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Phosphorylation of the *Pseudomonas aeruginosa* Response Regulator AlgR Is Essential for Type IV Fimbria-Mediated Twitching Motility

Cynthia B. Whitchurch,[1] Tatiana E. Erova,[2] Jacqui A. Emery,[1] Jennifer L. Sargent,[1] Jonathan M. Harris,[1] Annalese B. T. Semmler,[1,3] Michael D. Young,[1,3] John S. Mattick,[1,3] and Daniel J. Wozniak[2]*

ARC Special Research Centre for Functional and Applied Genomics, Institute for Molecular Bioscience,[1] and Department of Biochemistry, University of Queensland,[3] Brisbane, Queensland 4072, Australia, and Department of Microbiology and Immunology, Wake Forest University School of Medicine, Winston-Salem, North Carolina[2]

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The response regulator AlgR is required for both alginate biosynthesis and type IV fimbria-mediated twitching motility in *Pseudomonas aeruginosa*. In this study, the roles of AlgR signal transduction and phosphorylation in twitching motility and biofilm formation were examined. The predicted phosphorylation site of AlgR (aspartate 54) and a second aspartate (aspartate 85) in the receiver domain of AlgR were mutated to asparagine, and mutant *algR* alleles were introduced into the chromosome of *P. aeruginosa* strains PAK and PAO1. Assays of these mutants demonstrated that aspartate 54 but not aspartate 85 of AlgR is required for twitching motility and biofilm initiation. However, strains expressing AlgR D85N were found to be hyperfimbriate, indicating that both aspartate 54 and aspartate 85 are involved in fimbrial biogenesis and function. *algD* mutants were observed to have wild-type twitching motility, indicating that AlgR control of twitching motility is not mediated via its role in the control of alginate biosynthesis. In vitro phosphorylation assays showed that AlgR D54N is not phosphorylated by the enteric histidine kinase CheA. These findings indicate that phosphorylation of AlgR most likely occurs at aspartate 54 and that aspartate 54 and aspartate 85 of AlgR are required for the control of the molecular events governing fimbrial biogenesis, twitching motility, and biofilm formation in *P. aeruginosa*.

The bacterium *Pseudomonas aeruginosa* is a serious and life-threatening pathogen of immune-compromised patients, such as those suffering from AIDS or severe burns or who are undergoing cancer chemotherapy or organ transplantation, and is one of the most common causes of hospital-acquired infections. *P. aeruginosa* is also responsible for >95% of the mortality of cystic fibrosis (CF) patients (13, 31). Chronic infection of the lower respiratory tract of CF patients results in the emergence of mucoid strains of *P. aeruginosa* which overproduce the highly viscous exopolysaccharide alginate. Alginate production plays an important role in CF infections by serving an adherence function and by conferring protection to the bacterium from both antibiotics and the host immune system (13).

Colonization and establishment of infection by *P. aeruginosa* are dependent on the production of a number of virulence factors, including extracellular toxins, proteases, lipases, siderophores, and polar filamentous structures called type IV fimbriae. These fimbriae are essential for the attachment of the pathogen to host epithelial tissues and mediate a form of surface translocation known as twitching motility, which is implicated in the spread of infection (23) or the aggregation into microcolonies during biofilm formation (30). Mutants that either lack type IV fimbriae or produce nonfunctional fimbriae show reduced infectivity (18, 23). Twitching motility has been shown to occur by fimbrial extension and retraction, probably involving the assembly and disassembly of the fimbrial strand (25, 38).

Unlike *P. aeruginosa* strains commonly isolated from acute infection sites, isolates from chronic CF pulmonary infections characteristically produce colonies which are mucoid in appearance due to the production of copious amounts of alginate. High alginate production requires activation of the alternative sigma factor σ^{22} (otherwise referred to as AlgT or AlgU), which belongs to the RpoE family of extracytoplasmic function sigma factors. AlgT activity is modulated by the anti-sigma factor MucA, which is encoded within the *algTmucABCD* operon. Active AlgT induces the expression of a number of genetic loci required for alginate production including the *algT* operon itself, the *algD* operon which encodes most of the genes required for alginate biosynthesis, the *algB* operon, and *algR* (13, 46).

AlgR and AlgB belong to the superfamily of response regulators of prokaryotic two-component regulatory systems (6, 13, 16, 21). These systems are commonly comprised of two proteins: a sensor kinase and a response regulator. Typically at the N terminus of the sensor kinase is an input domain which functions to detect environment stimuli. Situated at the C terminus is a conserved domain known as a histidine protein kinase or transmitter. This domain is comprised of conserved motifs involved in nucleotide binding and a block of residues known as the H box, which contains a conserved histidine residue. Upon detection of a signal, the sensor kinase autophosphorylates this histidine by using the γ-phosphoryl group of ATP. The phosphate is then transferred from the sensor kinase to a conserved aspartate residue

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Wake Forest University School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157. Phone: (336) 716-2016. Fax: (336) 716-9928. E-mail: dwozniak@wfubmc.edu.

located in the receiver module or CheY-like domain of a second protein, the response regulator. The receiver domain is normally situated in the N terminus of this protein. Phosphorylation of the conserved aspartate of the receiver module usually activates the response regulator to elicit a response, commonly transcriptional activation of a target gene or genes via a domain located in the C terminus of the protein.

Both AlgR and AlgB control alginate levels by activating transcription of *algD*, the first gene of the alginate biosynthetic operon. Although AlgR lacks an obvious DNA binding domain, it is known to activate *algD* transcription by binding to three sites in the *algD* promoter region, two of which are located unusually far upstream of the *algD* transcription start site (12, 26). AlgR is also required for type IV fimbrial biogenesis and twitching motility in *P. aeruginosa*, yet the mechanism by which AlgR controls twitching motility is currently unclear (45). The gene *fimS* located immediately upstream of *algR* is also required for twitching motility and encodes an atypical sensor kinase that appears to be incapable of autophosphorylation as it lacks the conserved residues essential for ATP binding (45).

Aspartate 54 of AlgR is a highly conserved residue present in all members of the response regulator superfamily and is the predicted phosphorylation site of AlgR. Neither aspartate 54 nor a second conserved residue of the receiver domain of AlgR (aspartate 85) are required for alginate production in mucoid strains of *P. aeruginosa* (21), which typically have mutations in *mucA* (22). In this paper, we investigate the mechanism by which AlgR modulates type IV fimbria-mediated twitching motility in nonmucoid *P. aeruginosa*. We show that aspartate 54 of AlgR is the most likely site of phosphorylation and is critical for its role in twitching motility and in biofilm initiation.

MATERIALS AND METHODS

Strains, plasmids, and media. Bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* and *P. aeruginosa* liquid cultures were maintained in Luria-Bertani (LB) broth (4), and solid media were prepared by adding 1 to 1.5% agar. Light microscopy was performed using nutrient media (4 g of tryptone liter^{-1}, 2 g of yeast extract liter^{-1}, and 2 g of NaCl liter^{-1}) solidified with 8 g of GelGro (ICN) liter^{-1} for greater optical clarity. The following antibiotic concentrations were used for the selection of *E. coli*: 12.5 μg of tetracycline ml^{-1}, 100 μg of ampicillin ml^{-1}, and 50 μg of kanamycin ml^{-1}. The concentrations of antibiotics for selection in *P. aeruginosa* were 500 μg of carbenicillin ml^{-1}, 20 μg of rifampin ml^{-1}, and 200 μg of tetracycline ml^{-1}. Plasmid pJK223R1 (kindly supplied by A. Chakrabarty) was used for expression and purification of wild-type AlgR. To generate pJK223R1-derived plasmids for overexpression of AlgR mutants, the 545-bp *Xho*I-*Pst*I fragments from pUS152 (*algR7*), pUS164 (*algR10*), or pUS165 (*algR11*) (21) were used to replace the identical fragment in the wild-type *algR* allele of pJK223R1, resulting in pUS162, pUS170, and pUS172, respectively. All replacements were verified by DNA sequence analyses. Plasmids pUS150 (wild-type *algR*), pUS157 (*algR7*), pUS166 (*algR10*), and pUS168 (*algR11*) were used in gene replacements (21). Plasmid pRS1, which expresses CheA fused to a C-terminal His tag, was supplied by R. Bourret and used as a source of CheA in the phosphorylation studies. Plasmid pDJW220 (5) was used as the source of *algD* in the DNA binding studies. Plasmid pGemAlgRD54N was generated by PCR amplification of PAK*algR7* genomic DNA and subcloned into the pGemT-easy vector. pUCPAlgRD54N was generated by subcloning *algR7* as a *Not*I fragment from pGemAlgRD54N into pUCPKS.

Construction of isogenic mutants. Allelic exchange mutants of *algD* were constructed using the sucrose selection system described previously (1, 35). Briefly, *algD* was obtained by PCR amplification using the primers *algD*-Forward and *algD*-Reverse and subcloned into the vector pUK21. The tetracycline gene cartridge from pSM-TET was cloned into the *Eco*RI site within *algD* and to disrupt the gene. The resulting clone was then digested with *Spe*I, whose sites span the multiple cloning site of pUK21, and the disrupted gene was inserted into the suicide vector pRIC380 (1). This vector carries the genes *sacBR*, which promote sensitivity to sucrose, and *oriT*, which enables conjugal transfer. The constructs were then transformed into the *E. coli* donor strain, S17-1, in preparation for mating into *P. aeruginosa*. Following conjugation, the transconjugates were selected on 5% sucrose media containing tetracycline. This forces the excision of the plasmid while leaving the recombined mutated gene in the chromosome. Mutants were confirmed using Southern analysis. All *algR* mutants in strains PAO1 and PAK were obtained by allelic exchange techniques with plasmids pUS150 (wild-type *algR*), pUS157 (*algR7*; AlgR D54N), pUS166 (*algR10*; AlgR D85N), or pUS168 (*algR11*; AlgR D54N D85N) as previously described (21). *algR* allelic exchange mutants were confirmed by both Southern and sequence analyses. The primers *algR*-Forward and *algR*-Reverse were used to PCR amplify *algR* alleles from genomic DNA. PCR products were ligated into pGEMT-easy and recombinant clones were sequenced.

Recombinant DNA techniques and sequence analysis. The preparation of plasmid DNA, restriction endonuclease digestion (New England Biolabs), ligation reactions, PCR, Southern blotting, and radiolabeling of probe were carried out using standard protocols (4). Recombinant clone miniprep plasmid DNA was sequenced using big-dye terminator chemistries and *Taq* cycle sequencing kits from Perkin-Elmer Applied Biosystems and analyzed on an ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems). Automated DNA sequencing was performed by the Australian Genome Research Facility, University of Queensland, Brisbane, Queensland, Australia.

Western blotting. Bacterial cells from plates were resuspended to an optical density at 600 nm (OD_{600}) of 1.0 in 50 mM sodium carbonate buffer, pH 9.6. Samples (1.0 ml) were centrifuged, and the cell pellet was resuspended in 100 μl of sample buffer (60 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue). Samples were passed 10 times through a 27-gauge needle to shear chromosomal DNA, heated at 100°C for 5 min, separated by SDS-polyacrylamide gel electrophoresis (PAGE) (15% polyacrylamide gel with a 5% stacking gel [20]), and transferred electrophoretically to Hybond-C nitrocellulose (Amersham) with the Tris-glycine system (39). PilA was detected with anti-PilA antiserum (1:5,000) followed by goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (1:5,000; Boehringer Mannheim). Polyclonal antiserum against AlgR was elicited in New Zealand White rabbits (Immunodynamics, Inc.) with partially purified wild-type AlgR (approximately 500 μg). Anti-AlgR antibodies were used in immunoblottings at a dilution of 1:5,000 with chemiluminescent reagents by procedures outlined by the manufacturer (Amersham), and film was exposed for 5 min prior to development.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was based on a method described by Engvall and Perlmann (11). The cells were resuspended in 50 mM sodium carbonate buffer, pH 9.6, at an OD_{600} of 1.0, and 200 μl was loaded into wells of a 96-well ELISA plate. After overnight incubation at 4°C, the wells were washed with phosphate-buffered saline containing 0.1% Tween 20, blocked with 3% bovine serum albumin for 1 h and then exposed to an anti-PilA antibody at a starting dilution of 1:500 for 2 h at 37°C. After removal of antiserum, the wells were again washed with phosphate-buffered saline containing 0.1% Tween 20, and then goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase was added (1:5,000) and incubated for 2 h at 37°C. Detection was carried out using 20 mg of *p*-nitrophenyl phosphate (Sigma) ml^{-1} in 1 M Tris buffer (pH 8.0), and the tray was read at 405 nm using an ELISA reader (Bio-Rad).

Twitching motility assay. Twitching motility was assayed as described previously (3). Briefly, the *P. aeruginosa* strain to be tested was stab inoculated through a 1% agar plate, and after overnight growth at 37°C the zone of twitching motility between the agar and petri dish interface was visualized by staining with Coomassie brilliant blue R250 stain.

Light microscopy. Light microscopy was performed as described previously (36). Sterile microscope slides were submerged in molten GelGro media to obtain a thin layer of media coating the slide. The slides were allowed to set in a horizontal position and air dried briefly prior to use. The slides were then inoculated with a small loopful of bacteria taken from an overnight plate culture. A sterile glass coverslip was placed over the point of inoculation, and the slide was transferred to a large petri dish containing a moist tissue and sealed with Nescofilm (Bando Chemical Industries) to maintain humid conditions. Incubation times ranged from 2 to 20 h at 37°C. Slide cultures were examined using a Zeiss microscope Axioskop 50 with Nomarski facilities at a 200× to 400× magnification. Images were captured via a JVC TK-CI38IEG video camera connected directly to a Macintosh 8600 using the software NIH Image (National Institutes of Health). Time-lapse video microscopy was performed in a room

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

Strain, plasmid, or oligonucleotide	Description or sequence[a]	Source or reference
E. coli		
DH5α	*hsdR recA lacZYA* ϕ80*lacZ*ΔM15	Promega
JM109	e14$^-$ *mcrA recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ZΔM15]	Promega
MV1184		41
P. aeruginosa		
PAK	Nonmucoid *P. aeruginosa* strain K	D. Bradley
PAK*pilA*::Tc^r	Previously referred to as AWK	44
R306	PAK*pilV*::Tn*5*-B21	2
S39	PAK*algR*::Tn*5*-B21	45
PAK*algD*::Tc^r	Tc^r cassette insertion in *Eco*RI site of *algD*	This study
PAK*algR7*	PAK encoding AlgR D54N	This study
PAK*algR10*	PAK encoding AlgR D85N	This study
PAK*algR11*	PAK encoding AlgR D54N D85N	This study
PAO1	Nonmucoid *P. aeruginosa* strain O1	
WFPA1	*algD*::*tet* in PAO1	This study
WFPA12	*algR*::*aac1* in PAO1	This study
WFPA8	*algR7* in PAO1 (encoding AlgR D54N)	This study
WFPA13	*algR10* in PAO1 (encoding AlgR D85N)	This study
WFPA16	*algR11* in PAO1 (encoding AlgR D54N D85N)	This study
WFPA6	*algR* gene replacement of WFPA12	This study
Plasmids		
pSM-TET	Source of Tc^r cassette	27
pRIC380	*P. aeruginosa* suicide vector	1
pUK21	Km^r cloning vector	42
pUCPKS	*P. aeruginosa-E. coli* shuttle vector	43
pGEMT-easy	*E. coli* cloning vector	Promega
pUCPAlgR	*algR* cloned into pUCPKS	45
pGemAlgRD54N	*algR7* cloned into pGEMT-easy	This study
pUCPAlgRD54N	*algR7* cloned into pUCPKS	This study
pDJW106	*algR* cloned in pALTER-1	21
pJK223R1	Source of AlgR	19
pUS152	*algR7* in pALTER-1	21
pUS164	*algR10* in pALTER-1	21
pUS165	*algR11* in pALTER-1	21
pUS162	*algR7* in pJK223R1 (source of AlgR D54N)	This study
pUS170	*algR10* in pJK223R1 (source of AlgR D85N)	This study
pUS172	*algR11* in pJK223R1 (source of AlgR D54N D85N)	This study
pUS150	Wild-type *algR* in gene replacement vector pEX100T	21
pUS157	*algR7* in gene replacement vector pEX100T	21
pUS166	*algR10* in gene replacement vector pDJW525	21
pUS168	*algR11* in gene replacement vector pDJW525	21
pRS1	Source of His-CheA	R. Bourret
pDJW220	*algD* cloned in pALTER-1	5
Primers		
algR-Forward	5′-TAAAGCGAGTCTCAGCGTCG-3′	This study
algR-Reverse	5′-GGGACGACATGGGATATTCC-3′	This study
algD-Forward	5′-CGCGGATCCGAGGTGAATGCGATGCG-3′	This study
algD-Reverse	5′-TGCTCTAGACTAGGAGCAGATGCCCTC-3′	This study
*algD*33	5′-AGCCCTTGTGGCGAATAGGC-3′	This study
*algD*36	5′-GAATTGGGGAAAAGTCTGTG-3′	This study

[a] Tc^r, tetracycline resistance; *aac1*, gentamicin resistance; Km^r, kanamycin resistance.

heated to 30°C. Video images were recorded over a period of 2 to 4 h at speeds of either 1 field/3.22 s, 1 field/0.66 s, or real time (1 field per 1/50 s) using a JVC TK-CI38IEG video camera connected to a Sanyo TLS-S2500P time-lapse video recorder. Video images were edited and converted to Quick-time movies using Avid Videoshop version 3.0 and can be viewed online (http://www.cmcb.uq.edu.au/cmcb/PUBS/twitch.html).

Purification of proteins. *E. coli* MV1184 cells harboring pJK223R1 (19) and *E. coli* JM109 cells harboring pUS162, pUS170, and pUS172 (see above) were grown in LB or M9 minimal medium supplemented with ampicillin at 37°C. A 5-ml overnight culture was inoculated into 1,000 ml of the same medium and grown to an A_{600} of 0.3. For induction, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the cells were grown for another 3 to 4 h. The cells were harvested and resuspended in 10 ml of TMBG buffer (50 mM Tris-HCl [pH 7.6], 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10% [vol/vol] glycerol) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF). A French press (15,000 lb/in^2) was used to make cell extracts. Cell debris was removed by centrifugation at 13,000 × *g* for 20 min. Cell extracts were subjected to 60% ammonium sulfate and centrifuged as described above, and the pellets were resuspended in 4 ml of TMBG and dialyzed exhaustively against TMBG. The samples were applied to a heparin agarose column (bed volume, 10.0 ml;

Sigma) equilibrated with TMBG buffer. After washing, proteins were eluted with a linear NaCl gradient (0 to 1.0 M) prepared in TMBG buffer (flow rate of 1.0 ml per min). AlgR was found to elute in the 0.8 to 1.0 M NaCl fractions. When these preparations were examined by SDS-PAGE and image analyses, AlgR was estimated to be >85% pure.

A C-terminal hexahistidine-tagged CheA fusion protein was expressed and purified from RBB1296 (*E. coli* M15/pREP4/pRS1). Briefly, a 100-ml culture of RBB1296 was cultured in L broth (ampicillin and kanamycin) to an A_{600} of 0.3. IPTG was added (0.2 μM), and the cells were cultured for an additional 3 to 4 h, harvested by centrifugation, and suspended in 5 ml of lysis buffer (100 mM Na_2HPO_4, 10 mM Tris-HCl [pH 8.0], 6.0 M guanidine hydrochloride [GuHCl], 0.5 M NaCl, 10 mM imidazole). The cells were incubated with shaking for 1 h at 37°C and then centrifuged (15,000 × *g* for 30 min), and the supernatant was applied to a Ni-nitrilotriacetic acid agarose column equilibrated with lysis buffer. The column was washed with lysis buffer and CheA refolded with a gradient of decreasing ratios of buffer A (4.8 M GuHCl, 20% glycerol, 0.2 mM PMSF) to buffer B (200 mM Na_2HPO_4, 20 mM Tris-HCl [pH 8.0], 1.0 M NaCl, 20 mM imidazole, 20% glycerol, 0.2 mM PMSF). CheA was recovered in 0.5 ml of elution buffer (200 mM Na_2HPO_4, 20 mM Tris-HCl [pH 8.0], 1.0 M NaCl, 20% glycerol, 0.2 mM PMSF, 0.25 M imidazole) and dialyzed against FBG buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM $MgCl_2$, 5% glycerol).

Electrophoretic mobility shift assay (EMSA). DNA-binding assays were performed as described previously (5) using AlgR proteins purified as above. A radiolabeled *algD* fragment was prepared by PCR amplification of *algD* sequences in pDJW220 with *Taq* polymerase (Promega) and the oligonucleotides *algD*33 and *algD*36. Binding assays were performed using AlgR (100 to 200 ng) with buffers and conditions outlined elsewhere (5). Following electrophoresis, the gels were dried under a vacuum and subjected to autoradiography.

In vitro phosphorylation assays. The conditions used in the autophosphorylation of CheA and phosphotransfer from CheA to AlgR have been described previously (10). Autophosphorylation of CheA (1 μg) was performed with 50 μCi of [γ-^{32}P]ATP at 25°C in 10 μl of phosphorylation buffer (100 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 50 mM KCl) for 1 h. An aliquot (1 μl [100 ng of CheA]) was removed and added to 1.0 μg of purified AlgR, AlgR D54N, AlgR D85N, or AlgR D54N D85N in 10 μl of phosphorylation buffer. Phosphorylation of AlgR was carried out for 1 h at 37°C. The reaction was stopped by the addition of 10 μl of SDS-PAGE sample buffer (60 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.1 mg of bromophenol blue/ml, 5% 2-mercaptoethanol) and placing samples at −20°C. Radiolabeled products were detected by autoradiography after their separation by SDS-PAGE (12% polyacrylamide).

Biofilm assays. Biofilm formation was assayed in polyvinyl chloride microtiter plates and quantitated by crystal violet staining as previously described (29). Biofilm formation was assayed in triplicate every 2 h over an 8-h time course with the strains indicated in Fig. 4.

Homology modeling of AlgR. The AlgR receiver domain model was constructed using the homology modeling program SPDV3.7 (14, 15). A starting model was constructed by threading the primary sequence of the AlgR receiver domain onto the three-dimensional structure of NarL, the nitrate response regulator protein of *E. coli* (PDB ID1A04). Following threading, the two primary amino acid sequences were structurally aligned. Nonhomologous loops were then rebuilt from minithreading templates obtained by scanning a database of peptide fragments. The resulting model was subject to energy minimization using the program GROMOS (40). This was used to give a final minimized model that was subject to Ramachandran analysis (32) and further quality checking with the WhatIf suite of programs (www.cmbi.kun.nl/swift/whatcheck/). Electrostatic potentials for this finalized coordinate file were then calculated using the DELPHI (17) implementation within Insight II and displayed using the surface modeler GRASP (28).

RESULTS AND DISCUSSION

Aspartate 54 of AlgR is required for phosphorylation of AlgR by enteric CheA. While the source of AlgR phosphorylation within *P. aeruginosa* is unknown, it has been previously demonstrated that AlgR can be phosphorylated in vitro by the enteric histidine kinase CheA and by small phosphate donors, such as carbamyl phosphate and acetyl phosphate (10). However, the residue(s) at which this phosphorylation occurs has not yet been established. Considering that aspartate 54 of AlgR is highly conserved among response regulators (Fig. 1A) and is the site of phosphate acceptance in other response regulators such as NtrC and CheY (33, 34), we predict that Asp 54 is also likely to be the residue at which AlgR phosphorylation occurs in *P. aeruginosa*.

We used the technique of homology modeling to generate a putative molecular model of the AlgR receiver domain (Fig. 1B). Homology modeling of AlgR using the solved X-ray crystal structure of the receiver domain of NarL, the nitrate response regulator protein of *E. coli*, produced a structure with good statistical qualities as judged by Ramachandran analysis and WhatIf. Examination of the model AlgR structure shows that residues aspartate 54 and aspartate 85 are both exposed to solvent and lie in a plane at a common surface of AlgR (Fig. 1B). Given the similar positioning of these residues, we included both in our studies of AlgR phosphorylation.

We generated AlgR proteins with asparagine (N) substitutions in aspartate (D) residues D54 and D85 located in the N-terminal receiver domain (Fig. 1A) (21). Wild-type and mutant AlgR proteins (AlgR D54N, AlgR D85N, and AlgR D54N D85N) were partially purified and their capacity for phosphorylation by enteric CheA was examined. While CheA was capable of phosphorylating wild-type AlgR as well as AlgR D85N, no phosphorylation of AlgR D54N was observed (Fig. 2A).

The fact that AlgR D85N is phosphorylated by CheA indicates that replacing the acidic aspartate residue with neutral asparagine does not cause misfolding of this domain and does not significantly alter the accessibility of CheA to this region of the AlgR receiver domain. In support of this, we investigated the molecular consequences of mutating residues 54 and 85 of AlgR from aspartate to asparagine by using homology modeling to generate putative molecular models of the AlgR receiver domain with these mutated residues. These models predict that the aspartate-to-asparagine substitutions at these residues do not produce significant structural alterations to the AlgR receiver domain (Fig. 1C). This suggests therefore that the loss of phosphorylation of AlgR D54N is not due to misfolding of this domain and is likely a consequence of mutating the target residue of AlgR phosphorylation by CheA.

Two additional experiments provided strong evidence that the structural integrity of AlgR D54N is maintained. First, a competition assay was performed to determine if AlgR D54N would compete with wild-type AlgR for access to CheA. As can be seen in Fig. 2B, inclusion of AlgR D54N at increasing concentrations resulted in decreased phosphorylation of wild-type AlgR by CheA. Furthermore, when examined for binding to an *algD* fragment containing previously defined AlgR-binding sites, we found that each AlgR derivative retained DNA binding activity comparable to that of wild-type AlgR (Fig. 2C) (19, 26). This suggests that the mutations in AlgR did not dramatically alter the overall structure of the protein and also suggests that AlgR phosphorylation is not essential for its DNA binding activity at *algD*. It is possible that defective AlgR phosphorylation might affect binding of AlgR to other genetic targets, such as those involved in AlgR control of twitching motility. However, these targets of AlgR are unknown at this time and so cannot be tested. Taken together, these results strongly suggest that aspartate 54 of AlgR is the site of AlgR phosphorylation by enteric CheA.

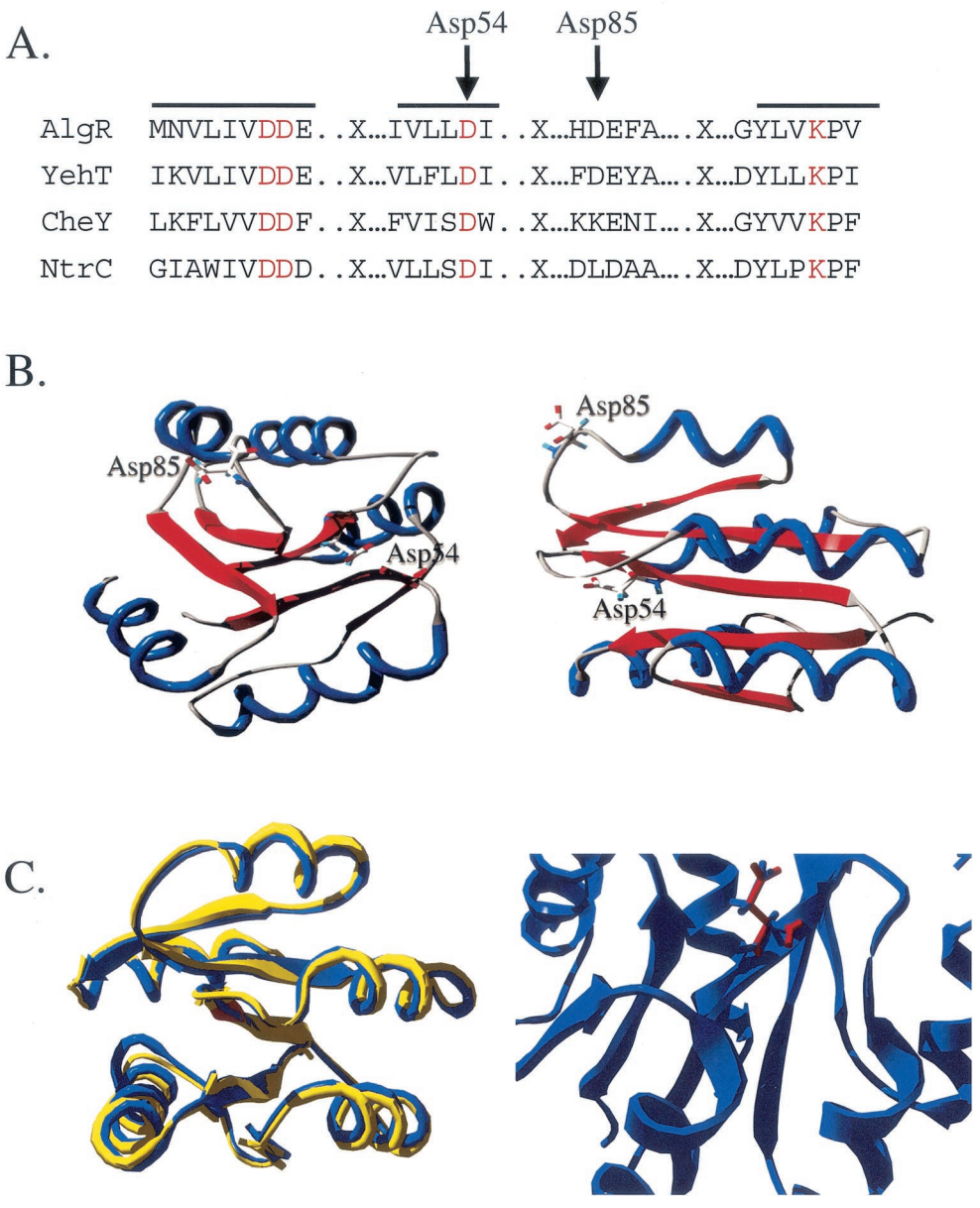

FIG. 1. (A) Alignment of the amino-terminal phosphorylation domains of AlgR, YehT, CheY, and NtrC. X, variable sequences between conserved domains of each protein. Overlined sequences are highly conserved among all response regulators, and conserved aspartates and lysines are depicted in red. In CheY and NtrC, the aspartate which aligns with AlgR D54 is the site of phosphorylation by CheA or NtrB, respectively (33, 34). (B) A ribbon diagram for AlgR model. Homology-based model structure for the AlgR receiver domain was constructed using the structure of the NarL nitrate response regulator protein as a template. The α-helices are blue, β-sheets are red, and loops are gray. Asp54 and Asp85 are depicted in stick form and colored according to standard CPK. Two views are shown: the first is down the axis of a barrel formed by the peripheral helices and the second is at right angles to this and shows the two aspartates lying in the same plane as the mouth of the barrel. (C) Comparison of wild-type AlgR and AlgR D54N receiver domain structures. The panel on the left is an overlay of wild-type AlgR receiver domain (yellow) with AlgR D54N (blue) showing very little structural disturbance of the domain structure. The position of residue 54 in these structures is indicated in red. The right panel is a ribbon diagram of the region surrounding residue 54 (shown in stick form) with the wild-type aspartate depicted in blue and the substituted asparagine overlaid in red.

Aspartate 54 of AlgR is required for twitching motility. We have previously demonstrated that neither aspartate 54 nor aspartate 85 of AlgR are required for alginate production in mucoid *P. aeruginosa*, indicating that the control of alginate biosynthesis in these strains is independent of the AlgR phosphorylation status (21). The mucoid *P. aeruginosa* parent strain (FRD1) used in this study does not exhibit twitching motility and therefore could not be used to investigate the role of AlgR residues D54 and D85 in fimbrial biogenesis and function. Using the same gene replacement strategy described previously (21), we introduced the *algR* mutant alleles into the nonmucoid *P. aeruginosa* strains PAK and PAO1.

We routinely assay twitching motility by stab inoculation of the strain to be examined through a thin 1% agar plate.

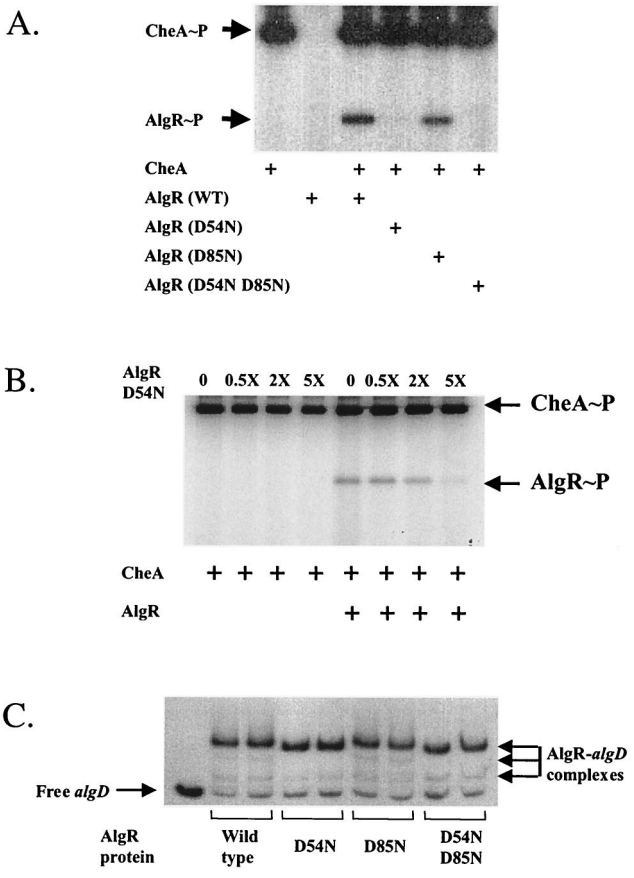

FIG. 2. (A) AlgR phosphorylation studies. Phosphorylation assays were conducted as described in Materials and Methods. Lanes contain 100 ng of CheA and/or 1 μg of AlgR or AlgR derivatives (+, addition of protein). The positions of the phosphorylated forms of CheA and AlgR are indicated on the side. Note that CheA can phosphorylate AlgR and AlgR D85N but not AlgR D54N or AlgR D54N D85N. (B) AlgR D54N competes with wild-type AlgR for access to CheA. A competition experiment was performed using 1 μg of wild-type AlgR and different amounts of AlgR D54N (0.5, 1, and 5 μg). The conditions used in the autophosphorylation of CheA, phosphotransfer from CheA to AlgR, and detection are identical to those described for panel A. Lane 1, 100 ng of CheA alone; lanes 2, 3, and 4, 100 ng of CheA and 0.5, 1, or 5 μg of AlgR D54N, respectively; lane 5, 100 ng of CheA and 1 μg of wild-type AlgR; lanes 6, 7, and 8, 100 ng of CheA, 1 μg of wild-type AlgR, and either 0.5, 1, or 5 μg of AlgR D54N, respectively. (C) EMSA of AlgR or AlgR derivatives binding to *algD* sequences. The fragment used in this assay contains two AlgR-binding sites and is located from −324 to −424 relative to the *algD* transcription start site. Duplicate samples of AlgR or the various AlgR derivatives (100 ng; protein source depicted below the gel) were tested for binding to *algD* sequences. The positions of unbound *algD* as well as the AlgR-*algD* complexes are indicated.

Twitching motility occurs at the agar-plastic interface and produces a halo of colony expansion surrounding the inoculated colony (Fig. 3A) (36). Nontwitching mutants, such as PAK*pilA*::Tc^r, which does not produce the major fimbrial subunit PilA, showed no zone associated with twitching motility (Fig. 3A). Twitching motility assays of the various AlgR mutants revealed that strains which express either AlgR D54N (*algR7*) or AlgR D54N D85N (*algR11*) behave similarly to the *algR* transposon null mutant (Fig. 3A), whereas strains expressing AlgR D85N (*algR10*) produce a wild-type twitching zone (Fig. 3A). These results indicate that D54 but not D85 of AlgR is required for wild-type twitching motility. Similar results were obtained in the PAO1 genetic background (see below; see Fig. 5A).

We have recently used time-lapse video microscopy to examine the dynamics of twitching motility at a cellular level (36). These studies have revealed that this is a complex multicellular process in which the bacteria move in a highly coordinated fashion, initially forming rafts of cells which move away from the colony edge, behind which an intricate lattice-like network of cells is formed (PAK sample) (Fig. 3B). Cells within this network follow each other closely and demonstrate frequent reversals of direction. Cells within the outgoing rafts also demonstrate some cell reversal but do so less frequently than those in the network. Nontwitching mutants demonstrate no differentiation of the colony edge (36). Light microscopy of the *algR* mutants confirms that PAK*algR10* (AlgR D85N) exhibits wild-type twitching motility, whereas PAK*algR7* (AlgR D54N) and PAK*algR11* (AlgR D54N D85N) (data not shown) behave identically to the *algR* transposon mutant S39 (Fig. 3B and data not shown). When examined 2 to 4 h postinoculation, the latter *algR* mutants lack the lattice formation which is characteristic of wild-type twitching zones and produce an undifferentiated colony edge similar to that of the *pilA* mutant (data not shown; 36 and data not shown). However, when examined after overnight incubation, the colony edges of the *algR* mutants S39, PAK*algR7*, and PAK*algR11* show clear differences to the *pilA* mutant (Fig. 3B). With extended incubation, the *algR* mutants developed very large lobes of cells at the colony periphery. These colonies were still devoid of the intricate lattice-like network which is characteristic of wild-type *P. aeruginosa*. Time-lapse video microscopy of the *algR* null mutant S39 showed that these rafts moved at rates of about 0.2 μm/min, which is approximately 20 times slower than the rate at which wild-type leading edge rafts move. This is about twice the rate of colony expansion due to cell division as calculated from the nonmotile PAK*pilA*::Tc^r mutant (36). This defective twitching motility may explain the aberrant colony expansion observed in these mutants upon extended incubation (Fig. 3A). Furthermore, unlike the wild-type situation wherein cells within these rafts can be seen to reverse direction and move against the overall direction of the raft, the *algR* mutant S39 shows no detectable cellular reversal. It appears that AlgR or genetic targets of this activator control both the rate of cellular movements and frequency of cell reversal during the process of twitching motility and that this may be dependent on the phosphorylation status of AlgR at Asp54.

***algR* mutants have defects in type IV fimbrial production.** Data in Fig. 2 demonstrate that Asp54 of AlgR is essential for type IV fimbria-mediated twitching motility. However, it was unclear whether the *algR* mutants failed to synthesize surface fimbriae or if the fimbriae were expressed but failed to function properly. To address this, the production of type IV fimbrial structures on the cell surface of the various *algR* mutants was assayed by both ELISA of whole cells and Western blotting of sheared surface fimbriae by using antiserum for the major fimbrial subunit PilA (Fig. 4). The ELISAs indicated that, like the *algR* null mutant S39 described previously (45), PAK*algR7* and PAK*algR11* lack detectable surface-assembled fimbriae (Fig.

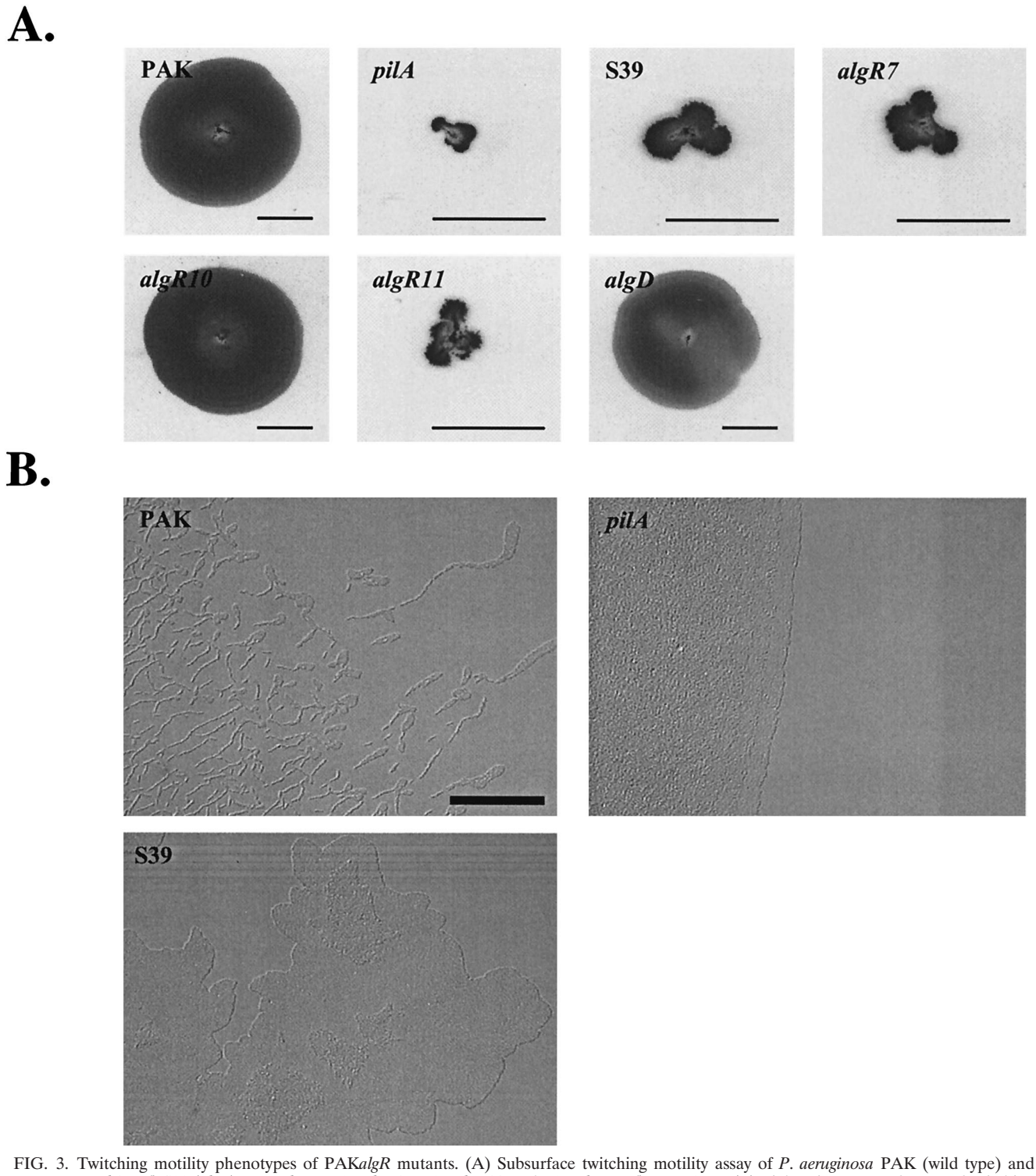

FIG. 3. Twitching motility phenotypes of PAK*algR* mutants. (A) Subsurface twitching motility assay of *P. aeruginosa* PAK (wild type) and mutants PAK*pilA*::Tc^r, S39 (*algR*), PAK*algR7*, PAK*algR10*, PAK*algR11*, and PAK*algD*::Tc^r. Bars, 1 cm. (B) Light microscopy of zones of twitching motility showing colony expansion zones obtained at the interstitial surface between the glass coverslip and Gelgro media for PAK (wild type), PAK*pilA*::Tc^r, and S39. Micrographs were taken after 2 to 4 h of incubation (PAK) or 20 h (PAK*pilA*::Tc^r and S39) at 37°C. Bar, 50 μm. In all images, the colony is situated to the left of the image.

4A). However, Western analyses of sheared surface fimbriae show that these strains do in fact produce trace amounts of surface-assembled fimbriae compared to that of the wild-type strain PAK (Fig. 4B). The levels of surface fimbriae detected in the *algR* mutants are significantly higher than in the *pilV* mutant (included to control for pilin subunit released by cell lysis) which shows almost no detectable fimbrial subunit in these Western analyses. The reduced amount of surface fimbriae is

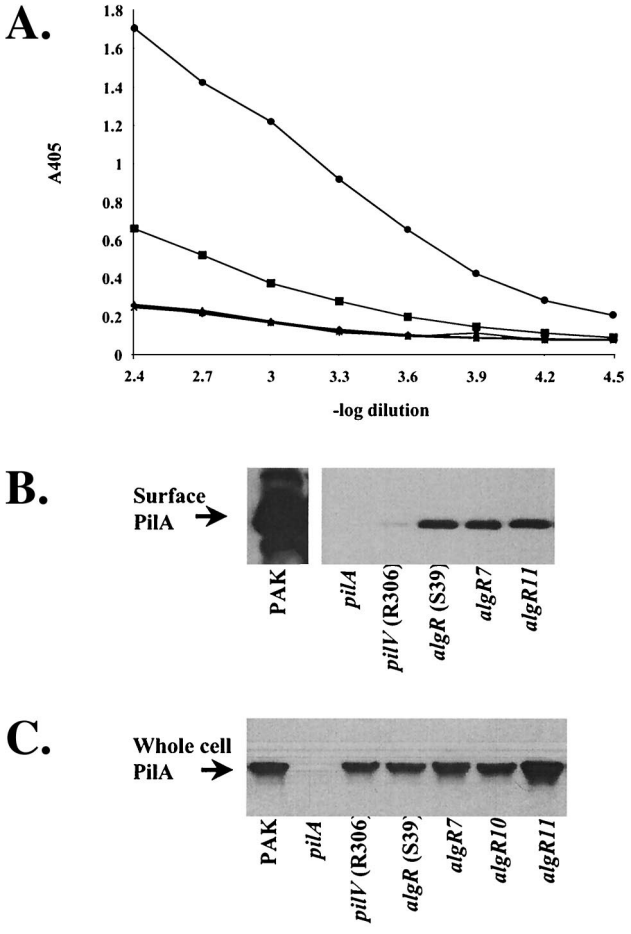

FIG. 4. Type IV fimbrial subunit production in *algR* mutants. (A) ELISA against whole-cell samples of the following *P. aeruginosa* strains: PAK (■), PAK*pilA*::Tc^r (▲), S39 (×), PAK*algR7* (|), PAK *algR10* (●), and PAK*algR11* (♦). Type IV fimbriae were detected using antipilin antiserum and are indicative of levels of surface fimbriae in these strains. Shown are immunoblots of the PilA subunit of sheared surface fimbriae (B) and PilA subunit (C) remaining in whole-cell samples after surface fimbriae had been sheared off indicated strains. The *pilV* mutant (R306), which is defective in assembly of the fimbrial structure, was included in these assays to control for fimbrial subunit contribution to surface samples as a result of cell lysis.

presumably sufficient to elicit the aberrant twitching motility observed with these *algR* mutants.

Interestingly, ELISA of whole cells (Fig. 4A) and Western analyses of sheared surface fimbriae (data not shown) showed that PAK*algR10* overproduces fimbriae on the cell surface. This hyperfimbriate phenotype was unexpected given the earlier observations that this mutant demonstrates wild-type twitching motility (see above). These results suggest that Asp85 of AlgR plays some role in controlling the amount of fimbriae assembled at the cell surface but is not required for twitching motility, at least under the conditions tested here.

Expression of the fimbrial subunit (PilA) was examined in whole cells of all strains by Western blot analysis using anti-PilA antiserum (Fig. 4C). These studies confirm that the various *algR* mutants produce relatively normal amounts of fimbrial subunit. This suggests that the defects in both the hyperfimbriate (*algR10*) and hypofimbriate (S39, *algR7*, and *algR11*) strains are occurring at some level in the fimbrial biogenesis pathway, perhaps indicating a role for AlgR in the control of fimbrial assembly or disassembly.

Alginate production is not required for twitching motility in *P. aeruginosa*. Other modes of bacterial surface translocation such as swarming motility and gliding motility are associated with the production of exopolysaccharide "slime" trails (7). Our time-lapse video analysis of twitching motility also implies the presence of "trails," which seem to confine bacterial movements in the expanding twitching zone (36). Given that AlgR is known to control both alginate production and twitching motility in *P. aeruginosa*, it is possible that alginate production affects twitching motility by contributing to the formation of these twitching trails. To investigate this possibility, we inserted a *tet* cartridge into the internal *Eco*RI site of *algD* and introduced this mutant allele into wild-type PAK via allelic exchange. This strain, PAK*algD*::Tc^r, demonstrates wild-type twitching motility both macroscopically and microscopically (Fig. 3A; data not shown), indicating that alginate is not essential for twitching motility nor is alginate likely to be a component of the twitching trails. Identical results were obtained with PAO1 *algD* mutants (data not shown). This suggests that AlgR is controlling twitching motility through a mechanism independent of alginate synthesis.

***P. aeruginosa* strains with *algR* mutations have defects in biofilm initiation.** It is believed that *P. aeruginosa* grows as microcolonies in the CF lung and forms a complex biofilm (8, 37). Biofilms are of considerable importance due to their innate resistance to antibiotics, phagocytic cells, and other agents (9). In a recent genetic screen, twitching motility was shown to be a critical determinant for controlling biofilm initiation (30). As AlgR controls twitching motility and cells expressing AlgR variants with a mutation in the predicted phosphorylation site (D54) are also altered in twitching motility, we examined whether AlgR played a role in biofilm formation. *P. aeruginosa* strain PAO1 has been a model strain for examining biofilm development (9), whereas strain PAK did not form biofilms in our hands (data not shown). We thus generated PAO1-derived strains with gene replacements of *algR* with the various *algR* alleles described above. As predicted from the results in Fig. 3A, PAO1 strains WFPA8 and WFPA16 which express AlgR D54N or AlgR D54N D85N, respectively, as well as the *algR* null strain WFPA12, were defective in twitching motility (Fig. 5A). These strains as well as appropriate controls were examined for biofilm initiation using the microtiter assay described elsewhere (30). As depicted in Fig. 5B, the *algR* null mutant (WFPA12) formed biofilms poorly compared with the wild-type strain PAO1 or WFPA13. Strains WFPA8 and WFPA16 had an intermediate biofilm initiation phenotype, suggesting that phosphorylation of AlgR at Asp54 plays a role but is not essential for biofilm initiation. A comparison of the biofilms formed by the *algR* null mutant versus mutants expressing AlgR proteins carrying the D54N mutation (WFPA8 and WFPA16; Fig. 5B) suggests additional AlgR targets essential for biofilm initiation may exist. An obvious choice would be *algD*, yet PAO1-derived *algD* mutants formed biofilms with kinetics similar to those of strain PAO1 (data not shown).

In summary, these studies have demonstrated that AlgR D54 is required for twitching motility and biofilm formation in

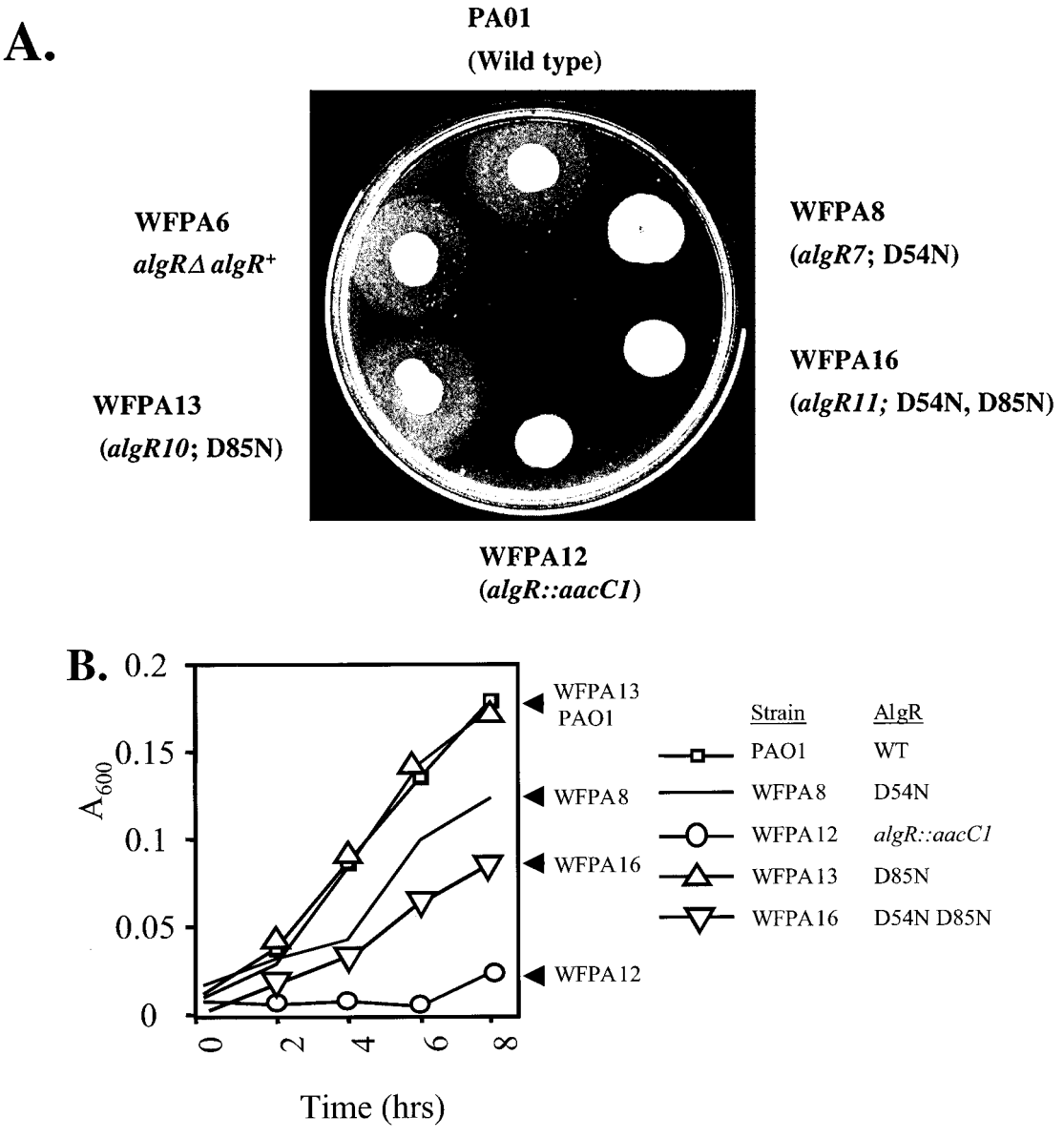

FIG. 5. AlgR is required for biofilm formation. (A) Subsurface twitching motility assay of *P. aeruginosa* PAO1 (wild type) and the indicated *algR* mutant strains. (B) Biofilm formation was assayed every 2 h during initiation using the microtiter plate assay (29). Surface-attached cells were stained with crystal violet, the stain was solubilized in ethanol, and the absorbance was analyzed at 600 nm (A_{600}). The wild-type (WT) strain and strains expressing the various AlgR mutant proteins are indicated.

P. aeruginosa. The in vitro studies suggest that this residue is the site of phosphorylation. AlgR phosphorylation in vivo could occur by small phosphate donors or through a transmitter histidine kinase, possibly FimS, although FimS itself must in turn be phosphorylated by an upstream phosphate donor, since it lacks the ATP binding site required for autophosphorylation (45).

Of notable interest is the fact that AlgR controls at least two pathways in *P. aeruginosa*. This response regulator was discovered in a genetic analysis of genes required for alginate production (see reference 13 and references therein). We showed earlier that mutations at Asp54 had no effect on alginate production or *algD* expression in mucoid *P. aeruginosa* (21). AlgR also controls twitching motility, and AlgR D54N mutants have marked defects in fimbrial biogenesis, function, twitching motility, and biofilm formation. As AlgR phosphorylation at Asp54 appears to be required for biofilm initiation but not for alginate production, it is possible that AlgR phosphorylation represents a biofilm developmental checkpoint, as *P. aeruginosa* makes a transition from fimbria-mediated attachment and microcolony formation to polysaccharide-containing multicellular structures often associated with mature biofilms (8).

We have also determined that residue D85 also appears to be involved in fimbrial biogenesis, as strains expressing AlgR D85N are hyperfimbriate. Analysis of the AlgR model structure shows that an extensive negative electrostatic potential

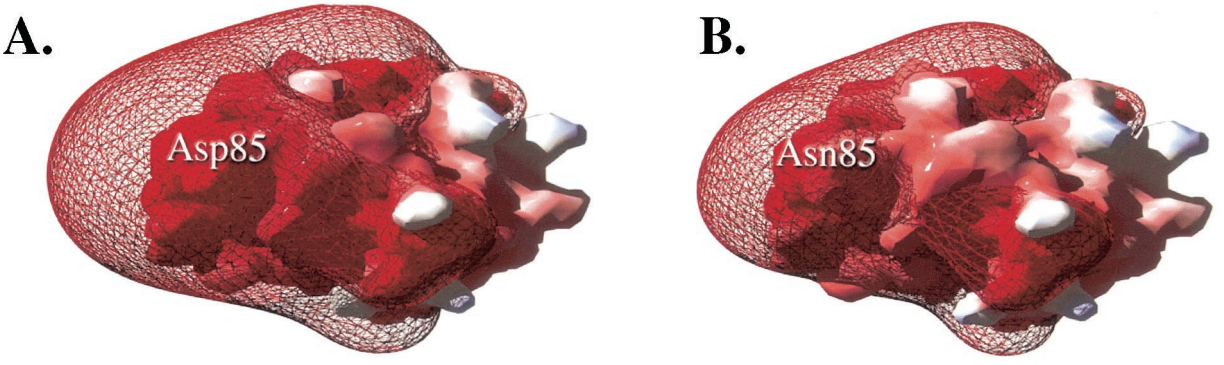

FIG. 6. Electrostatic potential diagrams for the wild-type and D85N mutant AlgR receiver domains. Homology-based model structure for the AlgR receiver domain was constructed using the structure of the NarL nitrate response regulator protein as a template. Electrostatic potential is represented as a mesh contoured to yield isosurfaces with charges of ±3.0. Negative potential is red and positive potential is blue. There is very little positive potential visible in this image. Electrostatic potentials have also been mapped to a solvent-accessible molecular surface constructed with a 1.4-Å probe to show AlgR surface topology. Accessible surface is colored as for electrostatic potential except that uncharged areas are white. The positions of Asp/Asn85 are indicated. Viewpoint is along the helical barrel.

network (Fig. 6, red isosurface) envelops D85. Electrostatic potential analysis gives an indication of the longer-range forces that are active in the immediate environment of a solvated protein. Complementation of electrostatic potential has been demonstrated for a number of protein-protein docking surfaces (24) and is evident between the regulator domain/DNA binding domain of NarL (data not shown). It is possible that the marked negative electrostatic potential of the AlgR receiver domain plays a role in determining protein-protein interactions. Mutation of D85 to N85 results in considerable disruption to this network as evident when the electrostatic potentials for wild-type AlgR and the D85 mutant are compared (Fig. 6). Thus, molecular modeling predicts that the D85N mutation alters the electrostatic potential of AlgR at this surface, a consequence of which could be altered protein-protein interactions of AlgR with some other component of the signaling pathway. This could be at the root of the AlgR D85N hyperfimbriate phenotype.

While the details of the mechanism at work here remain unclear, these studies have shown that both Asp54 and Asp85 are important in determining the molecular events governing AlgR target gene expression and twitching motility in *P. aeruginosa*.

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C.B.W. and T.E.E. contributed equally to this work.

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