Effect of Site-Specific Mutations in Different Phosphotransfer Domains of the Chemosensory Protein ChpA on Pseudomonas aeruginosa Motility

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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, 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 pili-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 pilGHIJK (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 one 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 DH5α (recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 680dlacZΔM15) 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 PA01 strain ATCC 15692 (American Type Culture Collection). 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...
TABLE 1. Strains and plasmids used in this study

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Plasmids

- **pBluescript II KS**: E. coli cloning vector with Ap selection
- **pOK2**: E. coli cloning vector with Km selection
- **pUC21**: E. coli cloning vector with Ap selection
- **pUCP19**: E. coli-P. aeruginosa cloning vector
- **pUCPSK**: P. aeruginosa-E. coli shuttle vector
- **pMMB206**: Pseudomonas controlled expression level vector with Cm selection
- **pMOB3**: Counterselectable sacB/sacR genes from Bacillus subtilis
- **pSM-TET**: Source of Tet’ cartridge

**MTP26**
- Cosmid containing *P. aeruginosa* PAO1 chromosomal DNA from bp 448166-472109

**pAL1**
- pBluescript II KS with modified multiple cloning site

**pAL5**
- 3,168-bp SacII/EcoRV fragment of chpA from MTP26 in pAL1
- 458-bp H912I PCR product in pUC21

**pAL6**
- 3,168-bp SacII/EcoRV fragment of chpA containing H1088 in pAL1
- 1,297-bp S1231H/H1410i PCR product in pUC21

**pAL7**
- 611-bp H1570i PCR product in pUC21

**pAL10**
- 872-bp BamHI/KpnI fragment of chpA containing H1717 in pUC21

**pAL11**
- 1,396-bp EcoRV/KpnI fragment of chpA from MTP26 in pAL5

**pAL13**
- 1,561-bp EcoRV/AatII fragment of chpA from MTP26 containing H54 in pOK12

**pAL14**
- ChpA H54Q in pAL13

**pAL16**
- ChpA H912Q in pAL6

**pAL17**
- ChpA H1088Q in pAL7

**pAL18**
- ChpA S1231A in pAL8

**pAL19**
- ChpA H1410Q in pAL8

**pAL20**
- ChpA H1570Q in pAL9

**pAL37**
- 2,839-bp BamHI/ClaI fragment of chpA from MTP26 containing D2406N in pUC21

**pAL40**
- 4,291-bp SacI/ClaI fragment in pUCPSK

**pAL41**
- Full-length chpA under Plac control in PUCPSK

**pAL42**
- ChpA D2406N in pAL37

**pAL49**
- Te’ cassette from pSM-TET (EcoRI/MluI) inserted into MfeI/AscI pAL11

**pAL52**
- 5-kb NotI fragment from pMOB3 ligated into pAL49

**pAL57**
- 7,972-bp NotI chpA fragment from MTP26 in pAL1

**pAL64**
- pAL41 with an internal double FLAG tag inserted between the native Nott/NruI sites of chpA

**pAL77**
- 3,862-bp ClaI/Sacl chpA fragment from pAL41 in pUCP19

**pAL78**
- Full-length chpA against Plac in pUCP19

**pAL79**
- 8-kb ClaI/Sacl fragment from MTP26 ligated into pMMB206 with PlacUV

**pAL82**
- 3,503-bp Stul fragment of chpA cut from pAL57 and ligated into pAL14

**pAL83**
- pAL79 with ChpA H54Q (mutation in HP1 domain)

**pAL86**
- pAL79 with ChpA H912Q (mutation in HP2 domain)

**pAL87**
- pAL79 with ChpA H1088Q (mutation in HP3 domain)

**pAL88**
- pAL79 with ChpA S1231A (mutation in SPt domain)

**pAL89**
- pAL79 with ChpA H1410Q (mutation in HP4 domain)

**pAL90**
- pAL79 with ChpA H1570Q (mutation in HP5 domain)

**pAL92**
- pAL79 with ChpA D2406N (mutation in CheY domain)

**pAL79FLAG**
- pAL27 with a double FLAG tag inserted into the NotI/KpnI sites of chpA

**pAL86FLAG**
- pAL66 with a double FLAG tag inserted into the NotI/KpnI sites of chpA

**pAL87FLAG**
- pAL87 with a double FLAG tag inserted into the NotI/KpnI sites of chpA

**pAL92FLAG**
- pAL92 with a double FLAG tag inserted into the NotI/KpnI sites of chpA

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 μg/ml tetracycline for plasmid selection and 40 μg/ml tetracycline for cosmid selection, 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, and 50 μg/ml kanamycin. The concentrations of antibiotics for the selection of *P. aeruginosa* were as follows: 250 μg/ml carbenicillin, 20 μg/ml rifampin, 250 μg/ml chloramphenicol, and 200 μg/ml tetracycline.

**Construction of PAO1ΔchpA**

The *chpA* deletion mutant PAO1ΔchpA 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 Tem 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 mutant 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 using either the Stratagene QuikChange site-directed mutagenesis kit (catalog no. 200518) or a sequential PCR method (24). Oligonucleotide primer sequences

**Construction of chpA expression vectors.** Cosmid MTP26 (10) was digested with SacI and Clal 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 pUCP19. A 3,862-bp Clal/SacI fragment was cut from pAL41 and ligated into Accl/Sacl-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 PlacUV5 promoter) was chosen in preference to pMMB207 (which uses a Plac promoter) primarily because of the relative strengths of the promoters (19). The PlacUV5 promoter has previously been reported to reach only 6.5% of the expression level of the Plac promoter in _Pseudomonas_ species (19), reducing the chance of detrimental effects of 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 H192Q, H1086Q, S1275A, H19211410Q, and H1757Q were subcloned from pAL16, pAL17, pAL18, pAL19, and pAL20, respectively, into pAL79 using the unique restriction enzyme sites for Mfel and KpnI to give pAL86, pAL78, pAL87, 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 Stul fragment was cut from pAL57 and cloned into Stul-digested pAL14, giving pAL82. This step increased the length of the inactivated gene in the chromosome. The genotype of the mutant was confirmed by Southern hybridization analysis. 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 NaCl, supplemented with 0.2% glucose (wt/vol), 0.05% glutamate or aspartate (wt/vol) (as the sole nitrogen source), 2 mM MgSO4, and trace elements (available upon request) and solidified with 0.5% agar (Becton Dickinson 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 PA01 (PA01ΔchpA) 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 _PlacUV5_ promoter (19). Eight phosphotransfer domains (HP1 to HP6, SP1, 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 HP6 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 _PlacUV_ in pAL79 (Table 1; also see Fig. S1 in the supplemental material) and transformed into PA01ΔchpA 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 PA01ΔchpA 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 HP1, SP1, HP4, and HP5 as well as the complemented _chpA_ mutant (PA01ΔchpA plus _chpA_) exhibit TM zones that are indistinguishable from those of the wild type. The _chpA_ deletion mutant (PA01ΔchpA) and the CheY point mutant both appear to completely lack TM, while the HP2 and HP3 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...
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

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**FIG. 1.** Diagram of chpA open reading frame detailing the location of phosphotransfer active-site codons (circles), a Tet' 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.

**FIG. 2.** (A) Subsurface twitching motility assay of *P. aeruginosa* strains PAO1, PAO1ΔchpA, PAO1ΔchpA 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 PAO1ΔchpA 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.
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 PAO1ΔpilA showed no zone of expansion over the agar surface (Fig. 5), confirming the requirement of type IV pili for the formation of this motility (16, 28). 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 PAO1ΔpilA 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 PAO1ΔpilA mutant except that some simplified branching is observed (Fig. 5). Interestingly, neither of these mutants showed any motility in the conventional twitching stab 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 pilA (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...
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 /H9004 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 PAO1ΔchpA 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 PAKΔchpA mutant with PAO1 chpA (A. J. Lecch, unpublished observation). It is also possible that the difference in pilA transcription between the PAO1ΔchpA 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 swimming 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 Chp 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 HPr2/3 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