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Cluster II *che* Genes from *Pseudomonas aeruginosa* Are Required for an Optimal Chemotactic Response

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***Pseudomonas aeruginosa*, a γ-proteobacterium, is motile by means of a single polar flagellum and is chemotactic to a variety of organic compounds and phosphate. *P. aeruginosa* has multiple homologues of *Escherichia coli* chemotaxis genes that are organized into five gene clusters. Previously, it was demonstrated that genes in cluster I and cluster V are essential for chemotaxis. A third cluster (cluster II) contains a complete set of *che* genes, as well as two genes, *mcpA* and *mcpB*, encoding methyl-accepting chemotaxis proteins. Mutations were constructed in several of the cluster II *che* genes and in the *mcp* genes to examine their possible contributions to *P. aeruginosa* chemotaxis. A *cheB2* mutant was partially impaired in chemotaxis in soft-agar swarm plate assays. Providing *cheB2* in *trans* complemented this defect. Further, overexpression of CheB2 restored chemotaxis to a completely nonchemotactic, cluster I, *cheB*-deficient strain to near wild-type levels. An *mcpA* mutant was defective in chemotaxis in media that were low in magnesium. The defect could be relieved by the addition of magnesium to the swarm plate medium. An *mcpB* mutant was defective in chemotaxis when assayed in dilute rich soft-agar swarm medium or in minimal-medium swarm plates containing any 1 of 60 chemoattractants. The mutant phenotype could be complemented by the addition of *mcpB* in *trans*. Overexpression of either McpA or McpB in *P. aeruginosa* or *Escherichia coli* resulted in impairment of chemotaxis, and these cells had smooth-swimming phenotypes when observed under the microscope. Expression of *P. aeruginosa cheA2*, *cheB2*, or *cheW2* in *E. coli* K-12 completely disrupted wild-type chemotaxis, while expression of *cheY2* had no effect. These results indicate that *che* cluster II genes are expressed in *P. aeruginosa* and are required for an optimal chemotactic response.**

Chemotaxis, the directed movement towards chemicals in the environment, is a behavioral response exhibited by most flagellated bacteria. *Escherichia coli* and *Salmonella enterica* serovar Typhimurium have served as prototype organisms for studying chemotaxis, and the signal transduction pathway used to effect a chemotactic response in these γ-proteobacteria is a paradigm for "two-component" and histidine kinase phosphosignaling pathways (5, 6, 54, 55). A set of six chemotaxis proteins acts in concert with receptors called methyl-accepting chemotaxis proteins (MCPs). The current model is that MCPs exist as homodimers that are physically associated with a CheW linker protein dimer and a CheA dimer. There is evidence that these dimeric signaling units exist in cells as supermolecular complexes that are arranged as trimers of dimers (30, 51). On binding an amino acid or other attractant, an MCP dimer undergoes a conformational change that initiates sensory signal transduction by altering the activity of CheA, which is a sensor histidine kinase. CheA-P is a phosophodonor for the response regulator protein, CheY. CheY-P is mobile in the cell and interacts with the rotational "switch" protein FliM in the flagellar motors. The flagellar motors of *E. coli* and *S. enterica* are in a default counterclockwise rotation status. In this condition, the peritrichous flagella form a bundle that propels the cell in a single direction (smooth swimming). When CheY-P binds to FliM, the flagella rotate clockwise. This causes the flagellar bundle to come apart; each flagellum pushes in a different direction, and the cell "tumbles" and changes direction. The phosphorylation status of CheY thus dictates whether *E. coli* runs or tumbles. As cells swim up a concentration gradient of attractant, they spend more time smooth swimming than tumbling; this modulation of swimming behavior is manifested as a chemotactic response.

While CheY phosphorylation relies on the activity of the histidine kinase CheA, its dephosphorylation is controlled by CheZ, as well as by an intrinsic dephosphorylation activity. To ensure proper periodic monitoring of the environment, the system is reset by methylation and demethylation of the MCPs. MCP methylation counterbalances the effect of attractant binding and contributes to adaptation by resetting the signaling activity of the receptors, despite the continued presence of stimulus (12). Two proteins regulate the level of methylation of MCPs. CheR, a methyltransferase, adds methyl groups to conserved cytoplasmic glutamate residues. CheB, a methylesterase, which is active when phosphorylated by CheA-P, removes the methyl groups.

The fundamental characteristics of signal reception and transduction that occur during chemotaxis by *E. coli* are likely conserved among bacteria and archaea. However, with the recent proliferation of genome sequences, we also now realize that there is much more diversity and complexity in chemotactic signaling pathways in prokaryotes than had been previously anticipated. Most motile bacterial species for which genome sequence information is available have multiple homologues of each of the *E. coli che* genes; most have many more methyl-accepting chemotaxis genes than the five found in the well-

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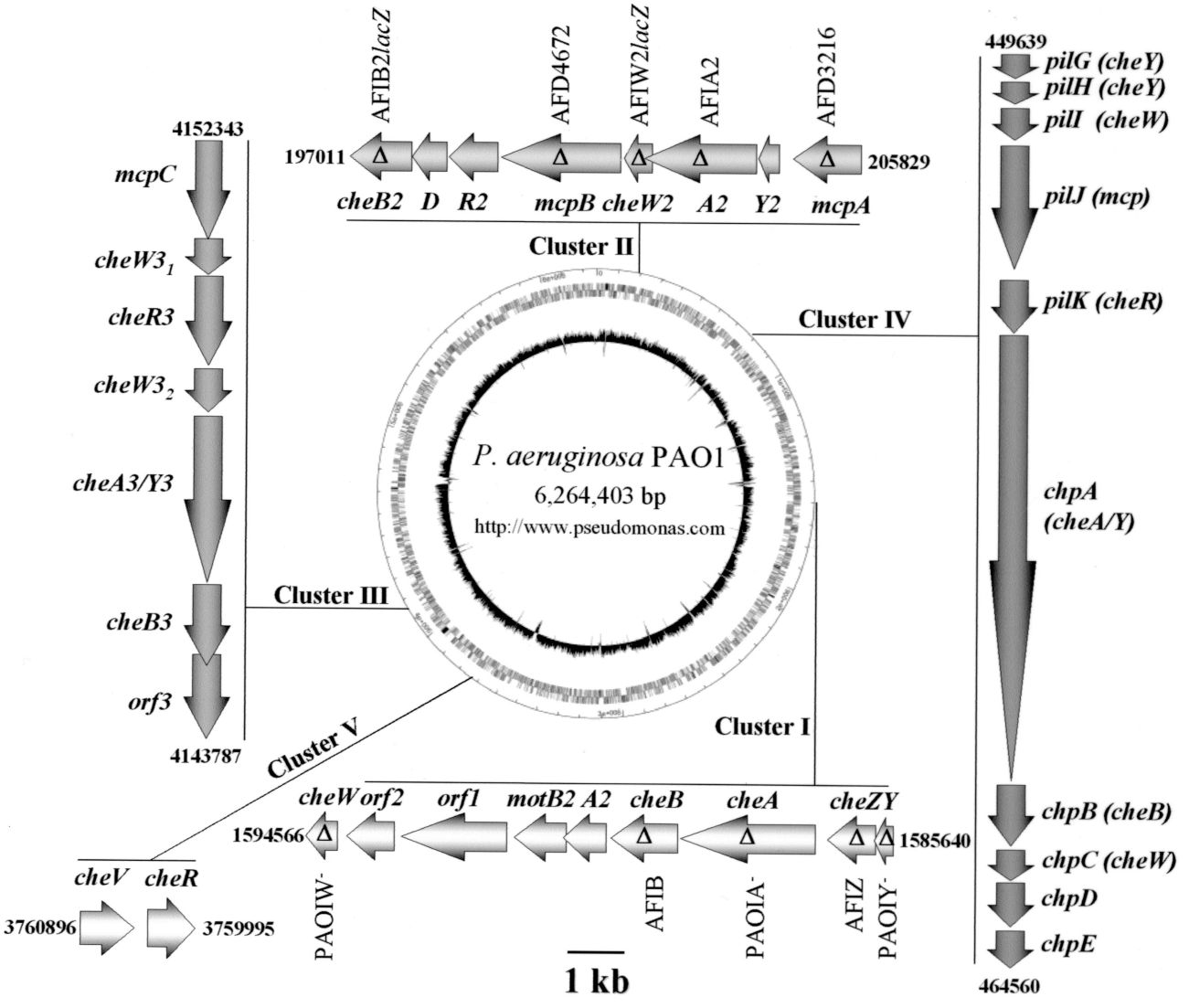

FIG. 1. Chemotaxis genes in *P. aeruginosa*. *P. aeruginosa* has five clusters of chemotaxis-like genes. Clusters I and V have been previously demonstrated to be involved in swimming motility chemotaxis (24, 37). Cluster IV is involved in twitching motility (8, 25). Mutations constructed in this study are indicated by a delta (Δ) within the arrow representing each gene. Names given to each mutant strain are indicated either above or below each mutation (Table 1 provides further information). The *P. aeruginosa* PAO1 genome map (center) was obtained from the Pseudomonas Genome Project website (http://www.pseudomonas.com), and the positional numbers flanking each cluster of genes are as previously described (*Pseudomonas aeruginosa* Community Annotation Project [http://www.pseudomonas.com]).

studied enteric species (5). *Bacillus subtilis* has chemotaxis genes (*cheC* and *cheD*) not found in enterics (22, 31, 45). The α-proteobacteria *Rhodobacter sphaeroides*, *Sinorhizobium meliloti*, and *Caulobacter crescentus* have several copies of genes encoding products homologous to those of *E. coli che* genes, but each lacks a *cheZ* homologue (2, 41).

Pseudomonas aeruginosa is an opportunistic human pathogen and member of the γ-*Proteobacteria*. It swims in liquid environments by means of a single polar flagellum, and it can also move on solid surfaces by means of swarming (32) and twitching (49). *P. aeruginosa* is chemotactic to most of the organic compounds that it can grow on, and several repellants have been identified (14, 23, 27, 39, 40, 42). *P. aeruginosa* has 26 genes that are homologous to *E. coli mcp* genes. It also has multiple copies of *E. coli*-like chemotaxis genes arranged in five clusters (Fig. 1) (56). Two *che* clusters, cluster I and cluster V, which encode homologues of the six *che* genes found in *E. coli*, have previously been shown to be essential for chemotaxis by *P. aeruginosa* (24, 37). Cluster IV has been shown to be involved in twitching motility (8, 25). Here, we investigate the role that cluster II chemotaxis-like proteins may play in *P. aeruginosa* chemotaxis.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown on rich medium, Luria-Bertani (LB) medium (46), at 37°C unless otherwise noted. Antibiotics were used at the following concentrations, where appro-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
P. aeruginosa		
AFD3216	*mcpA*::Gm^r derivative of PAO1-Ig; 873 bp deleted from gene	This study
AFD4672	*mcpB*::Gm^r derivative of PAO1-Ig; 234 bp deleted from gene	This study
AFIA2	Δ*cheA2* derivative of PAO1-Ig; 1,818 bp deleted from gene	This study
AFIB	Δ*cheB* derivative of PAO1-Ig; 1,065 bp deleted from gene	This study
AFIB2*lacZ*	*cheB2*::*lacZ*-Gm^r derivative of PAO1-Ig; 1,026 bp deleted from gene	This study
AFIW2*lacZ*	*cheW2*::*lacZ*-Gm^r derivative of PAO1-Ig; 402 bp deleted from gene	This study
AFIZ	Δ*cheZ* derivative of PAO1-Ig; 738 bp deleted from gene	This study
PAO1	Sequenced wild-type strain, which contains a frame shift mutation in the *pilC* gene; twitching motility$^-$	56
PAO1-Ig	Wild-type strain; twitching motility$^+$	44
PAOIA$^-$	Δ*cheA* derivative of PAO1-Ig; 2,226 bp deleted from gene	This study
PAOIW$^-$	Δ*cheW* derivative of PAO1-Ig; 433 bp deleted from gene	This study
PAOIY$^-$	Δ*cheY* derivative of PAO1-Ig; 327 bp deleted from gene	This study
E. coli		
CC118	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 phoA20 galE galK thi rpsE rpoB argE*(Am) *recA1*	28
DH5α	F$^-$ λ$^-$ *recA1* Δ(*lacZYA-argF*)*U169 hsdR17 thi-1 gyrA96 supE44 endA1 relA1* ϕ80*lacZ*ΔM15	Gibco-BRL
K-12	Wild-type strain	4
S17-1	*thi pro hdsR hdsM*$^+$ *recA*; chromosomal insertion of RP4-2 (Tc::Mu Km::Tn*7*)	52
Plasmids		
pAF27	Ap^r; *mcpB* PCR amplified and cloned into *Eco*RI/*Hin*dIII sites of pUC18	This study
pAF27B	Ap^r/Cb^r; *mcpB* PCR amplified and cloned into *Eco*RI/*Hin*dIII sites of pEX1.8	This study
pAF28	Ap^r; *Sma*I fragment from pUCGm (carrying the gentamicin cassette) cloned into pAF27 cut with *Ava*I and Klenow treated	This study
pAF29	Tc^r; *Eco*RI/*Hin*dIII fragment from pAF28 subcloned into pRK415	This study
pAF33	Ap^r; *mcpA* PCR amplified and cloned into *Bam*HI/*Hin*dIII sites of pUC18	This study
pAF35	Ap^r; *SmaI* fragment from pUCGm (carrying the gentamicin cassette) cloned into pAF33 cut with *Bss*HI and Klenow treated	This study
pAF37	Tc^r; *Bam*HI/*Hin*dIII fragment from pAF35 subcloned into pRK415	This study
pAF56	Ap^r; in-frame deletion of *cheW2* constructed by PCR and cloned into *Eco*RI/*Hin*dIII sites of pUC19	This study
pAF57	Ap^r; *Sma*I fragment from pUC*lacZ*Gm containing the *lacZ*-Gm^r cassette cloned into *Sma*I site of pAF56	This study
pAF58	Tc^r; *Hin*dIII/*Eco*RI fragment from pAF57 cloned into pRK415	This study
pAFB2T	Tc^r; in-frame deletion of *cheB2* constructed by PCR and cloned into *Eco*RI/*Hin*dIII sites of pEX19Tc	This study
pAFB2T*lacZ*Gm	Tc^r; *Sma*I fragment from pUC*lacZ*Gm containing the *lacZ*-gentamicin cassette cloned into *Sca*I site of pAFB2T	This study
pAFB2T*lacZ*Gm2	Tc^r; *Hin*dIII/*Eco*RI fragment from pAFB2T*lacZ*Gm cloned into pRK415	This study
pAFBG	Gm^r; in-frame deletion of *cheB* constructed by PCR and cloned into *Eco*RI/*Hin*dIII sites of pEX19Gm	This study
pAFZG	Gm^r; *Eco*RI/*Hin*dIII fragment from pUCZ subcloned into *Eco*RI/*Hin*dIII sites of pEX19Gm	This study
pEX1.8	Ap^r/Cb^r; high-copy-number cloning and expression vector; contains both ColE1 *ori* and *P. aeruginosa ori*; carries *lacI*^{q} and a P_{tac} promoter for expression of gene insert	44
pEX19Gm	Gm^r; *oriT*$^+$ *sacB*$^+$; gene replacement vector with MCS from pUC19	19
pEX19Tc	Tc^r; *oriT*$^+$ *sacB*$^+$; gene replacement vector with MCS from pUC19	19
pEXA2	Ap^r/Cb^r; *cheA2* PCR amplified from PAO1-Ig chromosome and cloned into *Eco*RI/*Hin*dIII sites of pEX1.8	This study
pEXB2	Ap^r/Cb^r; *cheB2* PCR amplified from PAO1-Ig chromosome and cloned into *Eco*RI/*Hin*dIII sites of pEX1.8	This study
pEXW2	Ap^r/Cb^r; *cheW2* PCR amplified from PAO1-Ig chromosome and cloned into *Eco*RI/*Hin*dIII sites of pEX1.8	This study
pEXY2	Ap^r/Cb^r; *cheY2* PCR amplified from PAO1-Ig chromosome and cloned into *Eco*RI/*Hin*dIII sites of pEX1.8	This study
pHAH128	Ap^r/Cb^r; *mcpA* PCR amplified and cloned into *Eco*RI/*Hin*dIII sites of pEX1.8.	This study
pRK415	Tc^r; broad-host-range cloning vector	26
pRK600	Cm^r; *ori* ColE1 RK2-Mob$^+$ RK2-Tra$^+$	28
pUC18	Ap^r; high-copy-number cloning vector	61
pUC18*Not*	Ap^r; identical to pUC18 except has *Not*I/polylinker from pUC18/*Not*I as MCS	17
pUC19	Ap^r; high-copy-number cloning vector	61
pUCGm	Ap^r Gm^r; pUC19 derivative carrying a Gm^r cassette	48
pUCGm*Not*	Ap^r Gm^r; 0.8-kb *Sma*I fragment from pUCGm cloned into *Hin*dII site of pUC18*Not*	This study
pUC*lacZ*Gm	Ap^r; high-copy-number *ori* ColE1 vector containing a promoterless *lacZ* followed by a Gm^r gene; 4-kb *lacZ*-Gm^r cassette can be excised as *Sma*I or *Bam*HI cassette useful for generating *lacZ* transcriptional fusions	This study
pUCZ	Ap^r; in-frame deletion of *cheZ* constructed by PCR and cloned into *Eco*RI/*Hin*dIII sites of pUC19	This study
pUT*lacZ*1 Gm	Gm^r; *Not*I fragment from pUCGm*Not* containing the Gm^r cassette cloned into *Not*I sites of pUTminiTn*5lacZ*1, replacing Km^r cassette	This study
pUTminiTn*5lacZ*1	Km^r; mini-Tn5-based promoter-probe vector	9

[a] Ap^r, ampicillin resistant; Cb^r, carbenicillin resistant; Cm^r, chloramphenicol resistant; Gm^r, gentamicin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant; MCS, multiple cloning site.

priate: carbenicillin, 300 μg per ml; chloramphenicol, 100 μg per ml; gentamicin (Gm), 100 μg per ml; and tetracycline (Tc), 100 μg per ml (*P. aeruginosa*) and ampicillin (Ap), 100 μg per ml; chloramphenicol, 100 μg per ml; gentamicin, 25 μg per ml; kanamycin, 100 μg per ml; and tetracycline, 25 μg per ml (*E. coli*).

Chemotaxis assays. Chemotaxis was examined qualitatively in soft-agar swarm plates. Strains were stab inoculated into the centers of plates that had been solidified with 0.3% agar and that contained one of the following: a defined minimal medium (11) plus 1 mM succinate; diluted LB medium (0.1% [wt/vol] tryptone, 0.05% [wt/vol] yeast extract, and 0.5% [wt/vol] NaCl); or tryptone (0.1% tryptone [wt/vol] and 0.5% NaCl [wt/vol] for *P. aeruginosa* or 1% tryptone [wt/vol] and 0.5% NaCl [wt/vol] for *E. coli*). Wild-type cells and the *mcpA* and *mcpB* mutants were screened for chemotaxis in minimal medium swarm plates that contained 1 mM concentrations of 60 different growth substrates. The 60 compounds that were tested as potential chemoattractants included amino acids, sugars, organic acids, and several different kinds of aromatic compounds. To test chemotaxis under anaerobic denitrifying conditions, minimal medium-succinate swarm plates were supplemented with 1 mM KNO_3 and incubated anaerobically in polycarbonate jars to which GasPak hydrogen plus carbon dioxide generator envelopes (BBL Microbiology Systems, Cockeysville, Md.) had been added. Carbon sources were sterilized separately and added to minimal medium after

being autoclaved. Soft-agar plates were incubated at 30°C for 18 to 24 h for *P. aeruginosa* and at 35°C for 5 to 6 h for *E. coli*. The assays were repeated at least three times.

Construction of a *lacZ*-Gm^r cassette. A 0.8-kb *Sma*I fragment containing a Gm^r gene was excised and purified from plasmid pUCGm (48). This fragment was then ligated into *Hin*cII-digested pUC18*Not* (10), giving pUCGm*Not*. Plasmid pUTminiTn*5lacZ*1 (9) was digested with *Not*I, and the Km^r gene was removed. A *Not*I fragment that contained the Gm^r gene from pUCGm*Not* was ligated into pUTminiTn*5lacZ*1 digested with *Not*I, yielding pUT*lacZ*1Gm. A *lacZ*-gentamicin fragment was purified out of the latter by digesting it with *Eco*RI. This fragment was blunt ended by treatment with the Klenow fragment of DNA polymerase I (Roche Molecular Biochemicals, Indianapolis, Ind.) and ligated to pUC18*Not* that was digested with *Not*I and blunt ended by treatment with Klenow. Finally, the *Hin*dIII and *Pst*I restriction sites within the *lacZ* and the Gm^r genes were removed by partial digestion with *Hin*dIII and *Pst*I, Klenow treatment, and then religation. The resulting plasmid, pUC*lacZ*Gm, is a high-copy-number *ori* ColE1 vector containing a promoterless *lacZ* followed by a Gm^r gene. This roughly 4-kb fragment can be excised as either an *Sma*I or *Bam*HI cassette for the generation of *lac*Z transcriptional fusions.

Construction of *cheB2*::*lacZ*-Gm^r and *cheW2*::*lacZ*-Gm^r mutant strains. An in-frame deletion of *cheB2* was created by overlap extension PCR as described previously (18, 20, 21) with the following modifications. A region of DNA from the PAO1-Ig chromosome spanning from approximately 1 kb upstream of *cheB2* to the 5′ end of *cheB2* was PCR amplified using primers DcheB2 [1] and DcheB2 [2]. A second region, from approximately 1 kb downstream of *cheB2* to the 3′ end of *cheB2*, was PCR amplified using primers DcheB2 [3] (complementary to DcheB2 [2]), and DcheB2 [4]. A mixture of these two DNA fragments (100 ng each) was used as the template in a third PCR amplification including primers DcheB2 [1] and DcheB2 [4]. The product of the third amplification contained a 1,026-bp in-frame deletion in *cheB2* including an engineered *Sca*I site plus approximately 1 kb upstream and 1 kb downstream of *cheB2*, with engineered *Hin*dIII and *Eco*RI sites on its 5′ and 3′ ends, respectively. This product was digested with *Eco*RI and *Hin*dIII and was ligated into *Hin*dIII/*Eco*RI-digested pEX19Tc, yielding plasmid pAFB2T. The *cheB2-lacZ* fusion was generated by ligation of a *Sma*I *lacZ*-Gm^r cassette obtained from plasmid pUC*lacZ*Gm into *Sca*I-digested pAFB2T, yielding pAFB2T*lacZ*Gm. The *Hin*dIII/*Eco*RI insert from pAFB2T*lacZ*Gm was then subcloned into pRK415, giving pAFB2T*lacZ*Gm2. This plasmid was then mobilized from *E. coli* DH5α into PAO1-Ig, using *E. coli* CC118(pRK600) to provide the transfer functions. The recombinant strain, AFIB2*lacZ*, was identified by screening for Gm^r and Tc^s colonies and verified by Southern blot analysis (46). A *cheW2*::*lacZ*-Gm^r mutant (strain AFIW2*lacZ*) was constructed using a similar strategy.

Construction of *P. aeruginosa cheB*, *cheZ*, *cheY*, *cheW*, *cheA*, and *cheA2* in-frame deletion mutants. An in-frame deletion in *cheB* was constructed by overlap extension PCR, as described above, using primers DcheB [1]-DcheB [2] and DcheB [3]-DcheB [4]. The product of the third amplification contained a 1,065-bp in-frame deletion in *cheB* including an engineered *Sca*I site plus approximately 1 kb upstream and 1 kb downstream of *cheB*, with engineered *Hin*dIII and *Eco*RI sites on its 5′ and 3′ ends, respectively. This 2,125-bp fragment was cloned as an *Eco*RI/*Hin*dIII cassette into pEX19Gm to give plasmid pAFBG. pAFBG was mobilized from *E. coli* S17-1 into PAO1-Ig by conjugation. A single recombination event was selected by growth on selective medium containing gentamicin. The double recombinant was selected by growth on LB medium plus 5% sucrose. Correct colonies were identified by screening for Gm^s and the ability to grow on 5% sucrose. *cheB* mutations were screened by PCR and further confirmed by Southern blot analysis, yielding the nonpolar, in-frame deletion mutant AFIB. Similar cloning strategies and methods were used to construct in-frame deletion mutations in *cheZ*, *cheY*, *cheW*, *cheA*, and *cheA2* genes in *P. aeruginosa* strain PAO1-Ig.

Construction of cluster II *mcpB*::Gm^r and *mcpA*::Gm^r mutants. *mcpB* was PCR amplified from PAO1-Ig chromosomal DNA using the primers RPA04672F (which includes an engineered *Eco*RI site) and RPA04672R (which includes an engineered *Hin*dIII site). The product was cloned into pUC18 digested with *Eco*RI/*Hin*dIII, and the resulting plasmid was named pAF27. pAF27 was digested with *Ava*I, removing a 234-bp fragment from the center of *mcpB*; treated with Klenow and shrimp alkaline phosphatase (Roche Molecular Biochemicals), and ligated to the *Sma*I fragment containing a Gm^r cassette from pUCGm. The resulting plasmid was named pAF28. The *Eco*RI/*Hin*dIII fragment from pAF28 was cloned into pRK415, giving pAF29. This plasmid was transferred from *E. coli* DH5α to *P. aeruginosa* PAO1-Ig by conjugation, using *E. coli* CC118(pRK600) to provide the transfer functions. A double recombinant was selected as described above to give strain AFD4672. The mutation was verified by Southern blotting and PCR. An *mcpA*::Gm^r mutant (AFD3216) was constructed by a similar strategy.

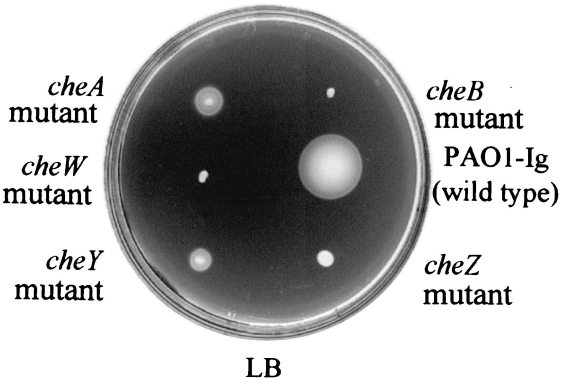

FIG. 2. Cluster I *che* gene mutants are nonchemotactic. Shown is a dilute LB soft-agar plate with cluster I mutant strains. Each strain was motile but deficient in chemotaxis.

Construction of plasmids for expression of cluster II genes. The coding sequences of *cheA2*, *cheB2*, *cheW2*, *cheY2*, *mcpA*, and *mcpB* were PCR amplified from PAO1-Ig chromosomal DNA using the appropriate primer pairs. The upstream primers for each PCR contained an additional sequence (CCGAATT CTGA*TTAACTTTA*TA**AGGAGG**AAAAACATATG. . .) containing an engineered *Eco*RI site (underlined), an optimized Shine-Delgarno sequence (in boldface) (38), a translational enhancer from gene 10 of phage 7 (in italics) (38), and the start codon of the gene being amplified (double underline). The products from PCR amplifications were digested with *Eco*RI/*Hin*dIII and cloned into the *Eco*RI/*Hin*dIII sites of pEX1.8. These new plasmids were designated pEXA2, pEXB2, pEXW2, pEXY2, pHAH128, and pAF27B and were used to express CheA2, CheB2, CheW2, CheY2, McpA, and McpB, respectively. Each plasmid was then introduced into either *P. aeruginosa* competent cells (58) or *E. coli* competent cells (The NEB Transcript, vol. 6, p. 7, New England Biolabs, 1994) and selected for by incorporation of carbenicillin (*P. aeruginosa*) or ampicillin (*E. coli*) into the growth medium. Transformants were verified by agarose gel electrophoresis of plasmid DNA isolated by alkaline lysis (3). Proteins were expressed under the control of the P_{tac} promoter and induced by the addition of 10, 100, or 1,000 μM isopropyl-β-D-thiogalactopyranoside (IPTG) (Research Product International Corp., Mt. Prospect, Ill.) to the growth medium.

RESULTS

Strains with mutations in cluster I *che* genes are generally nonchemotactic. Mutants with in-frame deletions in the *cheA*, *cheB*, *cheW*, *cheZ*, and *cheY* genes of cluster I were severely impaired in chemotaxis in LB medium, tryptone, and minimal medium soft-agar swarm plates (Fig. 2). They were also completely defective in chemotaxis when incubated anaerobically in soft-agar swarm plates that included succinate as an attractant and nitrate as a terminal electron acceptor. This is in agreement with the previously described results of Masduki et al. (37) and Kato et al. (24). The *cheB* and *cheZ* mutants reversed their swimming directions at high frequency when observed microscopically. The *cheA*, *cheW*, and *cheY* mutants had smooth-swimming phenotypes when observed under the microscope. *E. coli cheA*, *cheW* and *cheY* mutants also smooth swim. *E. coli cheB* and *cheZ* mutants have tumbly phenotypes.

A *cheB2* mutant is defective in chemotaxis. We constructed mutations in cluster II *cheB2*, *cheW2*, and *cheA2* genes and found that the *cheW2* and *cheA2* mutants behaved like the wild-type parent in swarm plate assays. The *cheB2* mutant, however, formed swarm rings that were smaller than those of the wild type (Fig. 3A). This general defect was observed in

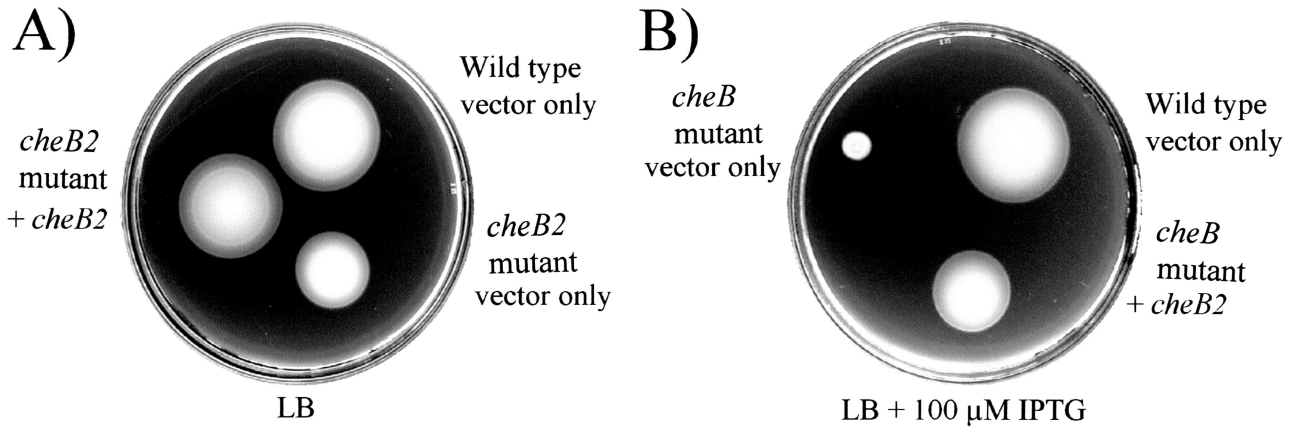

FIG. 3. CheB2 is required for optimal chemotaxis by *P. aeruginosa*, and overexpression of CheB2 partially restores chemotaxis to a nonchemotactic *P. aeruginosa cheB* mutant. (A) Dilute LB soft-agar plate showing chemotactic rings formed by *P. aeruginosa* PAO1-Ig (wild type) harboring only vector (pEX1.8), the *cheB2* mutant AFIB2*lacZ* harboring only vector (pEX1.8), and the *cheB2* mutant complemented with *cheB2* in *trans* (pEXB2). (B) Dilute LB soft-agar plate showing chemotactic rings formed by *P. aeruginosa* PAO1-Ig (wild type) harboring only vector (pEX1.8), the *cheB* mutant AFIB harboring only vector (pEX1.8), and the *cheB* mutant complemented with *cheB2* in *trans* (pEXB2). Expression of CheB2 from pEXB2 was controlled by the P_{tac} promoter and was induced by addition of 100 μM IPTG to the medium.

minimal swarm media as well as in rich medium. The *cheB2* mutant grew at the same rate as its wild-type parent in liquid medium. *cheB2* mutant cells did not have an obvious defect in swimming behavior when observed under the microscope. The *cheB2* mutant phenotype was complemented by the addition of *cheB2* in *trans* (Fig. 3A).

CheB2 restores chemotaxis to the nonchemotactic *cheB* mutant. To determine whether cluster I chemotaxis proteins can interact with cluster II proteins, four cluster II proteins, CheA2, CheB2, CheW2, and CheY2, were expressed in strains that contained a defect in the homologous cluster I gene. Of these, only CheB2 could complement a defect in the cluster I paralog, *cheB*. CheB2 partially restored chemotaxis to the nonchemotactic, cluster I, *cheB*-deficient strain AFIB (Fig. 3B).

***mcpA* and *mcpB* mutants have a general chemotaxis defect.** Cluster II contains two genes predicted to encode MCP-like proteins: *mcpA* and *mcpB*. Each gene was mutated by replacing part of the gene with a Gm^r cassette. The *mcpA* and *mcpB* mutants were then screened for chemotaxis to 60 carbon sources in minimal medium using the soft-agar plate assay (data not shown). Carbon sources that were tested as chemoattractants included amino acids, sugars, organic acids, and aromatic compounds. Wild-type cells formed well-defined chemotactic swarm rings in swarm plates that included any of the 60 carbon sources. The *mcpA* mutant strain, AFD3216, behaved like the wild type in minimal-medium soft-agar plates containing any of the organic chemoattractants that we tested (not shown), but it had a general chemotaxis defect in tryptone soft-agar plates (Fig. 4A). This phenotype was not due to a defect in the growth rate in tryptone media (data not shown). This led us to examine compositional differences between tryptone and our minimal medium. We determined that the presence of magnesium in the minimal medium allowed the *mcpA* mutant to overcome the mutant phenotype (Fig. 4B). Magnesium, in concentrations as low as 100 μM, added to the tryptone soft-agar plates as either $MgCl_2$ or $MgSO_4$, restored wild-type chemotaxis to the *mcpA* mutant. Overexpression of McpA in *P. aeruginosa* led to a nonchemotactic phenotype in soft-agar plates (Fig. 4C). The same phenotype was observed when McpA was overexpressed in *E. coli* K-12 (Fig. 4D). Individual *P. aeruginosa* or *E. coli* cells overexpressing McpA had a smooth-swimming phenotype.

The *mcpB* mutant strain, AFD4672, grew at wild-type rates (data not shown) but had a general chemotaxis defect on LB medium, tryptone, and minimal-medium soft-agar swarm plates containing any of 60 different attractants. This defect was observed when plates were incubated either aerobically or anaerobically under denitrifying conditions (Fig. 5A). The defect was complemented when *mcpB* was expressed in *trans* (Fig. 5A). Overexpression of McpB in *P. aeruginosa* led to a nonchemotactic phenotype on soft-agar plates (not shown). The same phenotype was observed when McpB was overexpressed in *E. coli* (Fig. 5B). Individual *P. aeruginosa* cells overexpressing McpB had a constantly smooth-swimming phenotype when observed microscopically. The same was observed when McpB was overexpressed in *E. coli*.

Cluster II proteins CheA2, CheB2, and CheW2 disrupt chemotaxis when expressed in *E. coli* K-12, but not when expressed in *P. aeruginosa* PAO1-Ig. CheA2, CheB2, and CheW2 were expressed in *E. coli* K-12 from a high-copy-number *ori* ColE1 plasmid (pEX1.8). Each of these proteins disrupted normal chemotaxis in 1% (wt/vol) tryptone soft-agar plates (Fig. 6). Overexpression of CheY2 had no effect on *E. coli* chemotaxis. Each strain was motile when a sample was removed from the plate and observed microscopically. Thus, the effect was not due to a disruption of motility. Overexpression of CheA2, CheB2, CheW2, and CheY2 in wild-type *P. aeruginosa* cells had no effect on chemotaxis as assayed in soft-agar swarm plates. Expression was induced by IPTG concentrations up to1,000 μM (data not shown).

DISCUSSION

The *P. aeruginosa* cluster I CheA, CheB, CheW, CheY, and CheZ proteins have 32, 36, 30, 58, and 32% amino acid identity with the corresponding *E. coli* Che proteins. CheR proteins

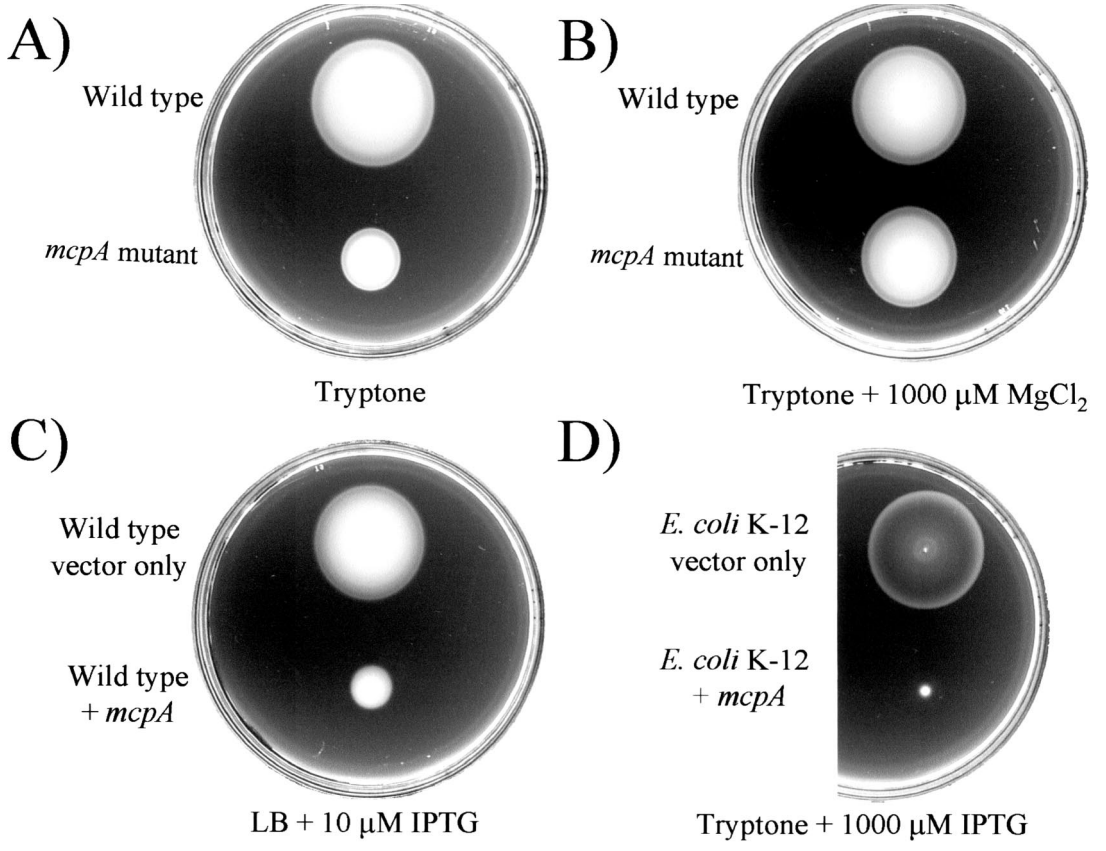

FIG. 4. McpA is required for a wild-type chemotactic response by *P. aeruginosa*, and overexpression of McpA inhibits the chemotactic response. (A) Tryptone soft-agar plate showing chemotactic rings formed by *P. aeruginosa* PAO1-Ig (wild type) and an *mcpA* mutant (AFD3216). (B) Tryptone soft-agar plate with 1,000 μM $MgCl_2$, comparing the chemotactic responses of wild-type cells and *mcpA* mutant cells. Addition of Mg^{2+} to the medium restores wild-type chemotaxis to the *mcpA* mutant. (C) Dilute LB soft-agar plate showing chemotactic rings formed by *P. aeruginosa* PAO1-Ig (wild type) harboring only vector (pEX1.8) and *P. aeruginosa* PAO1-Ig (wild type) overexpressing McpA (pHAH128). Expression of McpA from pHAH128 was induced by the addition of 10 μM IPTG to the medium. (D) Tryptone soft-agar plate showing chemotactic rings formed by *E. coli* K-12 harboring only vector (pEX1.8) and *E. coli* K-12 expressing McpA (pHAH128). Expression of McpA from pHAH128 was controlled by the P_{tac} promoter and was induced by the addition of 1,000 μM IPTG to the medium.

from *P. aeruginosa* (encoded in cluster V) and *E. coli* are 30% identical. Given that the cluster I *che* gene mutants are completely defective in chemotaxis, it was surprising to find, on inspection of the *P. aeruginosa* genome sequence, an additional cluster of *che* genes (cluster II) whose predicted protein products, with the exception of *cheY2*, had even higher overall sequence identities to the orthologous *E. coli* chemotaxis proteins (Table 2). Key amino acid residues shown to be important for *E. coli* chemotaxis protein function are conserved in both the cluster I and the cluster II Che proteins from *P. aeruginosa*.

Our data suggest that *P. aeruginosa* cluster II *che* genes participate in chemotaxis. A *cheB2* mutant is impaired in chemotaxis. Also, CheB2, when overexpressed, can complement a *P. aeruginosa cheB* mutant and restore it to a wild-type pattern of motile behavior and chemotactic response. Although the *cheA2* and *cheW2* mutants did not have a discernible chemotaxis defect, CheA2 and CheW2 proteins that were expressed in an *E. coli* K-12 background disrupted *E. coli* chemotaxis. This suggests that CheA2 and CheW2 can compete with endogenous *E. coli* chemotaxis proteins and interfere with the normal chemotactic response. There are a number of ways in which cluster II Che proteins might function in chemotaxis. They may contribute to an optimal chemotactic response by coexisting and participating in a major signal transduction pathway that is dominated by cluster I proteins. Alternatively, cluster II Che proteins may form complexes that are physically and functionally distinct from cluster I signaling complexes. The observation that overexpression of cluster II proteins interferes with *E. coli*, but not *P. aeruginosa*, chemotaxis favors the second possibility.

A distinct cluster II chemotaxis-signaling complex may be important for sensing some general parameter of cellular physiology through associated McpA and McpB proteins. Such a role is consistent with the observation that *mcpA* and *mcpB* mutants have general chemotaxis defects and unusual molecular architectures. Most *mcp* mutants that have been described are defective in chemotaxis to a subset of the chemoattractants that a particular bacterial species can detect. For example, mutants in the *P. aeruginosa mcp* genes *ctpH* and *ctpL* that are specifically nonchemotactic to inorganic phosphate have been described (59). Similarly, the *mcp* mutant *pctA* is defective in chemotaxis to L-serine but attracted to other amino acids (33). Most of the 26 predicted *P. aeruginosa* MCPs have a typical *E. coli*-like MCP architecture that consists of two transmembrane

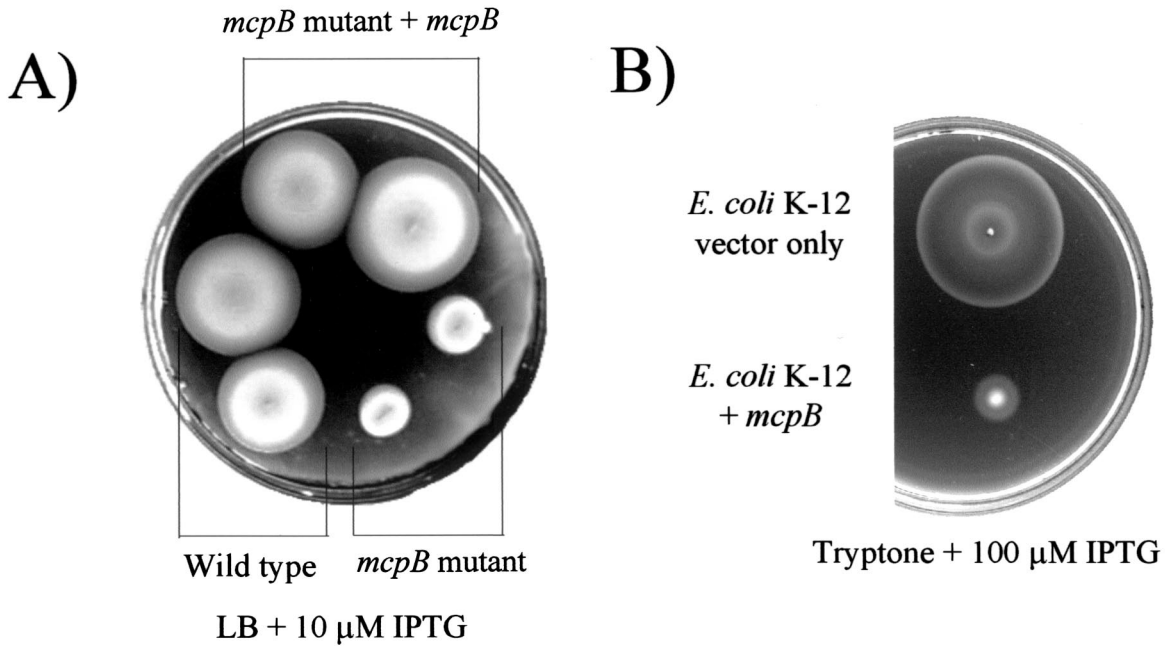

FIG. 5. McpB is required for optimal chemotaxis by *P. aeruginosa*, and overexpression of McpB inhibits the chemotactic response. (A) Dilute LB soft-agar plate showing chemotactic rings formed by *P. aeruginosa* PAO1-Ig (wild type) harboring only vector (pEX1.8), an *mcpB* mutant (AFD4672) harboring only vector (pEX1.8), and the *mcpB* mutant complemented with *mcpB* in *trans* (pAF27B). Expression of McpB from pAF27B was induced by the addition of 10 μM IPTG to the medium. (B) Tryptone soft-agar plate showing chemotactic rings formed by *E. coli* K-12 harboring only vector (pEX1.8) and *E. coli* K-12 expressing McpB (pAF27B). Expression of McpB from pAF27B was induced by the addition of 100 μM IPTG to the medium.

regions in the N-terminal half of the protein and a domain, called the highly conserved domain, that is involved in sensory signaling (Fig. 7). Analysis of the amino acid sequence of McpA using the Simple Modular Architecture Research Tool (SMART) (35, 47) indicates that McpA has a highly conserved domain (34) but only one predicted transmembrane domain (Fig. 7). McpB (35, 47) has a highly conserved domain (34), a PAS (for Per, ARNT, Sim) (57) domain, and no predicted transmembrane domains (Fig. 7). PAS domains are typically involved in sensing redox potential, oxygen, or light (57). McpA and McpB are the only *P. aeruginosa* MCPs that have C-terminal pentapeptides (EVELF in the case of McpA and GWEEF in the case of McpB) related to that found on the high-abundance receptors of *E. coli* (NWETF) (43, 60). This reinforces the concept that McpA and McpB may play a more general and central role in chemotactic signal transduction than do typical MCPs.

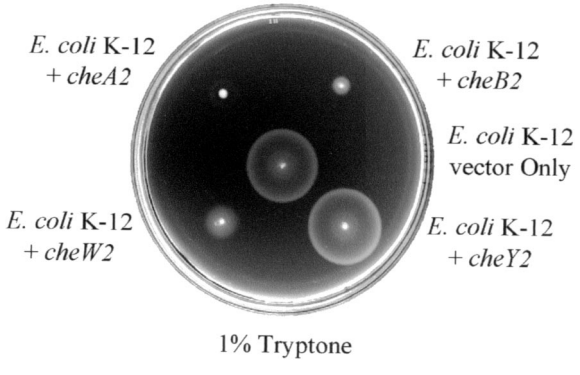

FIG. 6. Effect of expression of cluster II Che proteins on *E. coli* K-12 chemotaxis. Shown is a tryptone soft-agar plate with chemotactic rings formed by *E. coli* K-12 harboring only vector (pEX1.8) or expressing CheA2 (pEXA2), CheB2 (pEXB2), CheW2 (pEXW2), or CheY2 (pEXY2). No IPTG was added to the medium. CheY2 failed to inhibit *E. coli* chemotaxis even when expression was induced with 100 μM IPTG.

Cluster II has previously been referred to as the α-subgroup-like cluster of *che* genes (53). However, genetic organization and physiological observations suggest that α-proteobacteria and *P. aeruginosa* have different chemotaxis systems. None of the multiple clusters of chemotaxis genes identified in the genomes of the sequenced α-proteobacteria *R. sphaeroides*, *S. meliloti*, and *C. crescentus* resembles *P. aeruginosa* cluster II in gene organization (7, 15, 36). Furthermore, the proteins predicted to be encoded by cluster II genes, with the exception of CheY2, have the highest degree of sequence identity to *E. coli* rather than to α-proteobacteria orthologs (Table 2) (56). The best-described chemotaxis system in a bacterium belonging to the α subgroup of proteobacteria is that of *R. sphaeroides* (50). A model for *R. sphaeroides* chemotaxis has been proposed in which two chemotaxis-signaling complexes, one encoded by operon 1 and a second encoded by operon 2, contribute to an optimal chemotactic response (50). In the absence of operon 2, a repellent response to the attractant propionate was seen (50). An operon 1 deletion mutant had no obvious chemotaxis phenotype (50). Significantly, motor bias phenotypes (in the case of *R. sphaeroides*, either smooth swimming or stopped) were

TABLE 2. Percent identities of *P. aeruginosa* cluster II proteins to chemotaxis proteins from clusters in other organisms

P. aeruginosa cluster II protein	% identity				
	P. aeruginosa cluster I paralog	*E. coli* ortholog	*P. syringae* pv. tomato cluster II ortholog	*S. oneidensis* MR-1 cluster II ortholog	*V. cholerae* cluster II ortholog
McpA	NA[a]	NA	25	30	29
CheY2	33	36	49	60	50
CheA2	33	42	37	43	40
CheW2	30	63	35	59	37[b] 33[c]
McpB	NA	NA	29	49	37
CheR2	31	51	39	49	39
CheD	NA	NA	29	52	33
CheB2	39	60	47	53	NA[d]

[a] NA, not applicable; this cluster does not contain a paralog or ortholog. Identities have been rounded to the nearest whole percentage and were calculated with the ClustalW Multiple Alignment algorithm within the BioEdit sequence alignment editor (13).
[b] First *cheW2* in this cluster.
[c] Second *cheW2* in this cluster.
[d] *V. cholerae* has a frame shift mutation in the *cheB2* open reading frame (16).

seen only in mutants that had defects in both operon 1 and operon 2 (50). *P. aeruginosa* differs from *R. sphaeroides* and other α-proteobacteria in that a single set of chemotaxis genes clearly dominates the chemotactic response, as evidenced by the observation that cluster I mutants have profound effects on the rotational bias of the flagellar motor. This is likely to facilitate studies of the contribution of cluster II genes to the ability of *P. aeruginosa* to sense and respond to its environment.

One cannot, at this point, exclude the possibility that the major output from a cluster II signaling complex may be something other than a chemotactic response. Such a hypothetical output would likely be processed through CheY2. Overexpression and complementation data offer no support for the idea that CheY2 interacts with the flagellar motor. Moreover, CheY2 did not complement a *P. aeruginosa cheZ* mutant. These data suggest that CheY2 may not act as a phosphate sink, as has been reported for *R. sphaeroides* and *S. meliloti* CheY proteins (53).

The γ-proteobacteria *Pseudomonas syringae* pv. tomato, *Shewanella oneidensis* MR-1, and *Vibrio cholerae* each have a set of cluster II-like chemotaxis genes (Table 2 and Fig. 8). *Pseudomonas putida* (The Institute for Genomic Research [TIGR] unfinished genomes BLAST search [http://tigrblast.tigr.org/ufmg/]) and *Pseudomonas fluorescens* (*Pseudomonas fluorescens* Genome Project [http://www.jgi.doe.gov/JGI_microbial/html/pseudomonas/pseudo_homepage.html]), species that are closely related to *P. aeruginosa*, do not have cluster II chemotaxis genes. They do, however, have sets of cluster I, III, IV, and V chemotaxis genes with high amino acid sequence identity (on the order of 70 to 80% identity) to the orthologous *P. aeruginosa che* genes. The function of a cluster II signal transduction pathway could be associated with physiological capabilities that cluster II bacteria have in common. However, it is not immediately clear what these commonalities may be. *V. cholerae*, *S. oneidensis*, and *P. aeruginosa* are able to grow anaerobically by denitrification. Yet *P. syringae* is an obligate aerobe and *P. fluorescens*, which lacks cluster II genes, can denitrify. *P. syringae*, *V. cholerae*, and *P. aeruginosa* are each either an animal or plant pathogen. *S. oneidensis* strain MR-1 (formerly *Shewanella putrefaciens* MR-1) is not known to be pathogenic, but some *Shewanella* species are opportunistic human pathogens (29). This suggests that it might be worth exploring a possible connection between the activity of a cluster

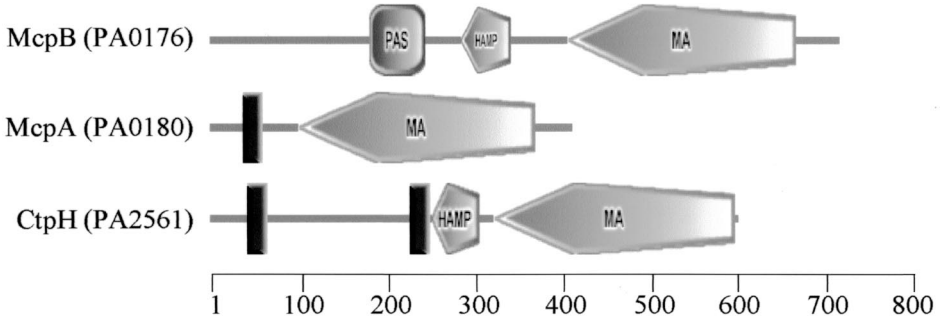

FIG. 7. Domain architectures of 3 of the 26 *P. aeruginosa* MCPs. The structures were created with the SMART server (http://smart.embl-heidelberg.de/) (35, 47). PA numbers are indicated in parentheses and are according to the *Pseudomonas aeruginosa* Community Annotation Project (http://www.pseudomonas.com). The roles of McpA and McpB in *P. aeruginosa* chemotaxis are discussed in the text. CtpH is an MCP for inorganic phosphate in *P. aeruginosa* and has been previously described (59). CtpH represents the common structural motif of an MCP and is similar to *E. coli* MCPs. Domain representations are as follows: rectangle, transmembrane domain; square, PAS domain; elongated pentagon, highly conserved domain of MCP (MA, methyl accepting); small pentagon, HAMP domain (1). The scale represents amino acid positions.

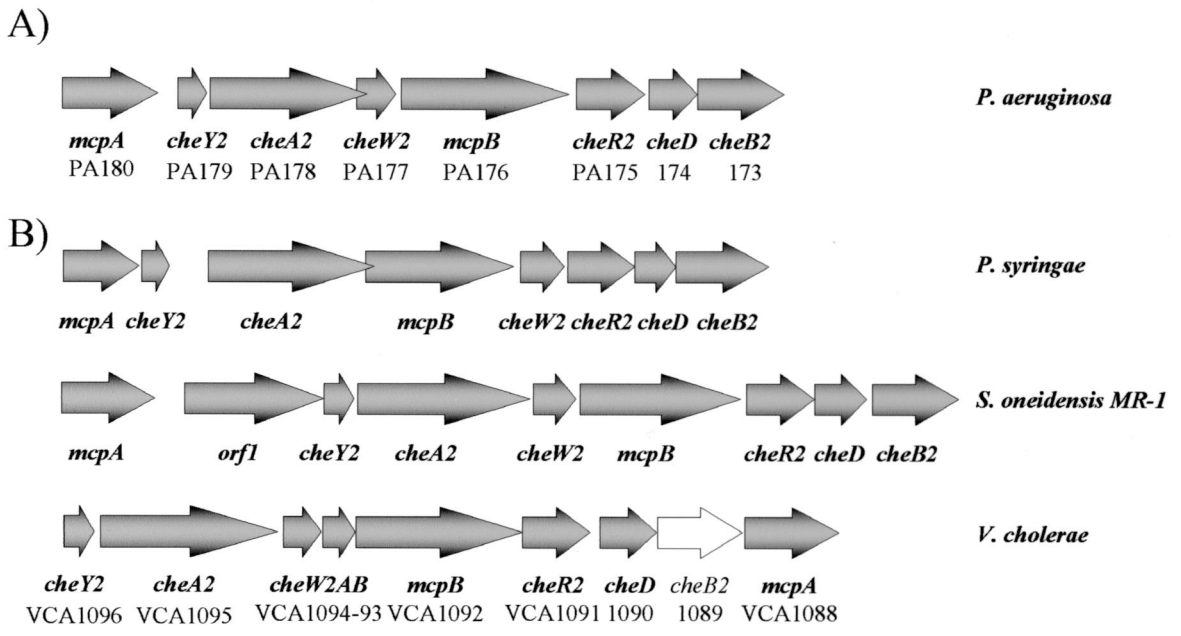

FIG. 8. Cluster II-like gene arrangements in γ-proteobacteria. (A) *P. aeruginosa* cluster II. PA numbers are according to the *Pseudomonas aeruginosa* Community Annotation Project (http://www.pseudomonas.com). (B) Cluster II-like gene arrangements in other γ-proteobacteria. *P. syringae* cluster II and *S. oneidensis* MR-1 cluster II were accessed through the TIGR Microbial Genome Database (http://www.tigr.org/tdbl). *V. cholerae* VCA numbers are according to the TIGR Complete Microbial Resource (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl). *V. cholerae cheB2* may not be functional due to a frame shift mutation (16).

II signal transduction pathway and environmental stress conditions of the type that pathogens are known to experience.

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