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Chemotaxis to Pyrimidines and Identification of a Cytosine Chemoreceptor in *Pseudomonas putida*▽†

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We developed a high-throughput quantitative capillary assay and demonstrated that *Pseudomonas putida* strains F1 and PRS2000 were attracted to cytosine, but not thymine or uracil. In contrast, *Pseudomonas aeruginosa* PAO1 was not chemotactic to any pyrimidines. Chemotaxis assays with a mutant strain of F1 in which the putative methyl-accepting chemotaxis protein-encoding gene Pput_0623 was deleted revealed that this gene (designated *mcpC*) encodes a chemoreceptor for positive chemotaxis to cytosine. *P. putida* F1 also responded weakly to cytidine, uridine, and thymidine, but these responses were not mediated by *mcpC*. Complementation of the F1 Δ*mcpC* mutant XLF004 with the wild-type gene restored chemotaxis to cytosine. In addition, introduction of this gene into *P. aeruginosa* PAO1 conferred the ability to respond to cytosine. To our knowledge, this is the first report of a chemoreceptor for cytosine.

Motile bacteria are capable of detecting chemical gradients in the environment and swim toward or away from them, a behavior known as chemotaxis. Historically, the enteric bacterium *Escherichia coli* has been the model organism for chemotaxis studies. *E. coli* has four transmembrane chemoreceptors called methyl-accepting chemotaxis proteins (MCPs), each of which binds a set of chemicals directly or in complex with specific periplasmic binding proteins. MCPs send signals to the flagellar motor via a complex signal transduction system that is composed of six soluble chemotaxis proteins, through which the bacterium modifies its swimming behavior based on the signal(s) received (for reviews, see references 5 and 15). The MCPs of *E. coli* sense a variety of stimuli, including amino acids, sugars, and dipeptides (30, 44). We recently reported that *E. coli* also responds to the pyrimidines thymine and uracil and demonstrated that Tap, the MCP known to mediate chemotaxis to dipeptides, is required for pyrimidine taxis (29).

Pseudomonads are environmental bacteria that are widespread in nature, and all *Pseudomonas* species are motile. They have conserved chemotaxis proteins that are homologous to those present in *E. coli*, but their chemosensory systems appear to be more complex (6, 39, 55). Unlike *E. coli*, which has only one set of chemotaxis (*che*) genes in a single gene cluster, *Pseudomonas* species have multiple *che* gene homologs organized in several unlinked gene clusters (39). In addition, genome sequence analyses have revealed that *Pseudomonas* strains have numerous putative MCP genes. For example, the genome of *Pseudomonas aeruginosa* PAO1 (46) encodes 26 MCP-like proteins, *Pseudomonas putida* KT2440 (34) has 27, and *Pseudomonas syringae* DC3000 (9) has 49 (39).

The best-studied chemotaxis system in *Pseudomonas* is that of the opportunistic pathogen *P. aeruginosa.* More than 75 different chemoattractants have been identified for *P. aeruginosa* (39), and 13 of its 26 MCP-like proteins have been functionally characterized. Eight MCPs have been shown to mediate positive responses to amino acids (PctABC), inorganic phosphate (CtpH and CtpL), malate (PA2652), ethylene (TlpQ), and chloroethylenes (McpA) (3, 25–27, 42, 47, 54). Two MCPs (McpA and McpB) were shown to be required for general optimal chemotaxis (16), and one MCP-like protein (Aer) was found to mediate energy taxis (22). The MCP-like proteins BdlA and PilJ were shown to be involved in biofilm formation and biosynthesis of type IV pili, respectively (10, 12, 32).

P. putida is a common soil bacterium and, unlike *P. aeruginosa*, is not known to be pathogenic. Although *P. putida* and *P. aeruginosa* each have approximately the same number of MCP-like genes in their genomes, most of the protein products show relatively low amino acid sequence similarity. Based on our BLAST searches, three putative *P. putida* F1 MCPs have no obvious counterparts in *P. aeruginosa* PAO1. Most of the others share between 30 and 70% amino acid sequence identity, with the highest sequence conservation in the C-terminal signaling domains. The most highly conserved MCP-like proteins in the two species are Aer and PilJ (both are 77% identical to the corresponding homologs). These observations suggest that the two organisms respond to different subsets of attractants, which most likely reflects their different lifestyles and environmental niches. *P. putida* is known for its catabolic versatility (45), and we expect that members of the species are capable of responding to a correspondingly wide range of organic attractants. We are interested in defining the range of attractant and repellent responses and the functions of the MCPs present in *P. putida* compared to those of *P. aeruginosa.* In this study, we used *P. putida* strains F1 and PRS2000 and *P. aeruginosa* strain PAO1 to investigate the chemotactic responses to pyrimidines.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* was cultured on LB agar (11) at

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristics or sequence (5′–3′)[a]	Source or reference
E. coli strains		
DH5α	Δ(*lacZYA-argF*)*U169 hsdR17 relA1 supE44 endA1 recA1 thi-1 gyrA96* ϕ80d*lacZ*ΔM15	Life Technologies, Gaithersburg, MD
DH5α λ*pir*	Δ(*lacZYA-argF*)*U169 hsdR17 relA1 supE44 endA1 recA1 thi-1 gyrA96* ϕ80d*lacZ*ΔM15 λ*pir*$^+$	William W. Metcalf
HB101	F$^-$ *thi-1 hsdS20*(r_B^- m_B^-) *supE44 recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Str^r) *xyl-5 mtl-1*	40
P. aeruginosa strain		
PAO1	Wild type	46
P. putida strains		
F1	Wild type	18, 20
PRS2000	Wild type	37
PRS4086	PRS2000 *cheA*::mini-Tn*5*; Kan^r	14
F1 *cheA*	F1 *cheA*::mini-Tn*5*; Kan^r	This study
XLF004	F1ΔPput_0623 (Δ*mcpC*)	This study
XLF028	F1ΔPput_2527 (Δ*codA*)	This study
XLF104	F1ΔPput_0623 ΔPput_2527 (Δ*mcpC* Δ*codA*)	This study
Plasmids		
pAW19	*sacB*-containing cloning vector; Ap^r Kan^r	53
pRK2013	ColE1 *ori*, RP4 mobilization function; Kan^r	17
pRK415	Cloning vector, pRK290 derivative; Tet^r	24
pHJD215	pRK415 carrying a 12-kb fragment that contains PRS2000 genes *flhf*, *orfC*, *fliA*, *cheY*, *cheZ*, and *cheA* with a mini-Tn*5* insertion in *cheA*; Kan^r Tet^r	14
pXLF004	Gene Pput_0623 upstream and downstream 1-kb PCR products ligated and cloned into SpeI-SacI-digested pAW19; Ap^r Kan^r	This study
pXLF204	Gene Pput_0623 (*mcpC*) PCR product cloned into XbaI-EcoRI-digested pRK415; Tet^r	This study
pXLFcodA	Gene Pput_2527 (*codA*) PCR product cloned into HindIII-EcoRI-digested pRK415; Tet^r	This study
pXLFΔcodA	Gene Pput_2527 upstream and downstream 1-kb PCR products ligated and cloned into SpeI-digested pAW19; Ap^r Kan^r	This study
Primers		
0623 SpeI up-for	GACTGTCACTAGTACCGCAGCAAACTCAATGTC	This study
0623 up-rev (p)	p-GCAACCGAAAATCAGAGCTTC	This study
0623 dn-for (p)	p-GATGCTCATCGACGCTCCC	This study
0623 SacI dn-rev	GGACCTATGAGCTCCGATGTGCACGCCGAAGTG	This study
0623 XbaI-for	GTACATTCTAGACAGTTGCTACCTGCCTCGTGTG	This study
0623 EcoRI-rev	GTACTCGAATTCACAGGCCATACGTCAGGTGC	This study
CytoHindFor	ATGCAAGCTTCGGTCGCCATGCTGG	This study
CytoEcoRev	ATATGAATTCCCTGTGCACCTGAGGCGAC	This study
CytoSpeFor	GTGAACTAGTATTCGTCACCGGCATCAATC	This study
UpCytoDEL	CACCTGAGGCGACAACAGCGGCTGTTACTCCTTGCCGTGG	This study
DnCytoDEL	CGCTGTTGTCGCCTCAGGTG	This study
CytoSpeRev	GGTGACTAGTGCGTGAAGTCGGCCTACATC	This study

[a] Underlined bases indicate the positions of restriction sites. Ap^r, ampicillin resistant; Kan^r, kanamycin resistant; Tet^r, tetracycline resistant; (p) indicates that the primer carries a 5′ phosphate.

37°C. For chemotaxis assays, *P. putida* PRS2000, F1, and F1 derivatives were grown in minimal medium (MSB) (45) with 10 mM sodium succinate. *P. aeruginosa* PAO1 was grown in MSB with 27.5 mM glucose. For pyrimidine utilization studies, *P. putida* strains were grown in modified MSB containing one-tenth the standard concentration of Hutner's mineral base and no ammonium sulfate. Succinate (10 mM) was provided as the carbon source, and pyrimidines were provided at 1 mM. For growth yield studies, cells were grown in the medium described above, but the nitrogen source was either a pyrimidine or ammonium sulfate provided at 0.1, 0.2, or 0.3 mM. For *E. coli* and *P. putida* F1 derivatives, kanamycin and tetracycline were used at 100 μg/ml and 20 μg/ml, respectively. For PAO1 derivatives, tetracycline was used at 60 μg/ml when needed.

DNA methods. Standard methods were used for isolation and manipulation of plasmid DNA from *E. coli* (7). Genomic DNA from strain F1 was purified using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). Plasmids were purified with a QIAprep miniprep kit (Qiagen, Valenica, CA), and DNA fragments were purified with a QIAquick gel extraction kit (Qiagen). Fluorescent automated DNA sequencing was carried out at the University of California, Davis, sequencing facility on an Applied Biosystems 3730 automated sequencer. Sequence analyses were performed using Vector NTI software (Invitrogen, Carlsbad, CA) and BLAST programs on the National Center for Biotechnology Information server.

Construction of a *P. putida* F1 *cheA* mutant. pHJD215, which carries the *P. putida* PRS2000 *cheA* gene and flanking DNA with a mini-Tn*5* insertion in *cheA*, was obtained following isolation of the generally nonchemotactic mutant of PRS2000, PRS4086 (14). Because the *cheA* genes from strains F1 and PRS2000 share approximately 91% nucleotide sequence identity, we used pHJD215 to construct a *P. putida* F1 *cheA* mutant. This plasmid was introduced into *P. putida* F1 by conjugation from *E. coli* DH5α(pHJD215) by triparental mating in the presence of the *E. coli* HB101(pRK2013) helper strain, as previously described (43). Kanamycin- and tetracycline-resistant colonies were selected and then grown in liquid MSB containing 10 mM succinate and 100 μg/ml kanamycin. To screen for an F1 *cheA* mutant that arose from double-crossover events, cells were plated, and kanamycin-resistant, tetracycline-sensitive colonies were identified.

The mini-Tn*5* insertion in the *cheA* gene in the F1 mutant was verified by PCR. The F1 *cheA* mutant exhibited the expected constantly smooth swimming phenotype and the inability to form chemotactic rings on dilute LB soft agar swarm plates (14) (data not shown).

Construction of a *P. putida* F1 *mcpC* deletion mutant. To construct the in-frame deletion mutant *P. putida* XLF004 (Table 1), primer pairs 0623 up-for/0623 up-rev and 0623 dn-for/0623 dn-rev (Table 1) were used to PCR amplify the 1-kb upstream and downstream regions of the gene Pput_0623, respectively. The resulting PCR fragments were fused by blunt-end ligation and then cloned into pAW19, resulting in pXLF004 (Table 1). The sequence of the insert in pXLF004 was determined to ensure that no PCR errors were present. Plasmid pXLF004 was introduced into *P. putida* F1 by conjugation from *E. coli* DH5α λ*pir* (pXLF004) by triparental mating in the presence of the *E. coli* HB101(pRK2013) helper strain, as previously described (43). Kanamycin-resistant exconjugants were selected and grown in MSB containing 10 mM succinate. To select for deletion mutants that arose from double-crossover events, cells were plated on MSB agar containing 10 mM succinate and 20% sucrose. Individual colonies were then screened for kanamycin susceptibility and analyzed by PCR using primers 0623 up-for and 0623 dn-rev.

To complement the mutation, gene Pput_0623 was amplified by PCR using primers 0623 Xbal-for and 0623 EcoRI-rev, ligated to vector pRK415 (24) to generate pXLF204, and sequenced to verify that no PCR errors were present. The plasmid was introduced into *P. putida* XLF004 by conjugation in the presence of HB101(pRK2013) (Table 1) as previously described (43), and tetracycline-resistant colonies were selected. The plasmids pXLF204 and pRK415 were also individually introduced into *P. aeruginosa* PAO1 by conjugation as described above.

Cloning of the cytosine deaminase gene from *P. putida* F1 and construction of cytosine deaminase mutants. A BLAST search of the *P. putida* F1 genome sequence was carried out with the deduced amino acid sequence of the *E. coli codA* gene encoding cytosine deaminase. Pput_2527 was identified as a putative cytosine deaminase, as its product was 59% identical in amino acid sequence to CodA. The gene Pput_2527 was amplified by PCR from *P. putida* F1 genomic DNA using *Pfu* DNA polymerase and primers CytoHindFor and CytoEcoRev, which contain HindIII and EcoRI restriction sites, respectively (Table 1). The 1.4-kb product was purified from an agarose gel, digested with HindIII and EcoRI, and cloned into pRK415, generating pXLFcodA. The insert was verified by restriction digestion and sequence analysis. Approximately 35 times more cytosine deaminase activity was detected in cell extracts of the recombinant *E. coli* strain than in DH5α(pRK415) cell extracts (data not shown) using a standard cytosine deaminase assay detecting the release of ammonia (19, 33). These results verified that Pput_2527 encodes a cytosine deaminase. The gene was therefore designated *codA*. To construct the in-frame *codA* deletion mutants XLF028 and XLF104 (Table 1), primer pairs CytoSpeFor/UpCytoDEL and DnCytoDEL/CytoSpeRev (Table 1) were used to PCR amplify the ~1-kb upstream and downstream regions of the gene Pput_2527, respectively. The amplified fragments were then used in PCR overlap extension (23), and the resulting product was cloned into pAW19, resulting in pXLFΔcodA (Table 1). The sequence of the insert was determined to ensure that no PCR errors were present. Plasmid pXLFΔcodA was mobilized from *E. coli* DH5α λ*pir* into *P. putida* F1 and XLF004, and the deletion mutations were verified as described above. The *codA* mutant XLF028 (ΔPput_2527) and the *codA mcpC* double mutant were unable to use cytosine as the sole nitrogen source but still grew with uracil (data not shown), confirming the role of CodA in cytosine utilization.

Chemotaxis assays. Bacterial cells were harvested in mid-exponential phase (optical density at 660 nm [OD_{660}] of ~0.3 to 0.45) by centrifugation and washed once with chemotaxis buffer (50 mM potassium phosphate buffer [pH 7.0], 10 μM disodium EDTA, 0.05% glycerol for *P. putida* strains [38]; 50 mM potassium phosphate buffer [pH 7.0] for strain PAO1).

Modified capillary assays were carried out as previously described (21). For this assay, microcapillaries contained chemotaxis buffer or attractant dissolved in chemotaxis buffer solidified with 2% low-melting-temperature agarose. Microcapillaries were introduced into suspensions of motile cells (in chemotaxis buffer at an OD_{660} of approximately 0.1), and the response was visualized under the microscope at a magnification of ×40.

We adapted the traditional quantitative capillary assay (1) into a high-throughput capillary assay, using a 96-well microtiter plate format (see Fig. 1A in the supplemental material). This assay is a variation of the high-throughput assay described by Bainer et al. (8). Our assay utilizes standard 1-μl capillaries, and bacteria are quantified by determining the number of CFU, as in the traditional quantitative capillary assay. Although this method is more labor-intensive than the assay developed by Bainer et al., the results can be directly compared to results using the traditional capillary assay (see Fig. S1B in the supplemental material). One-microliter capillaries were sealed at one end and sterilized, and the sealed ends were inserted into microtiter plate wells containing solidified 3% agar. The plate was then inverted, inserting capillaries into the wells of a new 96-well plate. Each well contained 100 μl of chemotaxis buffer or an attractant dissolved in chemotaxis buffer. This unit was placed under vacuum to fill the capillaries as previously described (31). The top plate was removed, and the capillaries were washed en masse with chemotaxis buffer. To start the assay, the washed capillaries (still held in the agar in the original 96-well plate) were inserted into the wells of another plate that had been prefilled with 300 μl of bacterial suspension (prepared as described above), with an empty sterile pipette tip tray inserted between the plates as a spacer (see Fig. S1A in the supplemental material). After incubation of this assembly at room temperature (1 h for *P. putida* or 30 min for *P. aeruginosa*), the plate containing the capillaries was removed. The capillaries were briefly rinsed with chemotaxis buffer, and the contents of each capillary were individually collected, diluted in a fresh 96-well plate, and enumerated as CFU by plate counts on LB plates. In all experiments, negative controls (chemotaxis buffer) and positive controls (10 mM succinate or 0.01% Casamino Acids for *P. putida*; 10 mM L-arginine for *P. aeruginosa*) were included.

TABLE 2. Yields of *P. putida* F1 and PRS2000 grown with various nitrogen sources

Compound[a]	No. of N atoms present	*P. putida* F1		*P. putida* PRS2000[c]	
		OD_{660} per mM compound[b]	OD_{660} per mM N atom	OD_{660} per mM compound	OD_{660} per mM N atom
NH_4Cl	1	0.73 ± 0.07	0.73 ± 0.07	0.66 ± 0.08	0.66 ± 0.08
Cytosine	3	2.10 ± 0.06	0.70 ± 0.02	1.91 ± 0.08	0.64 ± 0.03
Thymine	2	1.56 ± 0.05	0.78 ± 0.03	–	–
Uracil	2	1.49 ± 0.09	0.75 ± 0.04	–	–

[a] The inoculum was grown in MSB containing 10 mM succinate, washed once, and then transferred into modified MSB containing one-tenth the standard concentration of Hutner's mineral base and no ammonium sulfate, with 10 mM succinate and a range of growth-limiting concentrations of nitrogen sources (0.1 to 0.3 mM). Duplicate cultures were aerated until stationary phase at both 30°C and at room temperature (~21°C). The results were the same for both temperatures; results with cultures grown at 30°C are presented.

[b] Average final yield for at least four different cultures containing various nitrogen source concentrations grown at 30°C. Standard errors are indicated.

[c] –, no growth.

RESULTS

Utilization of pyrimidines as nitrogen sources by *P. putida* strains. To test whether *P. putida* strains F1 and PRS2000 were able to use each of the pyrimidines as the sole carbon or nitrogen source, we carried out growth experiments. Neither strain was capable of using any of the pyrimidines as the sole source of carbon (data not shown). However, *P. putida* F1 was able to utilize each of the pyrimidines as the sole nitrogen source, while PRS2000 utilized only cytosine. To determine how many moles of nitrogen each of the strains obtained per mole of pyrimidine, we compared the growth yields in the presence of limiting amounts of pyrimidines to those with ammonium (Table 2). As assessed by measuring the OD_{660}, the yield per mM N atom of pyrimidines indicated that all three nitrogen atoms of cytosine were assimilated by strains F1 and PRS2000, and both nitrogen atoms of thymine and uracil were assimilated by F1 (Table 2).

Wild-type *P. putida* strains are attracted to cytosine. We examined *P. putida* strains F1 and PRS2000 and *P. aeruginosa* PAO1 for chemotactic responses to pyrimidines using the high-throughput capillary assay described in this study. Both *P. putida* F1 and PRS2000 responded to cytosine at concentrations from 1 mM to 50 mM (Fig. 1). For both strains, the peak

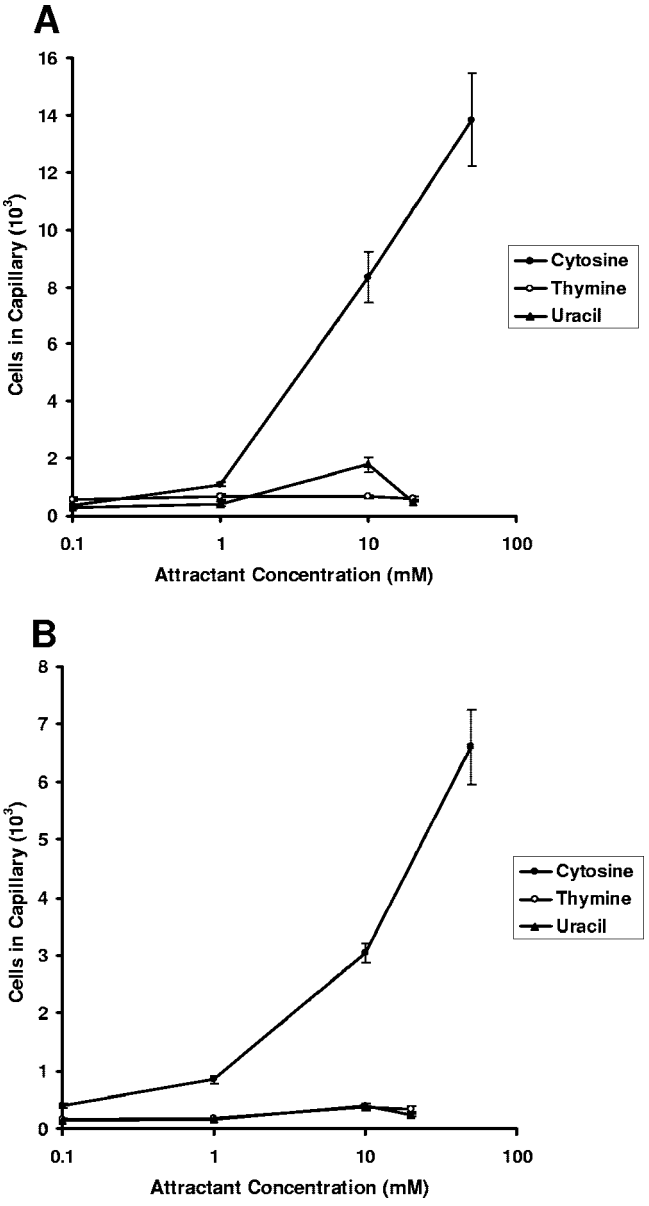

FIG. 1. Concentration-response curves for chemotaxis to pyrimidines by *P. putida* strains F1 (A) and PRS2000 (B). Cells were grown at 30°C in MSB containing 10 mM succinate. Assays were performed at room temperature with various concentrations of each pyrimidine, up to its limit of solubility. Results are the averages of at least 15 capillaries from at least two independent experiments; error bars indicate standard errors. The background accumulations in capillaries containing buffer only were 450 and 200 cells for *P. putida* F1 and PRS2000, respectively.

response concentration was 50 mM, and the response was approximately 30-fold over background. No response to thymine or uracil was detected for either strain (Fig. 1). We validated the results of the high-throughput capillary assay by directly comparing results with those of a traditional capillary assay. Although the overall numbers of cells that accumulated in capillaries in the high-throughput assay were approximately 10-fold lower than those in the traditional assay, the fold responses were comparable (see Fig. 1B in the supplemental material). We also verified that generally nonchemotactic *cheA* mutants of *P. putida* F1 and PRS2000 (F1 *cheA* and PRS4086; Table 1) did not show responses to known attractants in the high-throughput assay (351 ± 54 cells in capillaries containing Casamino Acids and 352 ± 68 cells in capillaries containing buffer for F1 *cheA*; 152 ± 22 cells in capillaries containing Casamino Acids and 324 ± 45 cells in capillaries containing buffer for PRS4086). *P. aeruginosa* PAO1 did not show a detectable response to any of the pyrimidines (data not shown). These results indicate that the soil bacterium *P. putida* has a different attractant profile than the opportunistic pathogen *P. aeruginosa* does.

Since the complete genome sequence of *P. putida* F1 (but not PRS2000) is available, we focused the remainder of our chemotaxis studies on *P. putida* F1. To test whether cytosine taxis was inducible, F1 was grown in the presence of 1 mM cytosine in addition to ammonium sulfate and assayed for its chemotactic ability. The number of cells that accumulated in capillaries containing 50 mM cytosine was 14,200 ± 1,700, which is similar to the level of the response with cells grown without cytosine (13,900 ± 1,600 cells per capillary). These results indicate that the cytosine chemotactic response in F1 is not induced in the presence of cytosine.

Identification of the cytosine receptor in *P. putida* F1. To identify the cytosine chemoreceptor in *P. putida* F1, we analyzed the F1 genome sequence (http://genome.jgi-psf.org/finished_microbes/psepu/psepu.home.html) and identified genes encoding 27 putative MCP-like proteins. Because Tap, which mediates chemotaxis to thymine and uracil in *E. coli* (29), is the only MCP known to be involved in pyrimidine chemotaxis, we compared the full-length and N-terminal periplasmic binding domains of all of the putative F1 MCPs with those of Tap. However, none of the *P. putida* F1 MCPs was identified as a Tap ortholog. Therefore, we systematically inactivated each of the MCP-like genes in *P. putida* F1 by generating in-frame deletions. The mutants were screened for loss of chemotaxis to cytosine using modified capillary assays. One mutant strain, *P. putida* XLF004, in which the gene Pput_0623 was deleted, showed no response to cytosine in the modified capillary assay (Fig. 2A). Growth with cytosine was not affected in XLF004 (data not shown), suggesting that this gene is not involved in cytosine metabolism. The *P. putida* F1 gene Pput_0623 was designated *mcpC*.

We confirmed the phenotype of the *mcpC* mutant using the quantitative high-throughput capillary assay. *P. putida* XLF004 had a significantly reduced response to cytosine compared to the wild type (Fig. 2B). However, a weak response to cytosine (~20% of the wild-type response) was still detected (Fig. 2B). This response could have resulted from a response to ammonia, which is released when cytosine deamination occurs, or there may be an additional chemoreceptor(s) present in *P. putida* F1 that also detects cytosine. To investigate this residual response to cytosine, we tested the chemotactic responses of *P. putida* F1 and XLF004 to 25 mM ammonium sulfate and 50 mM ammonium chloride (same molar concentration of nitrogen atoms as 50 mM cytosine). Both strains responded to ammonium sulfate and ammonium chloride, and the strength of these responses was similar (Fig. 2B and data not shown). These results indicate that ammonium is an attractant for F1 and that the response is not mediated by McpC. To examine the role of cytosine deamination in cytosine chemotaxis, we

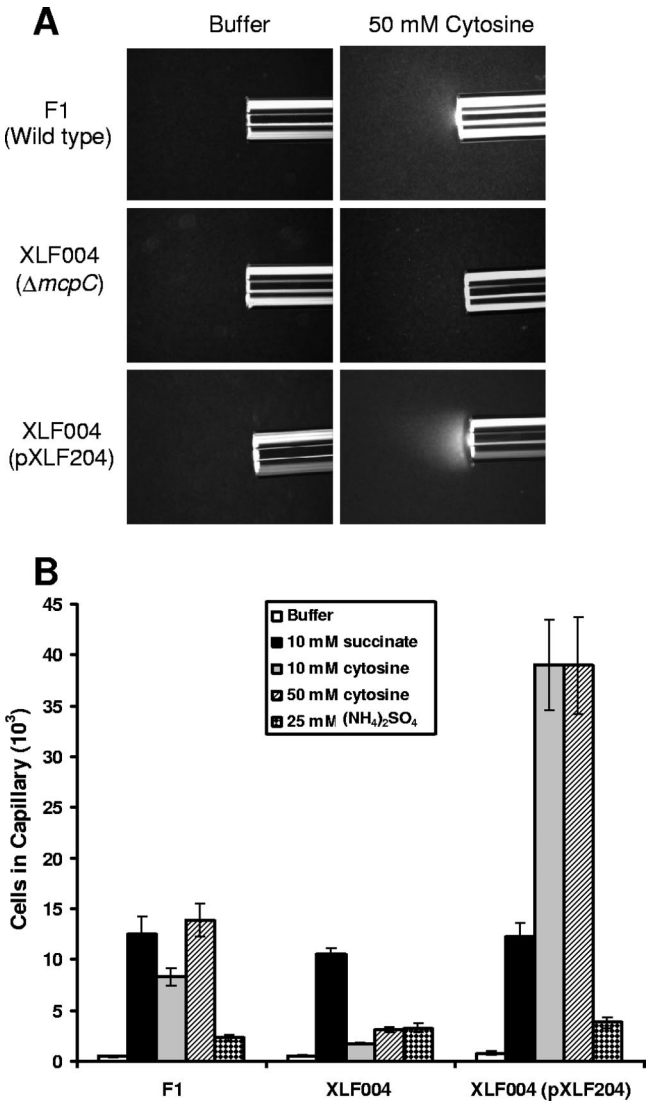

FIG. 2. Chemotactic responses of wild-type *P. putida* F1, the *mcpC* mutant (strain XLF004), and the complemented strain [XLF004 (pXLF204)] in qualitative and quantitative capillary assays. Cells were grown as described in the legend to Fig. 1, except that tetracycline was used at 20 μg/ml to maintain the plasmid in the complemented strain. (A) Chemotactic responses in modified capillary assays. Capillaries contained chemotaxis buffer or 50 mM cytosine in chemotaxis buffer solidified with 2% low-melting-temperature agarose. Assays were carried out at room temperature for 30 min. (B) Chemotactic responses in high-throughput quantitative capillary assays. Results are the averages of at least 15 capillaries from at least two independent experiments; error bars indicate standard errors. XLF004(pRK415) responded to cytosine at a level similar to that of XLF004 (data not shown).

identified the cytosine deaminase gene in F1 and constructed derivatives of strains F1 and XLF004 in which the *codA* gene was deleted. As described in Materials and Methods, strains lacking *codA* were unable to grow with cytosine as the sole nitrogen source. The chemotactic response of the double mutant XLF104 (Δ*mcpC* Δ*codA*) to 50 mM cytosine (2,970 ± 500 cells per capillary) was not significantly different from that of XLF004 (Δ*mcpC*; 3,140 ± 250 cells per capillary). The responses to 25 mM ammonium sulfate were also similar in these two strains (data not shown). These data rule out the possibility that the residual response to cytosine in the Δ*mcpC* mutant resulted from a response to ammonia released from cytosine and suggest that one (or more) additional chemoreceptor(s) besides McpC plays a minor role in the detection of cytosine by *P. putida* F1. However, none of the other single MCP deletion mutants showed an obvious defect in cytosine chemotaxis (data not shown).

The responses of the complemented strain XLF004(pXLF204) to both 10 and 50 mM cytosine appeared to be stronger than those of wild type, based on the increased number of cells that accumulated in the capillary (Fig. 2). We expect that this result was due to the increased *mcpC* gene copy number, although we did not confirm this hypothesis with further experiments. In contrast, the response to ammonium remained the same (Fig. 2B), further confirming that McpC is responsible for detecting cytosine and not ammonium.

Molecular characteristics of McpC. McpC contains 647 amino acids, with a predicted molecular mass of 70 kDa. It has the typical MCP domain structure: two hydrophobic membrane-spanning regions flanking a periplasmic sensing domain, a HAMP (*h*istidine kinase, *a*denylyl cyclase, *m*ethyl-accepting chemotaxis protein, and *p*hosphatase) domain (4), and a cytoplasmic signaling domain. The protein belongs to the MCP class 40H, which has 40 heptads in the cytoplasmic domain (2). McpC has at least two possible methylation sites based on sequence alignments, but it does not have the C-terminal pentapeptide tether based on the consensus motif -x-[HFWY]-x(2)-[HFWY]-, which has been shown to be important for adaptational modification in *E. coli* (2, 28). We compared the sequence of the periplasmic sensing domain of McpC (amino acids 30 to 291) with the sequences available in the public databases by use of BLAST. The closest match was PP_0584 in *P. putida* KT2440 (99% amino acid sequence identity), followed by *P. putida* GB1 (95%) and *Pseudomonas entomophila* L48 (75%). The *Pseudomonas fluorescens*, *Pseudomonas mendocina*, and *P. syringae* strains also have McpC homologs that share significant sequence identity (59 to 66%) with McpC in their respective periplasmic sensing domains. No other available sequenced genomes contained genes with strong homology to the McpC N-terminal domain; the next closest match was a protein from *Shewanella denitrificans* OS217, with 44% amino acid sequence identity.

McpC does not mediate chemotaxis to pyrimidine nucleosides. Pyrimidine nucleosides cytidine, thymidine, and uridine differ from the pyrimidines by the presence of the ribose moiety. We tested *P. putida* F1 for chemotactic responses to pyrimidine nucleosides at concentrations ranging from 1 mM to 100 mM (near saturation). F1 responded to all three nucleosides, with a peak response concentration of 100 mM for each. The responses to 100 mM pyrimidine nucleosides were relatively weak (3- to 6-fold) compared to that for 50 mM cytosine (30-fold) (Fig. 3). To investigate whether the responses to pyrimidine nucleosides are mediated by McpC, *P. putida* XLF004 (Δ*mcpC*) was assayed for chemotactic responses toward the three pyrimidine nucleosides. XLF004 responded to all three pyrimidine nucleosides at levels similar to those of wild-type F1 (Fig. 3). These data indicate that McpC does not mediate chemotaxis to pyrimidine nucleosides.

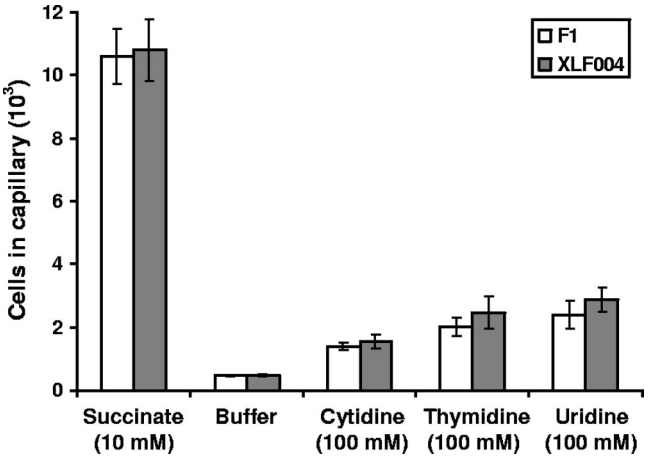

FIG. 3. Chemotactic responses of wild-type *P. putida* F1 and the *mcpC* mutant (strain XLF004) to pyrimidine nucleosides in high-throughput quantitative capillary assays. Cells were grown in MSB with 10 mM succinate. Results are the averages of at least 12 capillaries from at least two independent experiments; error bars indicate standard errors.

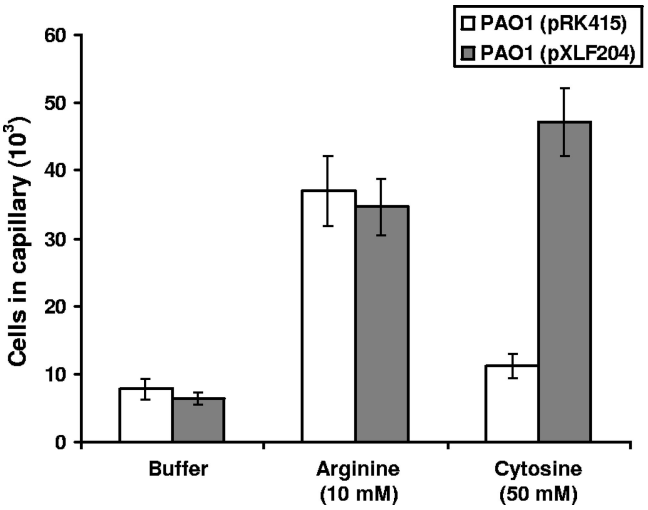

FIG. 4. Chemotactic responses of *P. aeruginosa* PAO1(pRK415) (wild-type vector control) and PAO1(pXLF204) (carrying *mcpC*) to cytosine in high-throughput quantitative capillary assays. Cells were grown in MSB with 27.5 mM glucose. Results are the averages of at least 15 capillaries from at least two independent experiments; error bars indicate standard errors.

Expression of McpC in PAO1. Wild-type *P. aeruginosa* PAO1 was not attracted to cytosine, so we tested whether the *P. putida* F1 cytosine chemoreceptor gene could be functionally expressed in PAO1 to allow the bacterium to gain the ability to respond to cytosine. The "complemented" strain PAO1(pXLF204) and the control strain PAO1(pRK415) were tested using quantitative high-throughput assays. PAO1 (pXLF204) and PAO1(pRK415) cells showed poor motility when grown in the presence of tetracycline, so the antibiotic was omitted from cultures grown for chemotactic assays. To check for plasmid maintenance, cells were plated on LB and LB containing tetracycline, following the assay. More than 50% of the cells retained the plasmid (data not shown). As expected, the background accumulation of cells in capillaries containing buffer only was about the same for both strains (~7,000 cells per capillary), and both responded to the positive control (10 mM L-arginine) at approximately the same level (Fig. 4). PAO1(pRK415) did not respond to cytosine (Fig. 4); however, PAO1(pXLF204) expressing *mcpC* showed a strong response to cytosine (Fig. 4). This result demonstrates an example of cross-species complementation by an *P. putida* MCP in a strain of *P. aeruginosa* and further confirms that the product of *mcpC* is indeed a cytosine chemoreceptor.

DISCUSSION

West and coworkers studied pyrimidine metabolism in *Pseudomonas* species and found that pyrimidine utilization varied significantly, depending on the species (48–52). For example, *Pseudomonas alcaligenes* ATCC 14909 was unable to utilize any of the pyrimidines as nitrogen sources, but *P. aeruginosa* PAO1, *Pseudomonas pseudoalcaligenes* ATCC 17440, and *Pseudomonas aureofaciens* used all three pyrimidines (48, 50, 52). *P. fluorescens* biotype F used only cytosine and uracil (49), while *P. putida* biotype B ATCC 17536 was reported to utilize uracil but used thymine only to a limited extent, which was suggested to be due to low-efficiency thymine uptake by cells of this strain (51). We found that *P. putida* F1 is able to utilize all three pyrimidines as the sole nitrogen source, whereas *P. putida* PRS2000 utilized only cytosine. The first step of cytosine metabolism in bacteria is deamination, which yields uracil and ammonia (35, 49). The inability of PRS2000 to grow with uracil while utilizing all three nitrogen atoms of cytosine suggests that PRS2000 may lack a uracil transporter. A thymine transporter may also be absent in PRS2000; alternatively, PRS2000 may lack the enzymes for thymine utilization. Therefore, even within a species, pyrimidine utilization seems to be strain specific. Of the three pyrimidines, cytosine appears to be most commonly utilized by pseudomonads, and therefore, chemotaxis to cytosine would appear to be advantageous to members of this genus. In addition, all three pyrimidines are likely to be found together in the environment as DNA and RNA from decaying cell material is degraded. Therefore, bacteria with the ability to sense and respond to at least one of the three pyrimidines should have the ability to move toward environments in which all three are present.

Although pyrimidine catabolism has been studied in many bacteria, only a few studies have reported pyrimidine chemotaxis. DeLoney-Marino et al. demonstrated that the marine bacterium *Vibrio fischeri* responded to the pyrimidine nucleosides thymidine, cytidine, and uridine (13). In addition, the strain also responded very weakly to relatively high concentrations (≥66 mM) of thymine and uracil, but not cytosine (13). However, no chemoreceptors for these responses were identified. We recently reported that *E. coli* was attracted to the pyrimidines thymine and uracil, but the response to cytosine was extremely weak (29). We have shown here that, of the three pyrimidines, *P. putida* responds only to cytosine, which is different from *V. fischeri* and *E. coli*, which respond to thymine and uracil. In general, the threshold and peak responses to pyrimidines were similar for *P. putida* and *E. coli* (approximately 1 mM and 10 to 50 mM, respectively) (Fig. 1) (29).

Our data clearly demonstrate that McpC mediates chemotaxis to cytosine in *P. putida* F1. To our knowledge, this is the first report of a chemosensory protein for chemotaxis to cytosine. We previously reported that Tap mediates the response to thymine and uracil in *E. coli* (29). However, sequence alignments revealed that there is no significant sequence identity between the periplasmic sensing domains of McpC and Tap. Tap is also known to be required for chemotaxis to dipeptides (30). *P. putida* F1 did not respond to the dipeptide Pro-Leu in modified capillary assays (data not shown), suggesting that McpC does not share this function with Tap. We also showed that F1 responded weakly to cytidine, uridine, and thymidine, but these responses were not mediated by McpC. This suggests that McpC recognizes cytosine only without the presence of the sugar moiety.

We have not determined whether McpC interacts directly with cytosine in the *P. putida* chemotactic response, although the interspecies complementation of *P. aeruginosa* PAO1 with only *mcpC* suggests that this might be the case. However, we cannot rule out the possibility that a periplasmic binding protein for cytosine transport in PAO1 is capable of binding cytosine and subsequently interacting with McpC.

The residual response to cytosine by the *mcpC* mutant prompted us to investigate the possible role of cytosine deamination in chemotaxis in *P. putida* F1. Our data indicate that the response to cytosine by the *mcpC* mutant is not due to a response to ammonia released from cytosine but is more likely due to the overlapping specificity of an additional chemoreceptor(s) that detects cytosine. It has been demonstrated with *P. aeruginosa* that certain chemicals are detected by multiple chemoreceptors. For example, the amino acid chemoreceptors PctA, PctB, and PctC all mediate chemotaxis to different but overlapping subsets of amino acids in *P. aeruginosa* PAO1 (27, 47). Similarly, CtpH and CtpL were shown to mediate chemotactic responses to high and low concentrations of inorganic phosphate, respectively, in PAO1 (54). Therefore, *Pseudomonas* strains appear to have complex chemosensory systems in which some chemoattractants are sensed by more than one MCP.

mcpC homologs are present in the sequenced genomes of *P. putida*, *P. fluorescens*, *P. mendocina*, and *P. syringae* strains, and the gene context is also conserved in these genomes. The neighboring genes have not been characterized but do not appear to play a direct role in chemotaxis. Their predicted products are a hypothetical protein (Pput_0626), a heavy metal-translocating P-type ATPase (Pput_0625), a MerR family transcriptional regulator (Pput_0624), a helix-turn-helix domain-containing protein (Pput_0622), and an acetyl-coenzyme A acetyltransferase (Pput_0621). The first three genes (Pput_0626 to Pput_0624) are transcribed in the same direction as *mcpC*, while the other two genes are transcribed in the opposite direction. It is likely that the McpC homologs present in other *Pseudomonas* strains also mediate cytosine chemotaxis in these organisms. Interestingly, we were unable to find any significant protein sequence matches to the periplasmic sensing domain of McpC in *P. aeruginosa* PAO1. The closest match to the full-length McpC in PAO1 is PA2652, which shares 42% amino acid sequence identity with McpC (most of the sequence identity resides in the C terminus). PA2562 mediates malate chemotaxis in *P. aeruginosa* (3). However, we demonstrated that McpC is not required for malate chemotaxis in *P. putida* F1, as the *mcpC* mutant still responded to malate (data not shown). Therefore, we conclude that there is no McpC ortholog in *P. aeruginosa* PAO1, which is consistent with the inability of PAO1 to respond to cytosine.

BLAST searches of public databases revealed that most putative MCP genes within *P. putida* species are highly conserved. Twenty-five out of the 27 putative MCPs in *P. putida* strains KT2440 and F1 share more than 95% amino acid sequence identity. Aer-like proteins are the only chromosomally encoded MCP-like proteins in *P. putida* that have been functionally characterized. Aer1 mediates aerotaxis in strain PRS2000, while another Aer homolog, Aer2, mediates aerotaxis and energy taxis in strain KT2440 (36, 41). The majority of the putative MCPs in *P. putida* have not been studied. Because both the predicted MCPs and the attractant/repellent profiles in *P. putida* and *P. aeruginosa* differ significantly, it is clear that further investigations into *Pseudomonas* chemotaxis should be carried out with additional species beyond the well-studied *P. aeruginosa*. We believe that this information will help us understand the lifestyles of different *Pseudomonas* species and provide insight into the ecological roles that these bacteria play.

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