

# Effect of Site-Specific Mutations in Different Phosphotransfer Domains of the Chemosensory Protein ChpA on *Pseudomonas aeruginosa* Motility<sup>∇‡</sup>

Andrew J. Leech<sup>†</sup> and John S. Mattick<sup>\*</sup>

*Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia*

Received 28 January 2006/Accepted 19 September 2006

**The virulence of *Pseudomonas aeruginosa* and other surface pathogens involves the coordinate expression of a wide range of virulence determinants, including type IV pili. These surface filaments are important for the colonization of host epithelial tissues and mediate bacterial attachment to, and translocation across, surfaces by a process known as twitching motility. This process is controlled in part by a complex signal transduction system whose central component, ChpA, possesses nine potential sites of phosphorylation, including six histidine-containing phosphotransfer (HPt) domains, one serine-containing phosphotransfer domain, one threonine-containing phosphotransfer domain, and one CheY-like receiver domain. Here, using site-directed mutagenesis, we show that normal twitching motility is entirely dependent on the CheY-like receiver domain and partially dependent on two of the HPt domains. Moreover, under different assay conditions, point mutations in several of the phosphotransfer domains of ChpA give rise to unusual “swarming” phenotypes, possibly reflecting more subtle perturbations in the control of *P. aeruginosa* motility that are not evident from the conventional twitching stab assay. Together, these results suggest that ChpA plays a central role in the complex regulation of type IV pilus-mediated motility in *P. aeruginosa*.**

*Pseudomonas aeruginosa* is a rod-shaped bacterium that is an opportunistic pathogen of plants and animals, including immunocompromised patients such as cystic fibrosis sufferers (14). The bacterium is equipped with a large arsenal of secreted and cell-associated virulence factors, including type IV pili, which are filamentous appendages located at the poles of the bacterium that facilitate attachment to host epithelial cells, and a form of surface translocation called twitching motility (TM) (16).

Twitching motility occurs on wet surfaces and is an important factor for host infection and colonization as well as other forms of complex colonial behavior (16). Twitching motility occurs by the extension and retraction of type IV pili and is distinct from swimming motility (as in *Escherichia coli* and *P. aeruginosa*), which is mediated by the rotation of polar flagella, and from orthodox “swarming motility” (as in *Proteus mirabilis*), which is mediated by the coordinated action of peritrichous flagella (23).

“Swarming motility” is, however, a loosely defined term that has been used somewhat indiscriminately to describe various forms of organized bacterial motility. A form of “swarming motility” in *P. aeruginosa* has been reported but differs from that described for other bacteria in that it appears to demonstrate a reliance on type IV pili rather than flagella (11). However, there is contradictory evidence, with another report

indicating that flagella are essential for swarming (22), which is consistent with the usual basis of swarming in other species.

In *P. aeruginosa*, around 40 genes whose products are required for TM, 16 of which code for proteins that are involved in transcriptional regulation and chemosensory control, have now been identified (16, 33). The chemosensory system that controls TM is encoded predominantly by the *pilGHJK* (5–7) and *chpABC* (33) gene clusters. Together, the proteins encoded by these genes appear to comprise a complex chemosensory signal transduction pathway that shares many modules with, but is significantly more complex than, the chemosensory systems that control flagellum rotation in bacteria and the Frz system that controls social gliding motility in *Myxococcus xanthus*, with which it shares many similarities (16, 28). The lynchpin of this chemosensory system appears to be ChpA, a complex signal transduction protein containing nine potential sites of phosphorylation, including six histidine-containing phosphotransfer (HPt) domains, one serine-containing phosphotransfer (SPt) domain, one threonine-containing phosphotransfer (TPt) domain, and the CheY-like receiver domain (33). Here, we begin to dissect the function of these domains in motility by site-directed mutagenesis.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** *Escherichia coli* strain DH5 $\alpha$  (*recA endA1 gyrA96 hsdR17 thi-1 supE44 relA1  $\phi$ 80dlacZ $\Delta$ M15*) was used in all genetic manipulations and in the preparation of DNA sequencing templates. *Escherichia coli* strain S17-1 was used as the donor strain in bacterial conjugations. The *P. aeruginosa* strain used was PAO1 strain ATCC 15692 (American Type Culture Collection). The MTP cosmid library (10) was used in the subcloning of the *chp* region. The preparation of *E. coli* competent cells and transformation protocols were performed according to methods described previously (25). The preparation of *P. aeruginosa* competent cells was performed as described previously (17). Plasmids used in this study are listed in Table 1. *E. coli* and *P. aeruginosa* liquid cultures were maintained in Luria-Bertani (LB) broth, and solid medium was

\* Corresponding author. Mailing address: Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia. Phone: 61-7-3346-2079. Fax: 61-7-3346-2111. E-mail: j.mattick@imb.uq.edu.au.

<sup>†</sup> Present address: Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, VA 23298-0678.

<sup>‡</sup> Supplemental material for this article may be found at <http://jb.asm.org/>.

<sup>∇</sup> Published ahead of print on 29 September 2006.

TABLE 1. Strains and plasmids used in this study

| Strain or plasmid         | Relevant characteristic(s)   | Source or reference |
|---------------------------|--|---------------------|
| <b>Strains</b>            |  |                     |
| DH5 $\alpha$              | <i>recA endA1 gyrA96 hsdR17 thi-1 supE44 relA1</i> $\phi$ 80 <i>dlacZ</i> $\Delta$ M15   | Lab collection      |
| S17-1                     | <i>thi pro hsdR recA chr::RP4-2</i>  | 29                  |
| GM2163                    | F <sup>-</sup> <i>ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galk2 galT22 mcrA dcm-6 hisG4 rfbD1 rpsL136</i> (Str <sup>r</sup> ) <i>dam13::Tn9</i> (Cm <sup>r</sup> ) <i>xyLA5 mtl-1 thi-1 mcrB1 hsdR2</i> | New England Biolabs |
| PAO1                      | Wild type <i>P. aeruginosa</i> strain ATCC 15692   | Lab collection      |
| PAO1 $\Delta$ <i>chpA</i> | PAO1 with Tc <sup>r</sup> insertion in <i>chpA</i>   | This study          |
| <b>Plasmids</b>           |  |                     |
| pBluescript II KS         | <i>E. coli</i> cloning vector with Ap selection  | Stratagene          |
| pOK12                     | <i>E. coli</i> cloning vector with Km selection  | 31                  |
| pUC21                     | <i>E. coli</i> cloning vector with Ap selection  | 31                  |
| pUCP19                    | <i>E. coli-P. aeruginosa</i> cloning vector  | 27                  |
| pUCPSK                    | <i>P. aeruginosa-E. coli</i> shuttle vector  | 32                  |
| pMMB206                   | <i>Pseudomonas</i> controlled expression level vector with Cm selection  | 19                  |
| pMOB3                     | Counterselectable <i>sacB/sacR</i> genes from <i>Bacillus subtilis</i>   | 26                  |
| pSM-TET                   | Source of Tet <sup>r</sup> cartridge   | 18                  |
| MTP26                     | Cosmid containing <i>P. aeruginosa</i> PAO1 chromosomal DNA from bp 448166-472109  | 10                  |
| pAL1                      | pBluescript II KS with modified multiple cloning site  | This study          |
| pAL5                      | 3,168-bp SacII/EcoRV fragment of <i>chpA</i> from MTP26 in pAL1  | This study          |
| pAL6                      | 458-bp H912i PCR product in pUC21  | This study          |
| pAL7                      | 3,168-bp SacII/EcoRV fragment of <i>chpA</i> containing H1088 in pAL1  | This study          |
| pAL8                      | 1,297-bp S1231i/H1410i PCR product in pUC21  | This study          |
| pAL9                      | 611-bp H1570i PCR product in pUC21   | This study          |
| pAL10                     | 872-bp BamHI/KpnI fragment of <i>chpA</i> containing H1717 in pUC21  | This study          |
| pAL11                     | 1,396-bp EcoRV/KpnI fragment of <i>chpA</i> from MTP26 in pAL5   | This study          |
| pAL13                     | 1,561-bp EcoRV/AatII fragment of <i>chpA</i> from MTP26 containing H54 in pOK12  | This study          |
| pAL14                     | ChpA H54Q in pAL13   | This study          |
| pAL16                     | ChpA H912Q in pAL6   | This study          |
| pAL17                     | ChpA H1088Q in pAL7  | This study          |
| pAL18                     | ChpA S1231A in pAL8  | This study          |
| pAL19                     | ChpA H1410Q in pAL8  | This study          |
| pAL20                     | ChpA H1570Q in pAL9  | This study          |
| pAL37                     | 2,839-bp BamHI/ClaI fragment of <i>chpA</i> from MTP26 containing D2406N in pUC21  | This study          |
| pAL40                     | 4,291-bp SacI/ClaI <i>chpA</i> fragment in pUCPSK  | This study          |
| pAL41                     | Full-length <i>chpA</i> under <i>Plac</i> control in pUCPSK  | This study          |
| pAL42                     | ChpA D2406N in pAL37   | This study          |
| pAL49                     | Tc <sup>r</sup> cassette from pSM-TET (EcoRI/MluI) inserted into MfeI/AscI pAL11   | This study          |
| pAL52                     | 5-kb NotI fragment from pMOB3 ligated into pAL49   | This study          |
| pAL57                     | 7,972-bp NotI <i>chpA</i> fragment from MTP26 in pAL1  | This study          |
| pAL64                     | pAL41 with an internal double FLAG tag inserted between the native NotI/NruI sites of <i>chpA</i>  | This study          |
| pAL77                     | 3,862-bp ClaI/SacI <i>chpA</i> fragment from pAL41 in pUCP19   | This study          |
| pAL78                     | Full-length <i>chpA</i> against <i>Plac</i> in pUCP19  | This study          |
| pAL79                     | 8-kb ClaI/SacI fragment from MTP26 ligated into pMMB206 with <i>PtaclacUV</i>  | This study          |
| pAL82                     | 3,503-bp StuI fragment of <i>chpA</i> cut from pAL57 and ligated into pAL14  | This study          |
| pAL83                     | pAL79 with ChpA H54Q (mutation in HPT1 domain)   | This study          |
| pAL86                     | pAL79 with ChpA H912Q (mutation in HPT2 domain)  | This study          |
| pAL87                     | pAL79 with ChpA H1088Q (mutation in HPT3 domain)   | This study          |
| pAL88                     | pAL79 with ChpA S1231A (mutation in SPT domain)  | This study          |
| pAL89                     | pAL79 with ChpA H1410Q (mutation in HPT4 domain)   | This study          |
| pAL90                     | pAL79 with ChpA H1570Q (mutation in HPT5 domain)   | This study          |
| pAL92                     | pAL79 with ChpA D2406N (mutation in CheY domain)   | This study          |
| pAL79FLAG                 | pAL79 with a double FLAG tag inserted into the NotI/KpnI sites of <i>chpA</i>  | This study          |
| pAL86FLAG                 | pAL86 with a double FLAG tag inserted into the NotI/KpnI sites of <i>chpA</i>  | This study          |
| pAL87FLAG                 | pAL87 with a double FLAG tag inserted into the NotI/KpnI sites of <i>chpA</i>  | This study          |
| pAL92FLAG                 | pAL92 with a double FLAG tag inserted into the NotI/KpnI sites of <i>chpA</i>  | This study          |

prepared by adding 0.5 to 1.5% agar (Becton Dickinson BBL agar grade A). The following antibiotic concentrations were used for the selection of *E. coli*: 12.5  $\mu$ g/ml tetracycline for plasmid selection and 40  $\mu$ g/ml tetracycline for cosmid selection, 100  $\mu$ g/ml ampicillin, 25  $\mu$ g/ml chloramphenicol, and 50  $\mu$ g/ml kanamycin. The concentrations of antibiotics for the selection of *P. aeruginosa* were as follows: 250  $\mu$ g/ml carbenicillin, 20  $\mu$ g/ml rifampin, 250  $\mu$ g/ml chloramphenicol, and 200  $\mu$ g/ml tetracycline.

**Construction of PAO1 $\Delta$ *chpA*.** The *chpA* deletion mutant PAO1 $\Delta$ *chpA* was constructed using standard allelic exchange techniques. The plasmid pBluescript

II KS was digested with SacI/KpnI, and a synthesized multiple cloning site was ligated in to form pAL1. A 3,168-bp SacII/EcoRV fragment of *chpA* from cosmid MTP26 (10) was cloned in to give pAL5. A 1,396-bp EcoRV/KpnI fragment from MTP26 was then also ligated in to give pAL11. This plasmid was then digested with MfeI/AscI, and a Tet<sup>r</sup> resistance cassette from pSM-TET (18) (EcoRI/MluI) was inserted to give pAL49. A NotI fragment from pMOB3 (that carries the *sacB/sacR* genes for counterselection) (26) was then inserted into pAL49 to give pAL52. This construct was then transformed into the *E. coli* donor strain S17-1 in preparation for mating into *P. aeruginosa*. Following conjugation, the

transconjugates were selected on 10% sucrose medium containing 200 µg/ml tetracycline, which forces the excision of the plasmid while leaving the homologously recombined inactivated gene in the chromosome. The genotype of the mutants was confirmed by Southern hybridization analysis.

**Construction of *chpA* point mutants.** Site-directed mutagenesis was performed using either the Stratagene QuikChange site-directed mutagenesis kit (catalog no. 200518) or a sequential PCR method (24). Oligonucleotide primer sequences are provided in the supplemental material.

**Construction of *chpA* expression vectors.** Cosmid MTP26 (10) was digested with SacI and ClaI, and the resulting fragments were separated by size on a 0.7% agarose gel. A 4,291-bp SacI/ClaI fragment was excised, purified, and ligated into pUCPSK (32) to give pAL40. A 3,739-bp SacI fragment was then ligated in to give pAL41, a full-length *chpA* clone regulated by the *Plac* promoter. A second *chpA* expression vector that would orientate *chpA* against the *Plac* promoter was then constructed in pUCP19. A 3,862-bp ClaI/SacI fragment was cut from pAL41 and ligated into AccI/SacI-digested pUCP19 to give pAL77. A 3,739-bp SacI fragment was then excised from pAL41 and ligated into pAL77 to give a full-length *chpA* clone orientated against the *Plac* promoter, pAL78. A third *chpA* expression vector was then created using a low-copy-number controlled expression vector, pMMB206, that contains the *lacI* repressor (19), which is essential for isopropyl-β-D-thiogalactopyranoside (IPTG) expression studies in *Pseudomonas*. This allows for the titration of ChpA activity by controlling the expression level. Vector pMMB206 (which utilizes a *PtaclacUV5* promoter) was chosen in preference to pMMB207 (which uses a *Ptac* promoter) primarily because of the relative strengths of the promoters (19). The *PtaclacUV5* promoter has previously been reported to reach only 6.5% of the expression level of the *Ptac* promoter in *Pseudomonas* species (19), reducing the chance of detrimental effects from the overexpression of the 270-kDa signal transduction protein. Partial EcoRI/HindIII digestion was performed on pAL78 to give an 8,064-bp fragment. This was ligated into EcoRI/HindIII-digested pMMB206 to give pAL79 (see Fig. S1 in the supplemental material).

The mutated phosphotransfer sites were then individually shuttled into pAL79 as described below to create full-length *chpA* expression constructs with point-mutated phosphotransfer domains. The mutated alleles H912Q, H1088Q, S1231A, H1410Q, and H1570Q were subcloned from pAL16, pAL17, pAL18, pAL19, and pAL20, respectively, into pAL79 using the unique restriction enzyme sites for MfeI and KpnI to give pAL86, pAL87, pAL88, pAL89 and pAL90, respectively (Table 1).

The mutated His54 site was subcloned into pAL79 in a three-step process. Plasmids pAL14 (which contains H54Q) and pAL57 were prepared from *E. coli* strain GM2163 to allow digestion at Dcm methylation-sensitive sites. A 3,503-bp StuI fragment was cut from pAL57 and cloned into StuI-digested pAL14, giving pAL82. This step increased the length of *P. aeruginosa* DNA contained in the vector and added a SacI site while retaining the mutated codon. A SacI fragment was then cut from pAL82 and ligated into SacI-digested pAL79, giving pAL83.

The mutated CheY site (D2406N) was cloned by first digesting pAL79 with HindIII, followed by blunting the 5' overhang of the DNA ends with T4 DNA polymerase and then a second digestion with NotI. A 682-bp fragment containing the mutated codon was excised from pAL42 with AfeI/NotI and then ligated into pAL79, creating pAL92 (Table 1).

**Twitching motility assay.** The subsurface twitching motility assay was carried out as described previously (28). Following stab inoculation of the *P. aeruginosa* strain to be tested through a 1% agar plate and overnight incubation at 37°C, the zone of motility between the agar and the petri dish interface was visualized by compressing the agar and then staining using 0.05% (wt/vol) Coomassie brilliant blue R250 stain (40% methanol, 10% acetic acid) as described previously (2).

**"Swarming motility" assay.** Swarm agar was prepared according to a method described previously by Kohler et al. (11). Medium was based on M9 salts without NH<sub>4</sub>Cl, supplemented with 0.2% glucose (wt/vol), 0.05% glutamate or aspartate (wt/vol) (as the sole nitrogen source), 2 mM MgSO<sub>4</sub>, and trace elements (available upon request) and solidified with 0.5% agar (Becton Dickinson BBL agar grade A). After solidification, plates were briefly dried and then inoculated with a single colony from a fresh LB plate by pipette tip. Incubation was performed at 37°C for 16 h.

**PilA Northern blot analysis.** RNA preparations were carried out using the QIAGEN RNeasy Mini kit. Northern blot analysis of *pilA* mRNA levels was performed as described previously (34), except that Ultrahybe was used as the hybridization solution.

**Rhamnolipid assay.** The amount of rhamnolipid was quantitated using the orcinol method (20). Briefly, 500 µl of cell-free supernatant was extracted twice with 1 ml of diethyl ether. The ether samples were pooled and evaporated to dryness, and 200 µl of water was added. To 100 µl of each sample, 900 µl of a solution containing 0.19% orcinol (in 54% H<sub>2</sub>SO<sub>4</sub>) was added, heated to 80°C for

15 min, and cooled. The *A*<sub>421</sub> was then measured, with higher rhamnolipid levels giving a higher absorbance.

**ChpA Western analysis.** Detection of FLAG-tagged ChpA was performed as follows. *P. aeruginosa* transformants were inoculated (1:50) into fresh 5-ml LB broth aliquots (in 50-ml tubes) from cultures grown overnight and were incubated at 37°C with shaking for 4 h. After the addition of 0.3 mM IPTG, cultures were grown for 1 h, and 2 ml of culture was then pelleted and resuspended in 400 µl of loading buffer. To reduce viscosity, whole-cell proteins were prepared by passage through a 27½-gauge needle three times and centrifugation at 45,000 rpm for 1 h to pellet chromosomal DNA. Samples were then boiled for 5 min. Proteins were then displayed on 8% sodium dodecyl sulfate-polyacrylamide gels (12) and electroblotted onto an Immobilon-P transfer membrane (Millipore Corp., Bedford, MA) in a Tris-glycine system described previously (30). Membranes were blocked with 3% skim milk and probed with a 1:500 dilution of primary anti-FLAG M2 monoclonal antibody (Sigma Aldrich) in 3% skim milk powder (0.1% Tween 20) in Tris-buffered saline. Membranes were then incubated with a 1:10,000 dilution of rabbit anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Bio-Rad) in 3% skim milk powder (0.1% Tween 20) in Tris-buffered saline followed by detection by enhanced chemiluminescence using the Supersignal chemiluminescence kit (Pierce).

## RESULTS

**ChpA point mutant twitching phenotypes.** Given the large size and complex structure of ChpA, our experimental strategy was to create a chromosomal deletion mutant of *chpA* in PAO1 (PAO1Δ*chpA*) and to complement this mutant in *trans* with point-mutated alleles of full-length *chpA* expressed from the low-copy-number plasmid pAL79, which contained the inducible *PtaclacUV5* promoter (19). Eight phosphotransfer domains (HPt1 to HPt6, Spt, and CheY) were targeted for mutagenesis using the Stratagene QuikChange kit, following a cassette mutagenesis and segment replacement strategy (Fig. 1; see also Fig. S1 in the supplemental material). Clones that showed the correct mutagenesis of the desired codon were obtained for seven of the domains, with only the mutation of HPt6 being unsuccessful, despite numerous attempts. DNA sequencing confirmed that all mutated alleles were correct.

Cassettes containing the mutated domains were then substituted into *chpA* under the control of the inducible promoter *PtaclacUV* in pAL79 (Table 1; also see Fig. S1 in the supplemental material) and transformed into PAO1Δ*chpA* to study the motility phenotypes of the various phosphotransfer domain mutations. The appropriate level of wild-type *chpA* expression was empirically determined by titrating the IPTG concentration to obtain wild-type levels of twitching motility for PAO1Δ*chpA* plus pAL79 (wild-type *chpA*) in the traditional twitching stab assay (16, 28). A concentration of 0.3 mM IPTG was found to give optimal complementation. The strains containing point mutations in the active sites of HPt1, Spt, HPt4, and HPt5 as well the complemented *chpA* mutant (PAO1Δ*chpA* plus *chpA*) exhibit TM zones that are indistinguishable from those of the wild type. The *chpA* deletion mutant (PAO1Δ*chpA*) and the CheY point mutant both appear to completely lack TM, while the HPt2 and HPt3 point mutants both display reduced TM (Fig. 2).

To confirm that the reduction in TM observed in the three mutants was not due to the instability of the mutated protein, we performed a Western blot analysis to visualize the level of ChpA protein in each strain. To circumvent the lack of a ChpA antibody, we modified the point mutant expression vectors (pAL79, pAL86, pAL87, and pAL92) to include

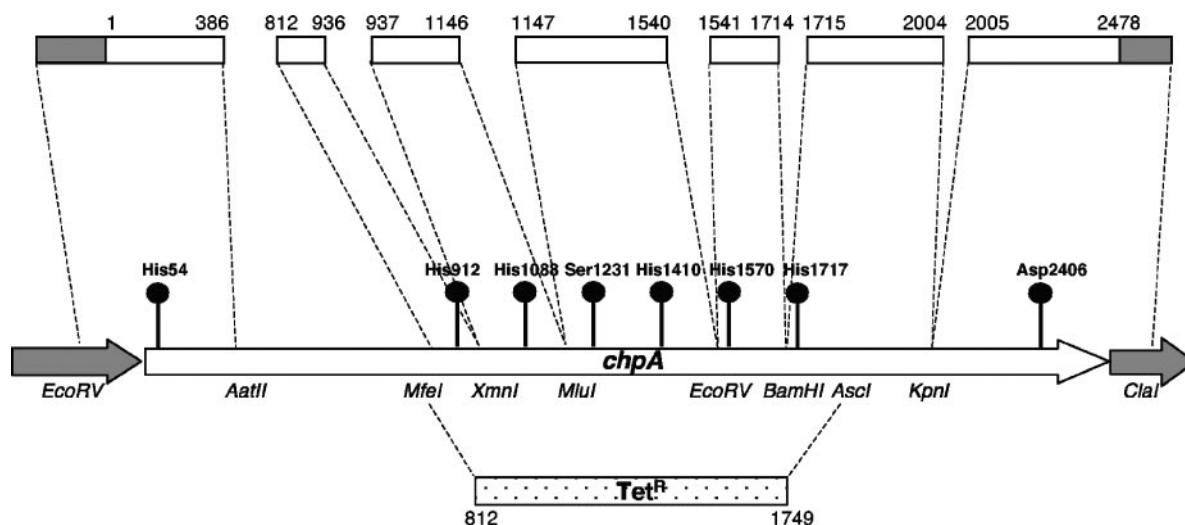


FIG. 1. Diagram of *chpA* open reading frame detailing the location of phosphotransfer active-site codons (circles), a *Tet<sup>R</sup>* insertional deletion spanning codons 812 to 1749 of *chpA*, and fragments of *chpA* (clear boxes) that were subcloned to isolate the phosphotransfer codons for mutagenesis. The numbers above the subcloned fragments indicate the codon regions of *chpA* contained in the fragment. The restriction enzyme sites used for the cassette substitution of the mutated alleles are listed. Full details of the mutagenesis and cloning strategies used to create the mutants referred to in this study may be found in Fig. S1 and Table S1 in the supplemental material, Table 1, and Materials and Methods.

the coding region for an internal double FLAG tag in frame at the C-terminal end of the sequences encoding ChpA and its variants (pAL79FLAG, pAL86FLAG, pAL87FLAG, and pAL92FLAG, respectively) (see Table 1 for details). A Western analysis was then performed using the anti-FLAG M2 monoclonal antibody (Fig. 3), which confirmed that the

introduction of the point mutations into ChpA did not affect protein stability and therefore that the observed phenotypic effects are most likely due to the cessation of phosphotransfer through the domains in question.

***pilA* expression in *chpA* point mutants.** Having shown that (at least) three of the phosphorylation domains of ChpA were

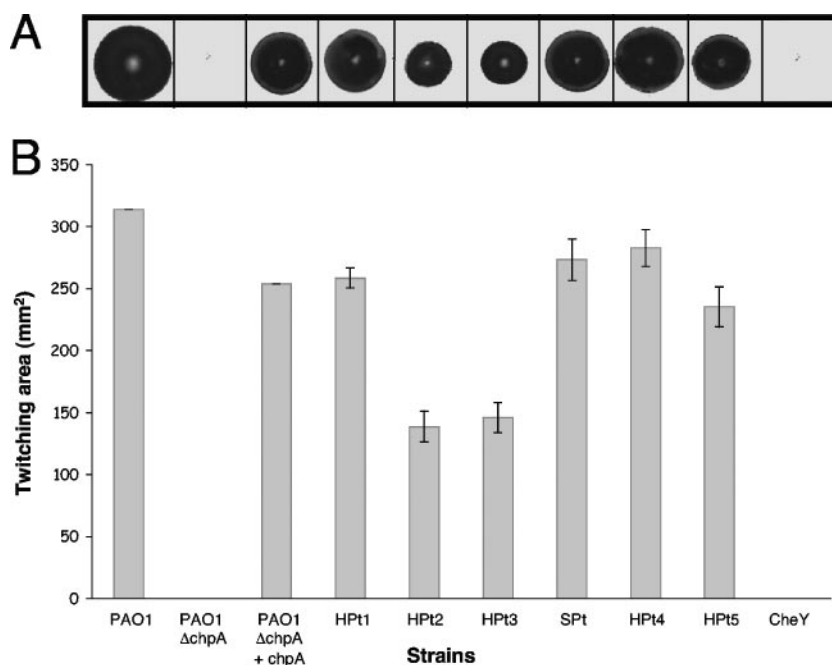


FIG. 2. (A) Subsurface twitching motility assay of *P. aeruginosa* strains PAO1, PAO1 $\Delta$ chpA, PAO1 $\Delta$ chpA plus *chpA*, HPt1, HPt2, HPt3, SPt, HPt4, HPt5, and CheY. The shorthand notation for HPt1, HPt2, HPt3, SPt, HPt4, HPt5, and CheY is used to designate PAO1 $\Delta$ chpA containing plasmids pAL83, pAL86, pAL87, pAL88, pAL89, pAL90, and pAL92, respectively, encoding *chpA* alleles with mutations in the corresponding domain. Twitching assays were performed by the subsurface stab method followed by Coomassie blue staining, as described previously (33). (B) Graph depicting twitching zone areas for the above-described strains. The average zone expansion from three independent experiments is displayed, with error bars representing the standard deviations from the means.



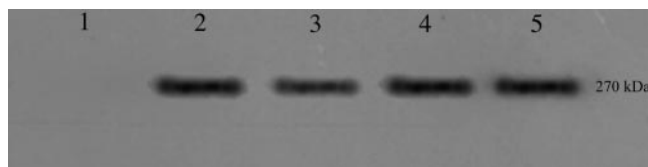


FIG. 3. Western analysis using anti-FLAG M2 monoclonal antibody to assess ChpA protein levels. Lanes: 1, PAO1 $\Delta$ *chpA*(pAL79); 2, PAO1 $\Delta$ *chpA*(pAL79FLAG); 3, PAO1 $\Delta$ *chpA*(pAL86FLAG); 4, PAO1 $\Delta$ *chpA*(pAL87FLAG); 5, PAO1 $\Delta$ *chpA*(pAL92FLAG). Bacterial samples were harvested from exponentially growing cultures 1 h after induction with 0.3 mM IPTG.

involved in the regulation of TM, we then examined whether this regulation is mediated through the transcriptional control of *pilA*. Northern analysis showed that mutations in the three domains involved in twitching motility (HPT2, HPT3, and CheY) have no discernible effect on the level of *pilA* mRNA (Fig. 4). This suggests that all three domains are directly involved in the chemosensory control of TM, presumably by controlling the rate of extension and retraction of the pilus (16, 28).

**Swarming motility phenotypes.** Recent studies have indicated that what has been described as “swarming motility” in *P. aeruginosa* is dependent on flagella, type IV pili, and the biosurfactant rhamnolipid (11). Under the conditions of the swarming assay, using a modified M9 medium with 0.2% glucose (wt/vol) and 0.05% glutamate (wt/vol) as sole carbon and nitrogen sources, respectively, supplemented with 0.3 mM IPTG, PAO1 produced the branching dendritic pattern that is normally characteristic of swarming motility (8, 11), while PAO1 $\Delta$ *pilA* showed no zone of expansion over the agar surface (Fig. 5), confirming the requirement of type IV pili for the process that we and others (11) observed under these conditions. It is important that we could observe this type of motility, which we will henceforth refer to as “pilus-mediated swarming,” only with Becton Dickinson BBL agar grade A and not with some other types of agar, suggesting the presence of stimulatory or inhibitory materials. The latter is indicated by the fact that the ability to detect this type of motility could be restored for some agars by dialysis of the agar prior to plate preparation, but this was not always the case. This problem may also account for some of the different gross manifestations of motility described in the literature.

Under the conditions of our assay, the PAO1 $\Delta$ *chpA* mutant produced a zone of expansion on the agar surface similar to if not greater than that of PAO1, but the colony did not possess the characteristic branching pattern. This suggests that none of the central HPT domains are required for this form of motility per se, and it is a moot point whether this motility is another manifestation of TM or in some way distinct from it, although we favor the former. It is unlikely that the truncated ChpA protein retains residual activity, as the deletion is not in frame and gives a phenotype similar to that of the mutant lacking the CheY effector domain (Fig. 5). Complementation of PAO1 $\Delta$ *chpA* with wild-type *chpA* restored the branching colony morphology, confirming that the mutation in *chpA* is responsible for the altered swarming phenotype seen in the PAO1 $\Delta$ *chpA* mutant. These data suggest that *chpA* is not required for the form of motility observed under these conditions

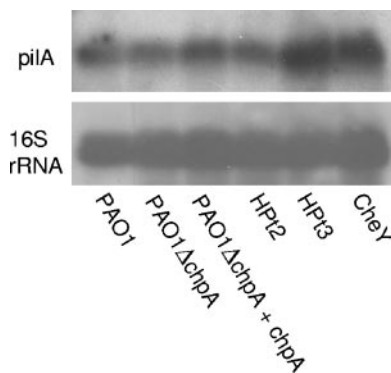


FIG. 4. Northern analysis of *pilA* transcript for ChpA point mutants. The top and bottom panels show the autoradiographs from the *pilA* and 16S rRNA probes, respectively. The shorthand notation for HPT2, HPT3, and CheY is used to designate PAO1 $\Delta$ *chpA* containing plasmids (pAL86, pAL87, and pAL92, respectively) encoding *chpA* alleles with mutations in the corresponding domain. The 16S rRNA probe was used to quantitate the total amount of RNA loaded.

(although *pilA* is) but that it does affect its characteristics, presumably by influencing chemosensory directional control.

The HPT1 and HPT4 point mutants produce a swarming colony morphology identical to that of the wild type and appear to have no effect on this process, at least at a gross level. Interestingly, the two HPT domains that regulate twitching motility, HPT2 and HPT3, both show an unusual swarming phenotype, where thick finger-like projections replace the normal dendritic pattern. The HPT5 point mutant produces a colony morphology that is identical to that of the PAO1 $\Delta$ *chpA* mutant, suggesting that the phenotype seen in the *chpA* deletion mutant is from inactivation of the HPT5 domain. The SPT point mutant produces a small irregular-shaped colony that appears to be restricted in its ability to swarm. The point mutant in the CheY domain exhibited an altered motility phenotype that resembles that of the PAO1 $\Delta$ *chpA* mutant except that some simplified branching is observed (Fig. 5). Interestingly, neither of these mutants showed any motility in the conventional twitching stab assay but did exhibit active motility in the swarming assay, indicating that ChpA is not required for *pilA*-dependent motility per se but rather that lack of chemosensory control of this process affects the former more than the latter. Additionally, although surfactants have been reported to affect swarming motility in *P. aeruginosa* (11) and other species (8), we did not detect any significant alteration in the level of rhamnolipid production in the *chpA* mutants under the conditions of the assay (data not shown).

## DISCUSSION

The results of the twitching complementation assay indicate that the HPT2, HPT3, and CheY domains of ChpA are directly involved in the signal transduction pathways that control TM. Given that the mechanism of TM is the extension and retraction of pili (16, 28), it is tempting to speculate that one of the HPT domains initiates the signal for extension, while the other initiates the signal for retraction, through PilH and PilG, respectively (both of which contain CheY domains), according to their predicted roles (5, 6, 33). However, there is a difference

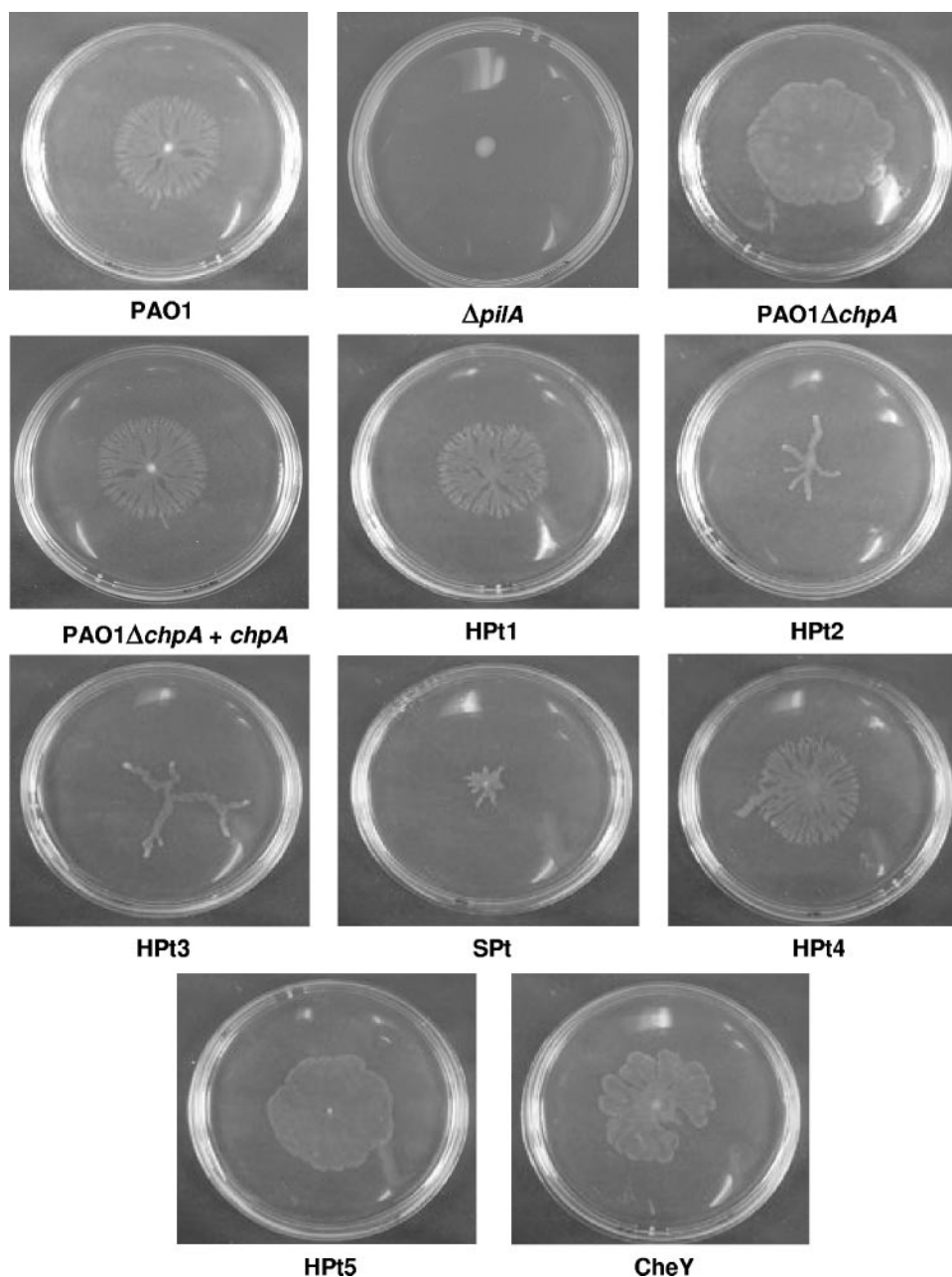


FIG. 5. Swarming motility phenotypes of *chpA* point mutants. The photographs are representative of three separate experiments. Swarm plates contained a modified M9 medium with 0.2% glucose (wt/vol) and 0.05% glutamate or aspartate (wt/vol) as sole carbon and nitrogen sources, respectively, and 0.5% agar (Becton Dickinson BBL agar grade A), supplemented with 0.3 mM IPTG and the appropriate antibiotics. Single colonies were inoculated onto the surface of the agar and incubated at 37°C for 16 h (11).

in twitching phenotypes between PilG/PilH and the HPT2/3 domains. A *pilG* mutant has little to no surface pili and is nontwitching, while a *pilH* mutant is hyperpiliated and is an aberrant twitcher (1, 5, 6). A *pilH* mutant also displays an aberrant swarming phenotype (data not shown) that resembles a  $\Delta$ *chpA* mutant but not the individual HPT2 and HPT3 mutants (Fig. 5). Therefore, it would seem unlikely that either HPT domain is interacting exclusively with PilG or PilH. An alternative hypothesis that is in agreement with the twitching and swarming phenotypes is that both HPT domains (and perhaps

other ChpA-HPT domains) can interact with either, or both, PilG and PilH. The notion that HPT domains can service multiple response regulators is supported by the chemotaxis phosphorelay of *Rhodobacter sphaeroides* (21) and the ArcB/ArcA/OmpR phosphorelay of *E. coli* (15).

Interestingly, the PAO1 $\Delta$ *chpA* mutant also exhibited wild-type levels of *pilA* transcription as observed by Northern analysis (Fig. 4). This is in contrast to our previous finding that some C-terminal transposon mutants of *chpA* in strain PAK resulted in reduced levels of *pilA* mRNA, although this effect

was variable (33). One explanation may be that there are significant strain differences between PAO and PAK in relation to ChpA, and indeed, we have been unable to complement a PAK $\Delta$ chpA mutant with PAO1 chpA (A. J. Leech, unpublished observation). It is also possible that the difference in pilA transcription between the PAO1 $\Delta$ chpA mutant and the C-terminal PAK transposon mutants may be caused by a dominant negative effect of truncated, nearly full-length ChpA protein in the transposon mutants (33).

The complete absence of surface translocation in the pilA deletion mutant (Fig. 5) indicates that what has been previously described as swarming motility is dependent on type IV pili, in agreement with the findings reported previously by Kohler et al. (11), and may be either a different manifestation of twitching motility at the gross colony morphology level under the assay conditions employed or a distinct motility that also requires type IV pili, analogous to the requirement of flagella (polar and peritrichous, respectively) for swimming and swarming motility in other bacteria. It is not known whether the surface deployment of type IV pili is altered under the "swarming" motility assay conditions in *P. aeruginosa*. The finger-like rafts seen for wild-type pilus-mediated swarming motility are, superficially at least, characteristic of swarming motility in other bacteria, although they resemble (on a larger scale) the motile rafts that are distinctive for the leading edge of a twitching zone (4, 6, 9).

The chemosensory system that controls type IV pilus-mediated motility in *M. xanthus* (*frz*) contains many modules that are similar to the *chp* system of *P. aeruginosa* (16). While there are a number of orthologs between the two systems (mimicking the Che proteins of *E. coli* and *Salmonella enterica* serovar Typhimurium), the closest match to ChpA in terms of domain type and organization (i.e., a CheA-CheY hybrid) is FrzE, although ChpA is substantially more complex. Mutational studies that assess motility phenotypes, similar to the ones completed here, have been carried out on *frzE*. There appear to be analogies between the phenotypes observed in the two studies. The swarm morphologies of the HPT2/3 mutations (which seem to lack the ability to divide the swarm stream) are similar to *frzA*, *frzE* in-frame, or *frzE(D709E)* mutations, which cause a hyporeversal phenotype in social gliding motility (3, 13), while the CheY-ChpA mutation (which seems to divide the swarm stream continuously) results in swarm morphologies similar to an *frzE(D709A)* mutation, which gives a hyperreversal phenotype in social gliding motility (13).

Regardless of the semantics of terminology for the observed motility, it is clear that the "swarming" assay is a sensitive indicator of perturbations in type IV pilus-mediated motility resulting from point mutations in phosphotransfer domains of ChpA. The sensitivity of the assay makes it potentially a good system to probe the subtleties of the chemosensory control of twitching/swarming motility in *P. aeruginosa* as well as perhaps to study such motility in other species. It is also apparent that the role of the different phosphotransfer domains, and the signals that they respond to and impart, remains to be elucidated. The mutants described here provide useful tools to enable future studies to dissect this complex system.

## ACKNOWLEDGMENT

This work was supported by National Health and Medical Research Council of Australia (NHMRC) grant number 143054.

## REFERENCES

1. Alm, R. A., and J. S. Mattick. 1997. Genes involved in the biogenesis and function of type-4 fimbriae in *Pseudomonas aeruginosa*. *Gene* **192**:89–98.
2. Alm, R. A., and J. S. Mattick. 1995. Identification of a gene, pilV, required for type 4 fimbrial biogenesis in *Pseudomonas aeruginosa*, whose product possesses a pre-pilin-like leader sequence. *Mol. Microbiol.* **16**:485–496.
3. Blackhart, B. D., and D. R. Zusman. 1985. "Frizzy" genes of *Myxococcus xanthus* are involved in control of frequency of reversal of gliding motility. *Proc. Natl. Acad. Sci. USA* **82**:8767–8770.
4. Bradley, D. E. 1980. A function of *Pseudomonas aeruginosa* PAO polar pili: twitching motility. *Can. J. Microbiol.* **26**:146–154.
5. Darzins, A. 1994. Characterization of a *Pseudomonas aeruginosa* gene cluster involved in pilus biosynthesis and twitching motility: sequence similarity to the chemotaxis proteins of enterics and the gliding bacterium *Myxococcus xanthus*. *Mol. Microbiol.* **11**:137–153.
6. Darzins, A. 1993. The pilG gene product, required for *Pseudomonas aeruginosa* pilus production and twitching motility, is homologous to the enteric, single-domain response regulator CheY. *J. Bacteriol.* **175**:5934–5944.
7. Darzins, A. 1995. The *Pseudomonas aeruginosa* pilK gene encodes a chemotactic methyltransferase (CheR) homologue that is translationally regulated. *Mol. Microbiol.* **15**:703–717.
8. Fraser, G. M., and C. Hughes. 1999. Swarming motility. *Curr. Opin. Microbiol.* **2**:630–635.
9. Henrichsen, J. 1972. Bacterial surface translocation: a survey and a classification. *Bacteriol. Rev.* **36**:478–503.
10. Huang, B., C. B. Whitchurch, L. Croft, S. A. Beatson, and J. S. Mattick. 2000. A minimal tiling path cosmid library for functional analysis of the *Pseudomonas aeruginosa* PAO1 genome. *Microb. Comp. Genomics* **5**:189–203.
11. Kohler, T., L. K. Curty, F. Barja, C. van Delden, and J. C. Pechere. 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signalling and requires flagella and pili. *J. Bacteriol.* **182**:5990–5996.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
13. Li, Y., V. H. Bustamante, R. Lux, D. Zusman, and W. Shi. 2005. Divergent regulatory pathways control A and S motility in *Myxococcus xanthus* through FrzE, a CheA-CheY fusion protein. *J. Bacteriol.* **187**:1716–1723.
14. Lyczak, J. B., C. L. Cannon, and G. B. Pier. 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes Infect.* **2**:1051–1060.
15. Matsubara, M., S. I. Kitaoka, S. I. Takeda, and T. Mizuno. 2000. Tuning of the porin expression under anaerobic growth conditions by His-to-Asp cross-phosphorelay through both the EnvZ-osmosensor and ArcB-anaerosensor in *Escherichia coli*. *Genes Cells* **5**:555–569.
16. Mattick, J. S. 2002. Type IV pili and twitching motility. *Annu. Rev. Microbiol.* **56**:289–314.
17. Mattick, J. S., M. M. Bills, B. J. Anderson, B. Dalrymple, M. R. Mott, and J. R. Egerton. 1987. Morphogenetic expression of *Bacteroides nodosus* fimbriae in *Pseudomonas aeruginosa*. *J. Bacteriol.* **169**:33–41.
18. Mongkolsuk, S., P. Vattanaviboon, S. Rabibhadana, and P. Kiatpapan. 1993. Versatile gene cassette plasmids to facilitate the construction of generalized and specialized cloning vectors. *Gene* **124**:131–132.
19. Morales, V. M., A. Backman, and M. Bagdasarian. 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. *Gene* **97**:39–47.
20. Ochsner, U. A., A. K. Koch, A. Fiechter, and J. Reiser. 1994. Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* **176**:2044–2054.
21. Porter, S. L., and J. P. Armitage. 2002. Phosphotransfer in *Rhodobacter sphaeroides* chemotaxis. *J. Mol. Biol.* **324**:35–45.
22. Rashid, M. H., and A. Kornberg. 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **97**:4885–4890.
23. Rauprich, O., M. Matsushita, C. J. Weijer, F. Siegert, S. E. Esipov, and J. A. Shapiro. 1996. Periodic phenomena in *Proteus mirabilis* swarm colony development. *J. Bacteriol.* **178**:6525–6538.
24. Reikofski, J., and B. Y. Tao. 1992. Polymerase chain reaction (PCR) techniques for site-directed mutagenesis. *Biotechnol. Adv.* **10**:535–547.
25. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. Schweizer, H. P. 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis* *sacB* marker. *Mol. Microbiol.* **6**:1195–1204.
27. Schweizer, H. P. 1991. *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* **97**:109–121.

28. **Semmler, A. B., C. B. Whitchurch, and J. S. Mattick.** 1999. A re-examination of twitching motility in *Pseudomonas aeruginosa*. *Microbiology* **145**:2863–2873.
29. **Simon, R., U. Reifer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. *BioTechnology* **1**:784–791.
30. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
31. **Vieira, J., and J. Messing.** 1991. New pUC-derived cloning vectors with different selectable markers and DNA replication origins. *Gene* **100**:189–194.
32. **Watson, A. A., R. A. Alm, and J. S. Mattick.** 1996. Construction of improved vectors for protein production in *Pseudomonas aeruginosa*. *Gene* **172**:163–164.
33. **Whitchurch, C. B., A. J. Leech, M. D. Young, D. Kennedy, J. L. Sargent, J. J. Bertrand, A. B. Semmler, A. S. Mellick, P. R. Martin, R. A. Alm, M. Hobbs, S. A. Beatson, B. Huang, L. Nguyen, J. C. Commolli, J. N. Engel, A. Darzins, and J. S. Mattick.** 2004. Characterization of a complex chemosensory signal transduction system which controls twitching motility in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **52**:873–893.
34. **Whitchurch, C. B., and J. S. Mattick.** 1994. Characterization of a gene, pilU, required for twitching motility but not phage sensitivity in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **13**:1079–1091.