

Identification of a chemotaxis gene region from *Pseudomonas putida*

Jayna L. Ditty, Ann C. Grimm, Caroline S. Harwood *

Department of Microbiology, The University of Iowa, 51 Newton Rd, 3-401 Bowen Science Bldg., Iowa City, IA 52242, USA

Received 7 November 1997; revised 12 December 1997; accepted 12 December 1997

Abstract

Pseudomonas putida is chemotactic to a range of organic compounds, including several aromatic compounds. Genes involved in this behavioral response were identified by Tn5 mutagenesis of *P. putida* PRS2000, resulting in a strain that was nonchemotactic to all chemoattractants tested. Cloning and sequencing of the DNA at the Tn5 insertion site revealed a 13-kb region that contained 12 open reading frames, 9 of which are homologous to chemotaxis, flagellar and motility genes in other bacterial species. This indicates that the basic chemotaxis machinery of *P. putida* is similar to that of other bacterial systems, even though some of the compounds that are sensed as attractants are different. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: Chemotaxis gene; *Pseudomonas putida*; Aromatic compound

1. Introduction

Motile bacteria can detect and swim towards organic compounds in the environment, many of which are used as sources of carbon and energy [1]. *Pseudomonas putida*, a ubiquitous soil bacterium, is attracted to organic compounds ranging from amino acids and TCA cycle intermediates to a wide variety of aromatic compounds including benzoate and 4-hydroxybenzoate (4-HBA) [2,3]. *P. putida* degrades these aromatic compounds to tricarboxylic acid cycle intermediates via the β -ketoadipate pathway. The

enzymology of the β -ketoadipate pathway has been particularly well studied in *P. putida*, making this a good model organism for the study of chemoattraction to aromatic compounds [4]. Initial work has demonstrated that *P. putida* modulates its swimming behavior in response to attractants in the same manner as *Escherichia coli*. As in *E. coli*, the addition of chemoattractants results in smooth swimming [5]. However, the positive chemotactic response to aromatic acids is not present in the enterics, and in fact various aromatic acids are chemorepellents for the enteric bacteria due to their properties as weak acids [6]. Recently a novel membrane protein, PcaK, was identified in *P. putida* that is required for both 4-HBA transport [7] and 4-HBA chemotaxis [3]. As a step towards elucidating how PcaK functions in chemoreception, we identified a gene cluster from

* Corresponding author. Tel.: +1 (319) 335-7783;
Fax: +1 (319) 335-7679;
E-mail: caroline-harwood@uiowa.edu

P. putida that is required for chemotaxis. The results reported here demonstrate that *P. putida* has genes that are homologous to chemotaxis, flagellar and motility genes from other bacteria, including *E. coli*. These genes are required for the general chemotactic response in *P. putida*.

2. Materials and methods

2.1. Strains, media and growth conditions

The bacterial strains and plasmids used are listed in Table 1. Strains were grown in Luria-Bertani (LB) medium for most experiments. *P. putida* strains were also grown, where indicated, in a defined minimal medium (basal medium), pH 6.8 [3]. Solid media contained 1.5% Bacto-agar (LB medium) or Noble agar (basal medium) (Difco Laboratories, Detroit, MI). Carbon sources were sterilized and added separately at the time of inoculation to a final concentration of 10 mM (succinate) or 5 mM (4-HBA or benzoate). Antibiotics were used at the following concentrations: kanamycin (Km), 100 µg ml⁻¹; tetracycline (Tc), 25 µg ml⁻¹; and ampicillin (Ap), 100 µg ml⁻¹.

LB soft agar swarm plates contained 0.1% tryptone, 0.05% yeast extract, 0.5% NaCl, and were solidified with 0.3% Bacto-agar. Basal medium swarm plates contained 0.5 mM chemoattractant and were solidified with 0.3% Noble agar.

P. putida broth cultures were incubated with shaking at 30°C. *E. coli* cultures were incubated at 37°C. Growth rates were measured spectrophotometrically at 660 nm.

2.2. Bacterial matings and mutagenesis

Wild-type *P. putida* strain PRS2000 was mutagenized with the transposon mini-Tn5 by conjugation with *E. coli* S17-λpir (pUT mini-Tn5 Km) [8]. General chemotaxis mutants were identified from the mating mixture by enriching for nonchemotactic cells as follows. Mutagenized cells were stabbed into the center of a basal medium swarm plate containing 4-HBA plus Km, and incubated overnight. Cells from the original site of inoculation, that had not migrated to the edge of the plate, were then transferred

to the center of a succinate plus Km swarm plate, incubated, and cells that failed to migrate to the edge were picked from the center and transferred to a second 4-HBA plus Km swarm plate. After two additional rounds of selection for cells that failed to migrate on 4-HBA and succinate swarm plates, cells from the center of the swarm were streaked to isolate colonies which were then individually screened on swarm plates. Cells that did not 'swarm' or migrate to the edge of soft agar plates in a sharp band were identified as possible nonchemotactic mutants.

To verify that the nonchemotactic phenotype of strain PRS4086 was caused by a mini-Tn5 insertion, a 12 kb *Kpn*I fragment from pHJD210 that conferred Km resistance was cloned into the broad-host range vector pRK415 and then transferred to PRS2000 by conjugation from *E. coli* S17-1 [9]. Recombinant strains were Km^r and Tc^s.

2.3. Quantitation of chemotaxis

PRS4086 swimming behavior was measured quantitatively using computer-assisted motion analysis to monitor changes in swimming direction in response to the addition of two chemoattractants, casamino acids and 4-HBA. For *P. putida*, chemoattraction is defined as less than 0.3 changes in direction of swimming per second. Greater than 0.4 changes in direction per second signifies random swimming behavior [5].

2.4. Cloning and sequencing

P. putida PRS4086 chromosomal DNA was partially restricted with *Pst*I, dephosphorylated, ligated to the cosmid vector pHC79, and then packing into λ phage heads using the Gigapack II Gold extract (Stratagene, La Jolla, CA). Transductants of *E. coli* strain JM109 were selected on Km and a cosmid clone (pHJD210) containing approximately 40 kilobases (kb) of *P. putida* DNA, was identified. Fragments of pHJD210 DNA were subcloned into pUC19 (Fig. 1) and sequenced at the University of Iowa DNA Core facility using universal and custom-synthesized primers.

The 13 kb *P. putida* chemotaxis and motility gene region that was sequenced has been assigned GenBank accession number AF031898.

3. Results and discussion

3.1. Identification of the *P. putida* chemotaxis and motility gene region

Mini-Tn5 mutagenized *P. putida* cells that did not swarm in soft agar plates were classified as possible nonchemotactic mutants. The majority of the non-swarming strains were nonmotile and presumed to be flagellar or flagellar motor mutants. One strain, PRS4086, was motile but did not swarm in LB, succinate, 4-HBA, or benzoate soft agar plates. PRS4086 exhibited wild-type growth rates on all chemoattractants. Unlike the wild-type strain, PRS4086 changed swimming direction infrequently in the presence of either buffer, casamino acids, or 4-HBA (Table 2). The mutant exhibited smooth swimming even in the absence of attractant, and thus the mutation in PRS4086 apparently resulted in an inability to change the direction of flagellar rotation. Consequently, the cells appeared to be stimulated whether or not an environmental stimulus was present. This constant state of attraction is characteristic of *E. coli* strains with mutations in one of three chemotaxis genes, *cheA*, *cheW*, or *cheY* [1]. The phenotype of PRS4086 suggested that it contained a mutation in a homologue of one of these genes.

Approximately 40 kb of PRS4086 chromosomal DNA surrounding the mini-Tn5 insertion was cloned to generate cosmid pHJD210. To be sure that the

mini-Tn5 interrupted DNA was responsible for the mutant phenotype of PRS4086, the Km insertion mutation was reconstructed on the wild-type PRS2000 chromosome by homologous recombination as described in Section 2. The recombinant strain had the same chemotaxis phenotype as the original PRS4086 mutant: motile and generally non-chemotactic.

Approximately 13 kb of DNA immediately flanking the Km insertion from pHJD210 was sequenced, revealing 12 open reading frames (orfs) which are all transcribed in the same direction (Fig. 1). Based on database searches, most of the orfs were identified as chemotaxis, motor or flagellar genes. Genes encoding five of the six cytoplasmic proteins (*cheY*, *cheZ*, *cheA*, *cheB*, and *cheW*) required for chemotaxis in many different bacterial systems were found within this region.

3.2. *CheA*

The orf interrupted by the mini-Tn5 was 2241 bp in length. The predicted gene product is 747 amino acids long, and is similar to CheA, a sensor histidine kinase that, in *E. coli*, forms a ternary complex with CheW and a methyl-accepting chemotaxis protein (MCP). Initiation of sensory signal transduction involves chemoattractant recognition at the cell surface by an MCP followed by autophosphorylation of CheA. CheA-P then transmits the sensory signal by

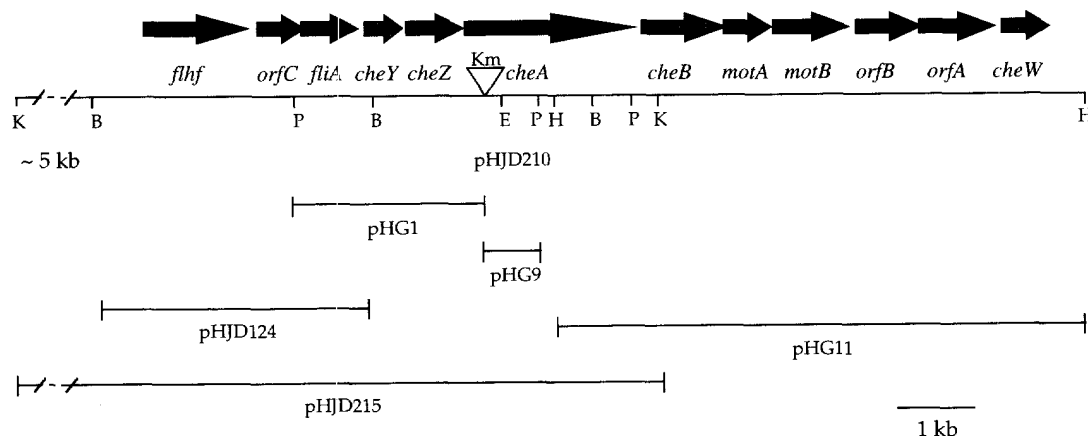


Fig. 1. The chemotaxis gene region of *P. putida* strain PRS2000. The open triangle indicates the site of the original mini-Tn5 insertion in the mutant strain PRS4086. H, *HindIII*; B, *BglII*; E, *EcoRI*; K, *KpnI*; P, *PstI*. (Not all *PstI* restriction sites are shown.) Plasmids shown are subclones of the pHJD210 cosmid clone.

Table 1
Bacterial strains and plasmids

Strain or plasmid ^a	Relevant characteristics ^b	Source or reference
Strains		
<i>P. putida</i>		
PRS2000	Wild-type	[3]
PRS4086	<i>cheA</i> ::mini-Tn5 Km	This study
Plasmids		
pHC79	Ap ^r , Tc ^r ; λ cos, cosmid cloning vector	Gibco-BRL
pRK415	Tc ^r ; IncP, broad host range cloning vector	[17]
pUC19	Ap ^r ; ColEI, <i>E. coli</i> plasmid cloning vector	Gibco-BRL
pUT mini-Tn5 Km	Km ^r ; vector pUT loaded with Km ^r mini-transposon	[8]
pHJD210	Tc ^r , Km ^r ; pHC79 carrying a ~40 kb <i>Pst</i> I partial fragment from PRS4086	This study
pHJD215	Tc ^r , Km ^r ; pRK415 carrying a 12 kb <i>Kpn</i> I fragment from pHJD210; used for recreating PRS4086 mutation	This study

^aSubclones of pHJD210, which were used for sequencing, are listed in Fig. 1.

^bAp^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

phosphorylating CheY, a response regulator protein that interacts with 'switch proteins' in the flagellar motor [1]. *P. putida* CheA was compared to CheA proteins from a wide variety of bacteria and one archaea. The best match (43% amino acid identity and 63% similarity) was to CheA from *Helicobacter pylori* [10]. Matches to CheA proteins from other organisms are listed in Table 3. At 747 amino acids, *P. putida* CheA is one of the largest of the known CheA polypeptides. When aligned with CheA peptides from other organisms, gaps arise in the putative CheY binding domain of the proteins, whereas the kinase and phosphorylation domains are well conserved. *P. putida* CheA has a histidine residue at position 50, which like histidine 48 in *E. coli* CheA, may serve as the site for autophosphorylation [1].

3.3. Upstream of *cheA*; identification of *cheY* and *cheZ*

In *E. coli*, the *cheY* gene encodes the response regulator that controls the direction of flagellar rotation [1]. Upstream from *P. putida* *cheA* was a homologue of *cheY* (Fig. 1). *P. putida* CheY was most similar to CheY from *P. aeruginosa* and *E. coli* CheY respectively (Table 3). By GAP analysis, *P. putida* CheY is similar throughout its length to CheY peptides from many other bacteria, which is interesting because *P. putida* CheA has large gaps (when compared to other homologues) in the putative CheY

binding region of the protein (see above). *P. putida* CheY has an aspartic acid residue at position 52, which based on sequence alignments with *E. coli* CheY, is likely to be the residue that accepts the phosphoryl group from CheA [1].

P. putida CheZ, a CheY dephosphorylase, again had the highest similarity to CheZ from *P. aeruginosa* with the second highest match to CheZ from *E. coli* (Table 3). To date, CheZ proteins have been identified in only the enteric bacteria *E. coli* and *Salmonella typhimurium* and in the two pseudomonads *P. putida* and *P. aeruginosa*, all members of the gamma subdivision of the proteobacteria. It is possible that CheZ will not be found in other bacterial families because it has recently been reported that

Table 2
Behavioral responses of wild-type and mutant *P. putida* strains to the attractants casamino acids and 4-hydroxybenzoate

Chemical stimulus ^a	Changes of swimming direction/s ^b	
	PRS2000 ^c	PRS4086 ^c
No addition	0.415	0.035
Casamino acids	0.130	0.040
4-Hydroxybenzoate	0.115	0.055

^aFinal concentrations of attractants were 0.01% casamino acids and 500 μ M 4-hydroxybenzoate.

^bBehavior was analyzed by computer-assisted motion analysis. Each value was determined from 45 s of analyzed behavior.

Chemoattraction is represented by <0.3 changes per second; >0.4 changes per second signifies random swimming behavior [5].

^cPRS2000 is wild-type *P. putida*; PRS4086 is the *cheA* mutant.

Strains were grown on 5 mM 4-hydroxybenzoate.

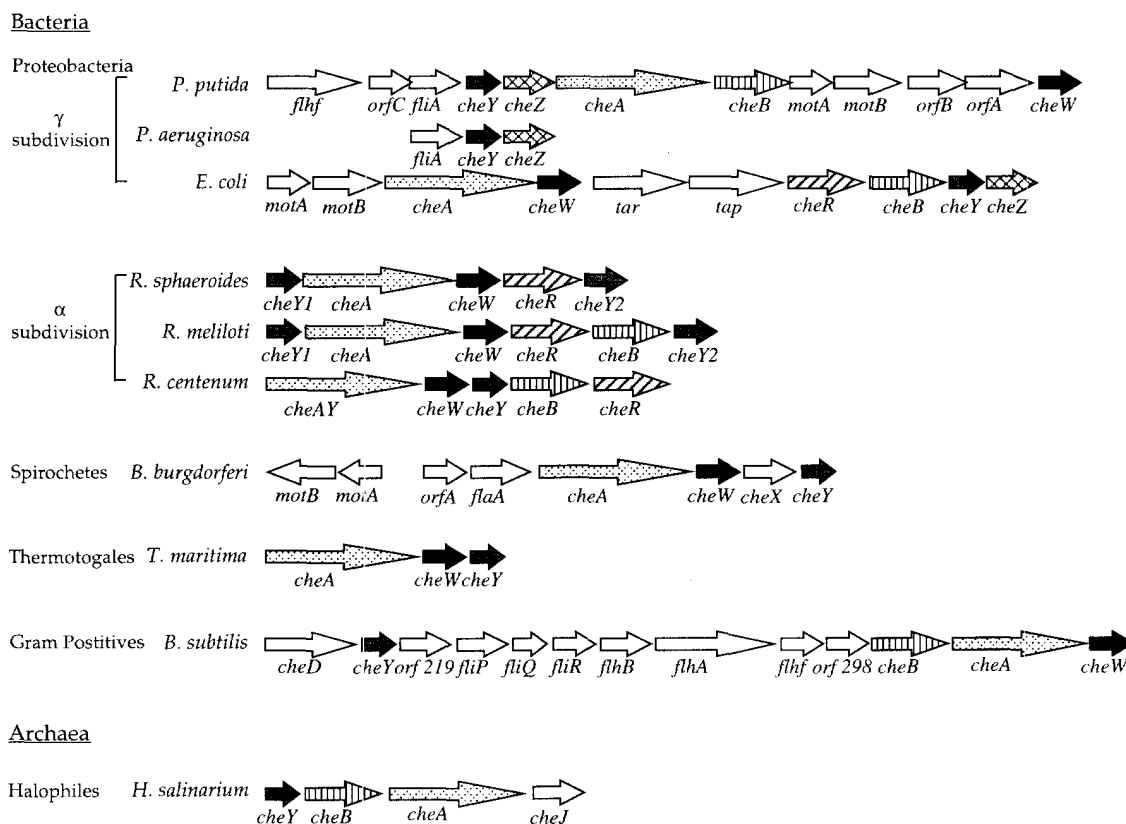


Fig. 2. Comparison of chemotaxis gene orders from various bacteria [1,12,18–25].

CheZ is not always required for chemotaxis to occur in *E. coli* [11].

Three orfs were identified upstream of *cheY*. Directly upstream of *cheY* is a homologue of *fliA*, the gene encoding an alternative sigma factor (σ^{28}) required for flagellin synthesis; the best match was to the *fliA* of *P. aeruginosa* [12]. Directly upstream of *fliA* is an orf (*orfC*) that is homologous to a gene within the chemotaxis region of the *Bacillus subtilis* genome which encodes for a 33 kDa protein that is not required for either motility or chemotaxis in *B. subtilis* [13]. Upstream of *orfC* is a homologue for a gene, *flhF*, that encodes a putative GTP binding protein required for motility in *B. subtilis* [14].

3.4. Downstream of *cheA*; identification of *cheB* and *cheW*

The *P. putida* homologue of CheB, the *E. coli*

chemotaxis protein that demethylates MCPs to allow appropriate adaptation to sensory stimuli [1], was found directly downstream of *cheA*. *P. putida* CheB has the highest amino acid identity to CheB from *Rhizobium meliloti*. However identities between *P. putida* CheB and the other CheB proteins are similar, around 40% (Table 3). Downstream of *cheB* were *motA* and *motB*, which in *E. coli* encode structural proteins of the flagellar motor [6]. The best match for both *P. putida* *motA* and *motB* gene products were to those reported from *H. pylori*; 34% identical and 61% similar and 28% identical and 51% similar respectively [10]. Immediately downstream of the two *mot* genes were two orfs of unknown function. *orfB* is homologous to a gene located at the origin of replication in the *B. subtilis* chromosome [15]. No function can be assigned to *orfA* based upon sequence homology. The homologue of *cheW* was located downstream of these two orfs. *P. putida* CheW

Table 3

Percent identity of *Pseudomonas putida* chemotaxis proteins to homologs from selected organisms^a

Organism	CheA	CheB	CheW	CheY	CheZ
<i>Pseudomonas aeruginosa</i>	— ^b	—	—	91	68
<i>Escherichia coli</i>	37	40	30	60	36
<i>Rhodobacter sphaeroides</i>	33	—	32	31 ^d	—
<i>Rhizobium meliloti</i>	33	42	31	34 ^d	—
<i>Rhodospirillum centenum</i>	39 ^c	38	27	35	—
<i>Helicobacter pylori</i>	43	—	26	50	—
<i>Borrelia burgdorferi</i>	31	—	30	25	—
<i>Thermotoga maritima</i>	35	—	28	28	—
<i>Bacillus subtilis</i>	34	41	32	34	—
<i>Halobacterium salinarum</i>	33	40	—	25	—

^aPercents are relative to 100% for *P. putida*. List includes the top matches for chemotaxis genes based on BLAST searches [26]. Percent amino acid identities are by GAP analysis [27]. GenBank accession numbers for represented chemotaxis proteins are as follows. *P. aeruginosa*: CheY, S20545; CheZ, D37810. *E. coli*: CheA, P07363; CheB, P07330; CheW, P07365; CheY, P06143; CheZ, P07366. *R. sphaeroides*: CheA, S49211; CheW, S47260; CheY1, S49210; CheY2, S47262. *R. meliloti*: CheA, S61834; CheB, S61837; CheW, S61835; CheY1, S61833; CheY2, S61838. *R. centenum*: CheAY, CheB, and CheW, U64519. *H. pylori*: CheA, AE000555; CheW, AE000555; CheY, U97567. *B. burgdorferi*: CheA, U28962; CheW and CheY, U61498. *T. maritima*: CheA, CheW, and CheY, U30501. *B. subtilis*: CheA, P29072; CheB, O05522; CheW, P39802; CheY, P24072. *H. salinarum*: CheA, S54304; CheB, S58646; CheY, X86407.

^b—, amino acid sequence not available.

^cIdentity was to the CheAY homolog from *R. centenum*.

^dIdentities are the same for both CheY1 and CheY2 proteins from these organisms.

is about 30% identical to CheW proteins from other bacteria (Table 3).

3.5. CheR, MCPs, and comparative gene organization

Genes encoding CheR, an MCP methyltransferase required for sensory adaptation, and MCPs have not yet been identified in *P. putida*. However, CheR and MCPs do seem to be involved in *P. putida* chemotaxis because proteins are methylated in response to the presence of various attractants during adaptation [16]. Outside of the enteric bacteria, CheR homologs have been identified only in the proteobacteria *Rhodobacter sphaeroides*, *R. meliloti*, and *Rhodospirillum centenum*.

Chemotaxis genes are typically found in clusters (Fig. 2). However, gene organizations are not absolutely conserved in different bacteria. In the alpha subdivision of the proteobacteria, the trend in gene organization is for *cheY*/*cheA* genes followed by *cheW*, but then the gene organization deviates. No *P. aeruginosa* *che* gene sequences, other than those shown in Fig. 2 have been reported. So it is not yet known if *P. putida* and *P. aeruginosa* have identical chemotaxis gene organizations. However, even between the closely related *E. coli* and *S. typhimurium*

the gene organization is not absolutely conserved, as the *S. typhimurium* cluster lacks the MCP gene *tap* [1].

4. Conclusions

Chemotaxis has been intensively studied in the enteric bacteria *E. coli* and *S. typhimurium* [1]. *P. putida* responds to a wider array of compounds than do the enteric bacteria. For example, it is attracted to the aromatic acids benzoate and 4-HBA [2], which are chemorepellents for the enteric bacteria [6]. Although they respond to different attractants, *E. coli* and *P. putida* modulate their swimming behavior in response to attractants similarly [5]. Here, the sequences of *P. putida* genes homologous to those encoding five of the six cytoplasmic proteins required for chemotaxis in *E. coli* are reported. *CheR* and *mcp* genes have not yet been identified, but they have been implicated in the *P. putida* chemotactic response [16]. Thus, the *P. putida* central chemotaxis machinery appears to be similar to those of other bacteria.

Chemoreception of aromatic acids by *P. putida* differs from chemoreception systems studied in en-

teric bacteria, as evidenced by the role that a transport protein, PcaK, plays as a receptor for the aromatic acid 4-HBA [3]. The hope is that the information described here will be valuable for future studies aimed at addressing how specific aromatic acid chemoreceptors, transporters, and the general chemotaxis machinery interact to transmit sensory input from aromatic acids to the *P. putida* flagellar motor.

Acknowledgments

This work was funded by grant MCB9603551 from the National Science Foundation.

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