formation. Sequence and promoter analyses indicate that AdnA affects at least 23 ORFs either directly or by polar effects. These results support the concept that AdnA regulates cell processes other than those directly related to flagellar synthesis and define a broader cadre of genes in *P. fluorescens* than that described so far for its homolog, FleQ, in *P. aeruginosa*.

The pseudomonads comprise a diverse group of bacteria found ubiquitously in heterogeneous environments. *Pseudomonas fluorescens*, a common soil microorganism, is a proposed agent for biocontrol, plant growth promotion, and bioremediation and is closely related to *Pseudomonas aeruginosa*, a human pathogen in immunocompromised individuals and cystic fibrosis patients. The discovery of a large number of genes for regulatory proteins in the *P. aeruginosa* genome suggests that this genus is finely tuned to respond to its environment (31, 34). Hence, the study of *P. fluorescens* in soil provides a model system to dissect genetic traits important for adaptation to various environments.

Our research studies of *P. fluorescens* and its activity in soil have led to the discovery of *adnA*, a gene affecting flagellar production, motility, and attachment to sand and seeds (9, 10). In addition, AdnA was shown to be required for biofilm formation and has 83% identity to FleQ in *P. aeruginosa* (6). Field studies showed that loss of *adnA* reduced the ability of *P. fluorescens* to spread and persist in soil (21). From their structure, AdnA and FleQ transcription factors appear to be members of the NtrC/NifA family of activators (6). In these systems, under the right environmental conditions, a sensor kinase phosphorylates an aspartate residue in the regulator protein that acts upon σ^{54}-dependent promoters. Other variations of this model that do not require phosphorylation include ligand binding, as reported for XylR activation, and antiactivation by protein-protein interaction as in the NifL/NifA system (25).

Synthesis of flagella seems to progress in a cascade manner, in which expression and assembly of early genes are required before activation of late genes (8, 16). FleQ and its antiactivator, FleN, are placed high in the cascade and control transcription of a number of structural genes of flagella and of *fleSR*, another two-component regulatory system in *P. aeruginosa* (8). FleQ regulates transcription of several flagellar genes including *fliD*, the flagellar cap protein that mediates attachment to respiratory mucin and is thus important in virulence of *P. aeruginosa* (1, 2).

Under certain environmental conditions, flagella are necessary for biofilm formation in *P. fluorescens,* but this effect is abolished when culture conditions include iron and/or citrate in the medium (27). Our observations that AdnA affects phenotypes important for soil activity and biofilm formation prompted the search to identify genes regulated by this factor and provide a genetic handle by which to study the effects of a specific regulon on biofilm formation. We generated random transcriptional fusions in the genome of a host in which the *adnA* gene had been deleted and screened for *adnA*-mediated change in expression. In addition to genes coding for early components of flagella and chemotaxis, as expected by the homology of AdnA to FleQ, we found open reading frames (ORFs) affected by AdnA with no clear connection to flagellar synthesis and other ORFs with no known function. The results of sequence and promoter analyses that also examined the flanking regions of the ORFs suggest a total of 23 genes affected by AdnA including at least two multiple gene operons. Phenotype studies of the strains with insertion mutations in the absence and presence of AdnA indicate that AdnA is required

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E. coli DH5α λ-*pir*	ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 gyrA96 thi-I hsdR17 supE44 relA1 deoR* λ-*pir*	19
E. coli S17-1 λ-*pir*	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn*7* λ-*pir*	11, 32
P. fluorescens Pf0-1	Wild type, Amp^r	7
P. fluorescens Pf0-2x	Pf0-1 *adnA*::Sm/Sp derivative	This study
Plasmids		
pRK2013	Helper plasmid, Tra^+ Km^r	13
pHRP315	Plasmid containing 2-kb *Dra*I Ω cassette, Sm^r Sp^r	28
pJB866	Broad-host-range vector, Tc^r	4
pPC100	1.6-kb *adnA* gene cloned into *Afl*III-*Eco*RI site of pJB866, Tc^r	6
pPC101	Deletion of 923-bp *Afl*III fragment from *adnA* on pPC100, Tc^r	6
pPF1B	6.5-kb *Eco*RI-*Bam*HI fragment cloned into pBluescript, Amp^r	6
pJS6DRA	pPF1B derivative with Ω cassette cloned into the *Pml*I site of *adnA*, Amp^r Sp^r Sm^r	This study
pSR47s	Broad-host-range suicide vector containing *sacB*, Km^r	22
pSUP101Tn*5*-B22	pSUP101 derivative with Tn*5* element containing promoterless *lacZ*, Tc^r Gm^r	32
pSUSM	pSR47s derivative containing *adnA*::Sm/Sp, Km^r Sp^r Sm^r	This study
pSRadnA	3-kb *Sac*I fragment containing *adnA* cloned in pSR47s	This study

for flagellar production and biofilm formation but that the biofilm formation defect can be suppressed by mutations in other genes in the AdnA regulon.

MATERIALS AND METHODS

Strains and media. *P. fluorescens* strains were grown on rich medium (Luria-Bertani [LB]), minimal medium (MMO) (33), and defined medium (*Pseudomonas* minimal medium) (18) at 30°C. *Escherichia coli* strains, used for plasmid construction and transposon mutagenesis experiments, were grown in LB medium at 37°C (Table 1).

Swim agar consisted of MMO with 0.3% Difco Bacto Agar. Swarm agar was made with 0.5% Difco Bacto Agar and Difco nutrient broth (8 g/liter), to which 5 g of glucose per liter was added (29). Both swim and swarm agar plates were allowed to dry overnight at room temperature before use.

Antibiotics were added at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; gentamicin, 25 μg/ml; streptomycin, 25 μg/ml; and tetracycline, 10 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl galactoside) was used at 50 μg/ml.

Construction of the *adnA* deletion strain. We disrupted *adnA* by inserting an omega cassette conferring streptomycin and spectinomycin resistance 500 bp from the translational start of *adnA*. The omega cassette was excised from pHRP315 (28) after *Dra*I digestion and cloned into the *Pml*I site in the *adnA*-containing plasmid pPF1B (6), giving rise to pJS6Dra. pJS6Dra, digested with *Sac*I, yielded a 5-kb fragment containing 1.1 kb upstream of *adnA*, the first 500 bp of *adnA*, the 2-kb streptomycin resistance cassette, and 1.3 kb including the 3′ end of *adnA*. The *Sac*I fragment was cloned into pSR47s (22), a broad-host-range suicide vector carrying kanamycin resistance as a selectable marker and *sacB* as a counterselectable marker, producing plasmid pSUSM. Replication of pSR47s is dependent on the π replication protein (19). pSUSM was introduced into *P. fluorescens* Pf0-1, the parent strain, by mating with *E. coli* DH5α λ-*pir*, using pRK2013 as a helper plasmid (13). Transconjugants resistant to ampicillin, kanamycin, and streptomycin indicated that the entire vector carrying the disrupted allele had inserted into the chromosome by recombination. The conjugation and recombination frequency was 4×10^{-5} per recipient. A few Ap^r, Km^r, and Sm^r colonies were grown overnight in LB broth containing ampicillin and plated on LB agar with ampicillin, streptomycin, and 5% sucrose, which demanded a second recombination event (1.3×10^{-3} per recipient) and replacement of the wild-type allele with the mutant allele, creating strain Pf0-2x. The replacement was confirmed by PCR using primers JK910 (GAGTGAGGCTTC GCTTGA) and JK6 (TGCTCCATGCGCGAGATCA) and by motility assays.

Identification of genes regulated by *adnA*. *P. fluorescens* Pf0-2x was mutagenized using *E. coli* S17-1 bearing pTn5-B22 (*lacZ* Gm^r) (32). Transposon insertion mutants were selected on LB agar supplemented with gentamicin (for the transposon) and streptomycin (for the cassette within *adnA*). Individual mutants were transferred to microtiter plates of LB broth with appropriate antibiotics and then transferred, using 96-prong metal replicators in LB broth without antibiotics, to the mating plates. *adnA* was introduced in *trans* into each of the mutants by a mass mating technique as follows. *E. coli* bearing pPC100 (Tc^r) was grown overnight in LB broth supplemented with tetracycline, and cells were pelleted, washed twice with LB broth without antibiotics, and resuspended in this medium to an A_{530} of 0.3. Two hundred microliters of the cell suspension was spread onto LB agar plates to form a lawn of the donor strain. *P. fluorescens* mutants were replica plated onto the lawn of the donor strain and grown overnight at 30°C. These mating spots were then replica plated onto LB agar containing appropriate antibiotics to select for the transconjugants carrying plasmid pPC100. Both collections of strains (with and without *adnA*) were inoculated into microtiter plates containing LB broth and appropriate antibiotics and incubated at 30°C until dense cultures had formed. Individual transposon mutants were then screened on LB plates supplemented with X-Gal and antibiotics for differences in reporter gene expression (blue color) with and without *adnA*. When a difference was observed, the corresponding patch was streaked to isolate colonies, and a single colony was retested for confirmation.

Complementation of mutants with *adnA*. The *adnA* gene was provided in *trans* on a plasmid, and reporter gene activity was measured using the β-galactosidase assay described below. For all *adnA* complementation experiments, two controls were used to confirm that AdnA was responsible for increases in reporter activity. In the first control, β-galactosidase was measured in each *aba* strain bearing the plasmid vector alone, and in the second control, *aba* strains contained pPC101, which has a 923-nucleotide deletion in *adnA*. Neither control affected β-galactosidase activity in any *aba* mutant.

Replacement of *adnA*::Sm with wild-type *adnA*. We replaced the *adnA*::Sm allele with a wild-type copy by allele exchange. A 3-kb *Sac*I fragment containing *adnA* was cloned from pPF1B into the pSR47s suicide plasmid. The resulting plasmid, pSRadnA, was transferred to *aba175* and *aba203* mutants by conjugation from *E. coli* S17-1. Strains in which pSRadnA had integrated into the genome by a single crossover were selected on the basis of kanamycin resistance. These strains were grown overnight in LB broth and plated on LB agar supplemented with ampicillin and 5% sucrose to select bacteria that had undergone a second crossover and therefore had lost the plasmid. These sucrose-tolerant colonies were screened for sensitivity to streptomycin, which indicated loss of *adnA*::Sm, and the presence of a wild-type *adnA* sequence was confirmed by PCR.

β-Galactosidase assay. Cultures from single colonies were grown overnight in LB broth; a 1-ml aliquot was inoculated into 12 ml of minimal medium broth and grown to an A_{600} of 0.4 to 0.6. Cells were permeabilized and assayed as described previously (24).

Arbitrary-primed PCR to locate Tn*5* insertions. DNA sequences adjacent to the transposon insertions in the mutants were determined by arbitrary PCR with Vent exo^- DNA polymerase (New England Biolabs, Beverly, Mass.) (27).

The first round of PCR was performed using chromosomal DNA as a template and primers TN5EXT (GAACGTTACCATGTTAGGAGGTC [unique to the right end of the transposon]) and an arbitrary primer ARB1 (GGCCAGCCGT CGACTCANNNNNNNNNGATA). The PCR reaction mixture was a solution

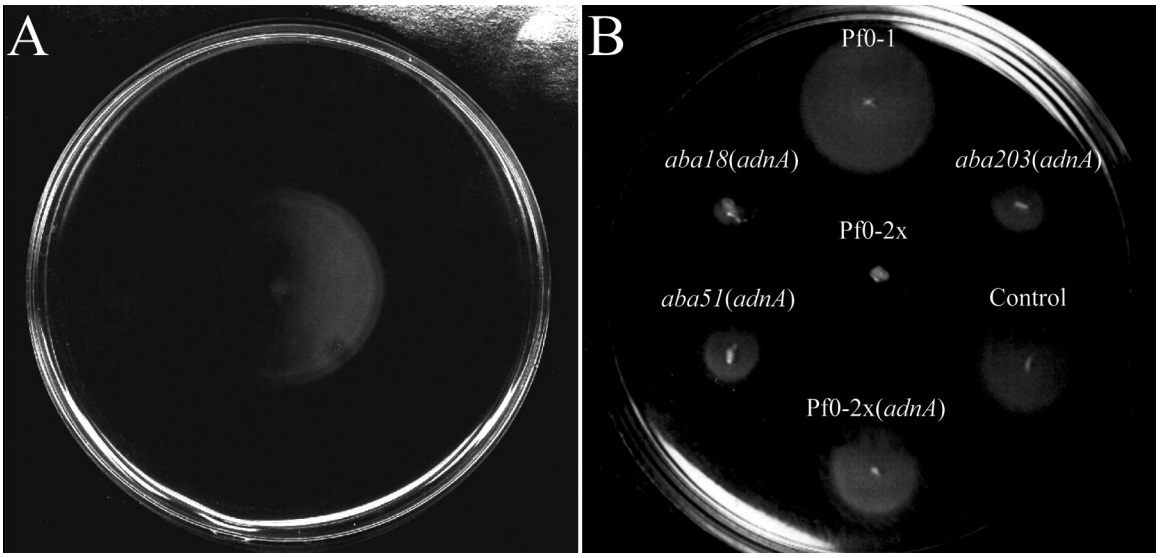

FIG. 1. Chemotaxis and swimming response of *P. fluorescens* Pf0-1. (A) Chemotactic response to Casamino Acids in a modified swarm plate assay. Pf0-1 was inoculated in the center of soft agar MMO and incubated at 28°C. The movement of inoculum was measured in millimeters. (B) Swimming phenotypes of *P. fluorescens* and *aba* insertion mutants. Plates containing 0.3% agar were inoculated and incubated for 24 to 48 h at 28°C. The control consists of an *adnA*-complemented transposon insertion which is not affected by AdnA; the response of the control is similar to that of *adnA*-complemented Pf0-2x in which *adnA* had been deleted.

containing 1 μg of template DNA, a 1 μM concentration of each primer, a 0.25 mM concentration of each deoxynucleoside triphosphate, 1× Vent polymerase buffer, and 1 U of Vent exo$^-$ DNA polymerase in a total volume of 50 μl. PCR was performed as follows: (i) 5 min at 95°C; (ii) six cycles, with one cycle consisting of 30 s at 95°C, 30 s at 30°C, and 1 min at 72°C; (iii) 30 cycles, with one cycle consisting of 30 s at 95°C, 30 s at 57°C, and 1 min at 72°C; and (iv) a final extension step of 5 min at 72°C.

A second round of PCR was performed using 5 μl of the product of the first round as a DNA template. PCR was performed as follows: (i) 5 min at 95°C; (ii) 30 cycles, with one cycle consisting of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C; and (iii) a final extension step of 5 min at 72°C. The primers used were TN5INT (CGGGAAAGGTTCCGTTCAGGACGC [specific to the rightmost end of the transposon]) and ARB3 (GGCCACGCGTCGACTAGTCA [specific to the 5′ end of ARB1]).

The PCR products were separated by electrophoresis, and the most-intense bands were purified (Qiagen gel extraction kit) and sequenced using the TN5INT primer at the Core Facility at Tufts Medical School. The sequences were analyzed and compared using BLAST programs from The Institute of Genomic Research (www.tigr.org) and The Pseudomonas Genome Project (www.pseudomonas.com). In addition, the sequences were compared with the preliminary DNA sequence of *P. fluorescens* Pf0-1 obtained from Department of Energy Joint Genomic Institute databases (www.jgi.doe.gov).

Motility assays. (i) Swimming. Swim agar plates (MMO and 0.3% agar) were inoculated with bacteria from colonies grown overnight on LB agar using a sterile toothpick. After incubation overnight at 30°C, the growth away from the inoculation point was compared to that of the parent strain (6).

(ii) Swarming. Bacteria were inoculated onto swarm agar plates (0.5% agar) with a sterile toothpick. The bacterial cells were taken from swim agar plates, and the plates were incubated overnight at 30°C. A positive result was recorded as a branching growth over the agar surface away from the inoculation point (29).

Biofilm. Biofilm formation was assayed by the method of O'Toole and Kolter (27) in MMO medium supplemented with 0.2% glutamate (6). An exponential-phase culture growing in this medium was inoculated in 1 ml of MMO with 0.2% glutamate. The tubes were incubated overnight without agitation at 30°C. They were then rinsed with distilled water, stained with a 1% crystal violet solution for 15 min, and rinsed again to eliminate excess stain. A positive result was recorded as the presence of a ring at the interface between air and medium. The stained tubes were allowed to dry at room temperature for 15 min and extracted with 1.5 ml of 95% ethanol. The quantification of biofilm formation was assessed measuring the A_{600}. Each assay included an experimental background consisting of cells incubated in the medium in test tubes for 10 min; the cells were then removed, and the tube was washed and stained. The results of the control experiments showed A_{600} values ranging between 0.020 and 0.030.

This method was used to evaluate the adhesion on different abiotic surfaces, such as borosilicate glass (Fisher Scientific), polystyrene (Falcon; Becton Dickinson), and polypropylene (Falcon; Becton Dickinson).

For strains exhibiting an increased biofilm phenotype, viable-cell counts were determined on selective media. Planktonic cells were removed from the culture tubes, and the tubes were then rinsed with sterile distilled water. One milliliter of sterile distilled water was added to the tube, which was sonicated for 30 s in a Bransonic 12 water bath. Cells recovered from the biofilm were 10-fold serially diluted and plated to enumerate viable cells. After recovery of the biofilm cells, the tubes were stained with crystal violet to confirm that the biofilm had been successfully removed. To allow comparison of viable-cell counts with biofilm formation, identical culture tubes were used to stain the biofilm with crystal violet and quantify the staining as described above.

Chemotaxis assay (swarm plate assay). The medium, MMO (without glucose) and 0.2% agar, was inoculated with 10 μl of washed cell suspension (A_{530} of 0.3) of bacteria at the center of the plate. On the left side of the plate, 3 drops (10 μl each) of phosphate-buffered saline (PBS) were used as a negative control, and on the right side, 3 drops (10 μl each) of a 10% Casamino Acids solution were used as a chemoattractant. The plates were incubated overnight at 30°C. The formation of rings in the direction of the chemoattractant was recorded as a positive result. The distance between the point of inoculation and the end of the rings was measured (Fig. 1A).

Sand attachment assays. Bacterial strains grown overnight in LB broth were inoculated into 12 ml of minimal medium broth and grown to an A_{530} of 0.4 to 0.5. Cells were centrifuged for 5 min at 6,000 rpm (Sorvall RC5b Plus), and the pellets were washed twice in PBS. Cells were resuspended in 4 ml of PBS and drawn into a syringe containing 12 g of Ottawa sand (Fisher Scientific catalog no. S23-3). An aliquot of the inoculum was used to determine cell density and allow an estimate of percent attachment. The sand column was allowed to equilibrate for 5 min, and the volume contained in the column was estimated by subtracting the amount that drained out of the column in the first 5 min. Columns were incubated for 1 to 1.5 h and washed with 1x equilibration volume. Washes were serially diluted, plated onto LB agar with appropriate antibiotics, and incubated at 28°C (10). Viable-cell counts from washes were used to estimate percent attachment on the basis of the initial number of viable cells in the inoculum.

Electron microscopy. For electron microscopy, a few drops of PBS were placed on single colonies of test cultures grown on LB agar plates. Copper grids were placed on the PBS drops and allowed to draw cells for 2 min. The grids were washed in PBS by placing them for a few seconds on fresh PBS drops and then

TABLE 2. Molecular characterization of *aba* mutants in *P. fluorescens*

Locus	Gene or homologs	Possible functions	*P. fluorescens* contig[a]	Homology in *P. aeruginosa* (%)[b]
aba18	MCP (*P. aeruginosa*)	Chemotaxis transducer	307 (gene 77, nt 1443)	PA1317 (88)
aba51	MCP (*P. aeruginosa*)	Chemotaxis transducer	294 (gene 29, nt 1609)	PA4633 (55)
aba120	*flgC* (*P. aeruginosa*)	Flagellar basal body rod	254 (gene 19, nt 4)	PA1078 (76)
aba160	*rfbE* (*Synechocystis* sp.)	Perosamine synthetase	274 (gene 14, nt 940)	PA3552 (35)
aba175	No match	Unknown	229	Not found
aba177	*flgI* (*P. aeruginosa*)	Flagellar P-ring protein	238 (gene 51, nt 399)	PA1084 (82)
aba203	No match	Unknown	311	Not found

[a] nt, nucleotide.
[b] The putative *P. aeruginosa* homolog of the *aba* locus and the percent homology (shown in parentheses) are shown.

dabbed dry using Whatman paper. The grids were then stained with urinidyl acetate for 30 s. Grids were observed using a Philips electron microscope at 80 kV (9).

RESULTS

Identification of genes whose transposon insertions were affected by *adnA*. Among 12,000 *lacZ* transcriptional fusion insertion mutants screened, seven contained insertions positively regulated more than threefold by AdnA. The DNA sequences of the junctions between the 3′ end of the transposon and the proximal DNA were determined by arbitrary-primed PCR. The sequences were used for comparisons with the sequences in databases using BLAST programs (Table 2). The fold change in transcription caused by *adnA* differed for each ORF affected (Table 3).

Two insertions, *aba120* and *aba177*, were in genes affecting flagellar synthesis. In the first case, the insertion took place in a gene with homology to *flgC* of *P. aeruginosa*. The product is a flagellar protein that forms part of the proximal basal body rod. The second locus showed similarity to *flgI* in *P. aeruginosa*, a gene also involved in flagellar synthesis (coding for the protein that constitutes the P ring) (20). The β-galactosidase assay showed that *adnA* caused a strong increase in the transcription of these two genes (Table 3).

Loci *aba18* and *aba51* occurred in genes which presented significant similarities with several methyl-accepting chemotaxis proteins (MCPs) of *P. aeruginosa*. MCPs are transmembrane proteins that act as receptors to environmental signals and recognize attractants and repellents (Table 2) (36, 39). The increase in the transcription of these genes after addition of *adnA* was six- to eightfold (Table 3).

Insertion in *aba160* revealed a gene with homology to *rfbE* in *Synechocystis* sp. and *Vibrio cholerae*, which encodes the enzyme perosamine synthetase (17, 35). This gene product participates in the synthesis of the O-antigen chain of the lipopolysaccharide (LPS) in these microorganisms (35). A low similarity value was found with a gene in *P. aeruginosa* encoding a protein of unknown function located downstream of the *alg* operon, which is implicated in adhesion and LPS biosynthesis (30) (Table 2). The gene expression of *aba160* was increased 56-fold by *adnA* (Table 3).

TABLE 3. Phenotypic characterization of *aba* mutants in *P. fluorescens*

Strain or *aba* mutant	*adnA*	Swimming	Biofilm	Sand column adhesion assay[a]	Chemotaxis[b]	Flagella[c]	Fold increase effect of AdnA on gene expression[d]
Pf0-1	+	+	+	99 ± 1	+ (33)	+	ND
Pf0-2x	−	−	−	65 ± 20	−	−	ND
Pf0-2x (*adnA*)	+	+	+	92 ± 0.7	+ (28)	+	ND
aba18	−	−	+	80 ± 15	−	−	
	+	+	+	97 ± 1	+ (10)	+	6
aba51	−	−	+	64 ± 6	−	−	
	+	+	+	94 ± 2	+ (12)	+	8
aba120	−	−	−	47 ± 23	−	−	
	+	−	+	63 ± 20	−	−	64
aba160	−	−	+	27 ± 8	−	−	
	+	−	+	58 ± 2	−	−	56
aba175	−	−	−	62 ± 8	−	−	
	+	−	+	76 ± 6	−	−	34 (11)
aba177	−	−	−	26 ± 12	−	−	
	+	−	+	80 ± 14	−	−	25
aba203	−	−	+	38 ± 24	−	−	
	+	+	+	88 ± 8	+ (16)	+	35 (7.8)

[a] Values are mean percentages of cells that attached ± standard errors.
[b] For strains exhibiting chemotaxis, the distance moved (in millimeters) is shown in parentheses.
[c] Confirmed by electron microscopy.
[d] Fold increase due to AdnA was estimated by β-galactosidase assays. All assays included the control consisting of *aba* strains harboring pPC101, which carries a deletion of the N-terminus domain of AdnA. Values shown are those obtained with *adnA* expressed from plasmid pPC100, apart from those in parentheses, which were determined from strains in which *adnA*::Sm/Sp was replaced by wild-type *adnA* (see Materials and Methods for details). LacZ activity for all insertion mutants was 2 to 7 Miller units, except for the *aba175* mutant, which was 17 to 18 Miller units. ND, not determined.

Two insertions (*aba175* and *aba203*) with different phenotypes were in genes without homology to any known sequences in the databases (Table 2). The results of β-galactosidase assays indicated that AdnA increased expression in the *aba175* mutant by 34-fold and led to a 35-fold increase in expression of the fusion in the *aba203* mutant (Table 3). To address the possibility that these novel ORFs were adversely influenced by AdnA expression from a multicopy plasmid, we constructed *aba175* and *aba203* strains that had single chromosomal copies of *adnA*. The results of β-galactosidase assays with these strains confirmed that AdnA activates transcription of the ORFs when *adnA* is present in its normal genomic context (Table 3). Since these ORFs have no similarity to known genes, we also used reverse transcription-PCR and confirmed that they are transcribed in the parental strain Pf0-1, lending support to the description of these ORFs as genes (data not shown).

Assay for chemotaxis. A swarm plate assay tested the chemotaxis behavior of the strains using Casamino Acids as the chemoattractant. The formation of migrating rings was observed in the wild-type strain Pf0-1 (Fig. 1A). A positive result was obtained in the wild-type strain and in all those mutants showing flagella after complementation with *adnA,* although in the mutants, the distance between the point of inoculation and the end of the rings was shorter than that observed in the wild type, indicating that the genes disrupted may be involved directly or indirectly in chemotaxis (Table 3).

Analysis of motility (swimming and swarming). For the swimming assay, we used a semisolid agar (0.3%) where cells swim through water-filled channels to form concentric rings away from the point of inoculation (29). *P. fluorescens* Pf0-2x, the *adnA* deletion mutant, exhibited defective swimming compared to Pf0-1, in accord with the central role in flagellar synthesis attributed previously to *adnA* (6). In the absence of *adnA,* all mutants were nonmotile. After complementation with *adnA*, only *aba18*, *aba51*, and *aba203* mutants showed a swimming phenotype, indicating that the insertions were not in genes essential for swimming. Note that *aba18* and *aba51* insertion mutants showed a decreased ability to swim compared to the wild type (Fig. 1B).

Swarming motility is a surface translocation that depends on extensive flagellation and cell-cell contact. Swarmer cells move over the surface of the agar within the slime that surrounds the colony. A positive result appears as a branching pattern of growth away from the point of inoculation when the strains are inoculated on 0.5% agar (29). *P. fluorescens* Pf0-2x and all mutants without *adnA* were deficient in swarming motility. Complementation of the insertion mutants with plasmid pPC100 restored swarming in *aba18*, *aba51* and *aba203* mutants, as seen in the swimming assay (data not shown).

Presence of flagella by electron microscopy. The formation of flagella appeared to be affected in four of seven insertion mutants. All the insertion mutants showed lack of flagella in the absence of *adnA*, whereas *aba120*, *aba160*, *aba175*, and *aba177* mutants lacked flagella in the presence of *adnA*. These results suggest that these insertions affected genes coding for components of the flagellum or its assembly (data not shown).

Analysis of biofilm formation. The strains were tested for their ability to form biofilms on three different abiotic surfaces, polypropylene, polystyrene (hydrophobic), and borosilicate glass (hydrophilic) (Fig. 2).

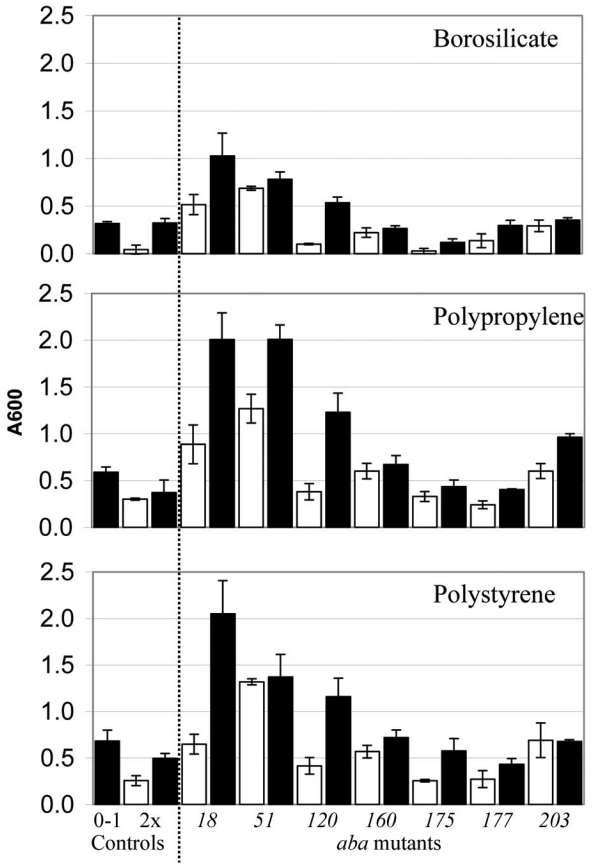

FIG. 2. Biofilm formation of *P. fluorescens* Pf0-1 and *aba* mutants on different substrates. Crystal violet rings were dissolved in ethanol, and A_{600} was read. Experiments were performed at least three times. Strains in which *adnA* had been deleted (white bars) and strains where *adnA* was carried on a plasmid or in wild-type Pf0-1 (black bars) are shown. Pf0-1 (0-1) and Pf0-2x (2x) were used as controls.

P. fluorescens Pf0-1 was able to form a biofilm on all surfaces tested. On the other hand, the ability of Pf0-2x to form a biofilm was reduced on all surfaces tested. Pf0-2x complemented with *adnA* showed similar adhesion values to Pf0-1 in borosilicate glass, whereas for the other surfaces tested, these values were lower.

Surprisingly, the insertion mutations *aba18*, *aba51*, *aba160*, and *aba203* restored biofilm formation to Pf0-2x in the absence of *adnA*. These mutants did not have flagella (and were nonmotile), a finding which indicates that flagella are not essential for biofilm formation and that other flagellum-independent mechanisms are involved in the adhesion properties of *P. fluorescens*.

Some of the mutants (*aba18* mutant complemented with *adnA*, *aba51*mutant with or without *adnA,* and *aba120* mutant with *adnA*) showed enhanced biofilm formation compared to the wild type (1.5- to 3-fold increase in crystal violet staining) on all surfaces tested. *aba18* mutant without *adnA* formed more biofilm than Pf0-1 on borosilicate glass and 5- to 10-fold-more crystal violet staining than Pf0-2x complemented with *adnA* on polypropylene. In contrast, *aba203* mutant comple-

mented with *adnA* was more adherent than Pf0-1 only on polypropylene (Fig. 2). Similar viable counts of cells from *aba51* and Pf0-1 biofilms suggest that the enhanced biofilm was not due to the presence of more cells (data not shown). These data concur with the results of preliminary microscopic analyses showing different architecture for biofilms formed by Pf0-1 and the *aba* mutants (unpublished data).

In most instances, the strains defective in biofilm formation on one of the materials were also affected in the ability to form biofilm on the other substrates. In most mutants (*aba18*, *aba120*, *aba175* and *aba177* mutants), the adhesion increased on all surfaces after the addition of AdnA. In *aba120* and *aba175* mutants, the increase was greater on borosilicate glass than on the other surfaces. In *aba51* and *aba203* mutants, the presence of AdnA increased adhesion only to polypropylene tubes. Complementation of *adnA* in *aba160* mutants, however, did not increase the biofilm formation on any of the surfaces tested. These findings suggest the existence of a common genetic pathway but also divergent processes to form biofilm on a variety of abiotic surfaces in *P. fluorescens* (Fig. 2). In this regard, complementation with *adnA* restored or increased biofilm formation in those mutants unable to form biofilms, including those which lacked complementation in the formation of flagella.

Sand column adhesion assay. The response of the insertion mutants in the sand adhesion assay varied between 26 and 97% in the presence and absence of AdnA, suggesting a high variability in the effect of the insertion on attachment (Table 3). Attachment of Pf0-2x was 65% compared to 99% for the wild-type Pf0-1. Complementation of Pf0-2x containing pPC100 brought the attachment value near the wild-type value (92%). Complementation of the insertion mutants with AdnA increased attachment to sand. In the absence of AdnA, insertion mutants *aba120*, *aba160*, *aba177*, and *aba203* exhibited impaired attachment to sand and had values lower than that for the *adnA* deletion strain, whereas insertion mutants *aba18*, *aba51*, and *aba175* showed little increase or were unaffected in sand attachment compared to Pf0-2x.

In the presence of AdnA, insertion mutants *aba51* and *aba203* showed attachment values similar to that of *adnA*-complemented Pf0-2x, indicating that those insertions do not have an effect on attachment. Insertion mutants *aba120*, *aba160*, *aba175* and *aba177* showed values lower than those of the *adnA*-complemented deletion strain, suggesting that the mutated *aba* genes are important for sand adhesion. Note that insertion mutant *aba18*, complemented or not complemented with *adnA*, was not significantly different from the wild-type strain (Table 3).

DISCUSSION

AdnA in *P. fluorescens* is a transcriptional regulator involved in motility, adhesion to sand columns and seeds, and survival in soil (21). The closest matches of AdnA are the transcriptional regulators FleQ (*P. aeruginosa*), FlrA (*Vibrio parahaemolyticus*), and FlaK (*V. cholerae*) that interact with σ^{54} and control early stages in the regulatory cascade of polar flagellar synthesis (2, 6, 23).

Using transposon mutagenesis, we identified seven mutants with insertions positively regulated by *adnA*. In the absence of *adnA*, all the mutants were nonmotile, consistent with the lack of flagella reported previously in strains without *adnA* expression (10).

Complementation with *adnA* did not restore the formation of flagella in four mutants (*aba120*, *aba177*, *aba160*, and *aba175* mutants), indicating that the insertions were in genes involved in flagellar synthesis. The insertion in the *aba160* mutant was in a putative *rfbE* gene, suggesting a role in LPS synthesis. Alterations in the expression of LPS can interfere with the assembly of flagella (14). Thus, it may not be surprising that AdnA affects flagellum formation at different levels, including the assembly step in which LPS seems to be involved. If this were true, the AdnA homologues would assume an essential role in pathogenesis, especially in bacterial pathogens such as *P. aeruginosa* and *V. cholerae* in which LPS is one of the most important virulence factors.

The complementation with *adnA* in *aba18*, *aba51*, and *aba203* mutants restored flagella and motility indicating that these genes are not involved in flagellar synthesis and may participate in novel aspects of cell physiology. The sequence interrupted in *aba203* has no similarity to any predicted genes. The flanking regions in the insertions of *aba18* and *aba51* gave matches to different MCPs in *P. aeruginosa*. These transmembrane receptor proteins detect environmental signals and activate the components of a cytoplasmic phosphorylation cascade that controls the direction of flagellar rotation (5). Our results suggest that *adnA* is also involved in the regulation of transmembrane components of the chemotaxis system. Chemotaxis assays were positive in the *adnA*-complemented mutants with defects in MCPs, although none of the mutants reached the value obtained with the wild type. Since the chemoattractant was a mixture of different amino acids, there are likely to be different chemoreceptors. The redundancy and/or overlapping of signaling receptors in *P. aeruginosa* have been described previously (36). Also, small-colony spontaneous mutants showed reduced chemotaxis, swimming, and twitching and increased ability to form biofilms (12). Insertion mutants *aba18* and *aba51* behaved similarly to the *P. aeruginosa* small-colony mutants; however, they did not present a small-colony morphology.

The study of adhesion using the biofilm and the sand column assays also provided interesting findings. First, the Tn*5* mutations in *aba18*, *aba51*, *aba160,* and *aba203* mutants suppressed the biofilm deficiency, which resulted from disrupting *adnA*. Insertions *aba18* and *aba51* affected the MCPs, and since MCPs are surface membrane proteins, their alteration may create modifications at the membrane level that increase the adhesive properties, or the mutations activate alternative paths to biofilm formation. Haugo and Watnick recently identified a mutant of *V. cholerae* which had a "superbiofilm" phenotype, similar to that of *aba18* and *aba51* mutants (15). In contrast to the findings of that study, the superbiofilm mutants identified here did not show a higher cell density than the wild type, indicating novel structural changes to the biofilm. In the *aba160* mutant, alterations in LPS from the insertion in the putative *rfbE* homologue may change the properties of the membrane and/or lead to alternative biofilm production. In this context, Williams and Fletcher (38) observed an increase in the adhesion of a mutant of *P. fluorescens* that lacked the O antigen. Interestingly, an *rfbE* transposon insertion mutant in

E. coli O157:H7 deficient in O-antigen expression was significantly more adherent than the parent strain (3). The *aba203* mutant was also able to form a biofilm on different surfaces in the absence of AdnA, indicating that the insertion affected a gene (yet unknown function) whose inactivation led to increased AdnA-independent adhesion.

Other mutants were able to form a biofilm in the absence of flagella, but they required AdnA, indicating that AdnA-dependent mechanisms other than flagella were involved. In this group were the *aba175* mutant and mutants affected in structural flagellar genes (*aba120* and *aba177* mutants).

Comparison of the sand column adhesion and biofilm results indicates that different underlying mechanisms affect these two adhesion assays. The insertions in *aba120*, *aba160*, *aba177*, and *aba203* mutants further exacerbate the sand adhesion defect that initially resulted from the *adnA* mutation. However, in the case of *aba160* and *aba203* mutants, this is perplexing because the Tn*5* insertions can suppress the requirement for AdnA in biofilm formation on plastic and glass surfaces, while no such effect was observed for *aba120* or *aba177* mutants. The data for *aba160* and *aba203* mutants also contrast with the data for *aba18* and *aba51* mutants; for the latter mutants, the insertion resulted in a similar suppression of the AdnA requirement in biofilm formation but had little impact on sand adhesion. While superficially the sand adhesion and biofilm assay systems are both measures of adhesion, there are clearly distinctions. The results make both systems valuable when used in concert to study attachment. These differences may reflect the characteristics associated with early and late events in biofilm formation, since in the sand assay the bacteria had a maximum of 1.5 h to attach, whereas the biofilm assay was typically carried out overnight (12 to 16 h).

Complementation with *adnA* reveals the important role of AdnA in biofilm formation. In *aba18*, *aba120*, *aba175*, and *aba177* mutants, the adhesion increased on all surfaces. In the first three mutants of this group, the difference was more significant on borosilicate glass. In *aba51* and *aba203* mutants, the biofilm formation increased only for polypropylene. These results suggest the existence of different pathways for biofilm formation, depending on the surface, or underline differences between early and late attachment events. In *aba160* mutants, *adnA* only minimally affected biofilm formation, but a greater effect was observed in the sand column assay, thus distinguishing between these two types of adhesion assays.

Previous studies in *P. fluorescens* reported that cells can form a biofilm on abiotic surfaces without flagella under certain environmental conditions, such as different carbon sources and/or iron levels (26). Also, Watnick and Kolter (37) demonstrated that in *V. cholerae* flagella facilitate, but are not absolutely required for, attachment. Our findings show that flagella, while important, are not necessary for adhesion to sand and biofilm formation and that another AdnA-dependent mechanism is involved in this process. We found mutants with insertions in genes involved in flagellar synthesis or chemotaxis and others in genes without known physiologic consequences which still formed biofilms. The first group validates the results of our screen and confirms the role of *adnA* in the control of the flagellar synthesis like its homologues in other microorganisms. The other mutants present AdnA as an activator regulating new functions in the bacterial physiology. The results of this first approach to the AdnA regulon reveal that the transcriptional regulators of the polar flagellar system may be involved in other aspects of cell function.

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