

Identification of the *pcaRKF* Gene Cluster from *Pseudomonas putida*: Involvement in Chemotaxis, Biodegradation, and Transport of 4-Hydroxybenzoate

CAROLINE S. HARWOOD,* NANCY N. NICHOLS, MIN-KYUNG KIM,
JAYNA L. DITTY, AND REBECCA E. PARALES

*Department of Microbiology and Center for Biocatalysis and Bioprocessing,
University of Iowa, Iowa City, Iowa 52242*

Received 27 June 1994/Accepted 18 August 1994

***Pseudomonas putida* PRS2000 is chemotactic to 4-hydroxybenzoate and other aromatic acids. This behavioral response is induced when cells are grown on 4-hydroxybenzoate or benzoate, compounds that are degraded via the β -ketoadipate pathway. Isolation of a transposon mutant defective in 4-hydroxybenzoate chemotaxis allowed identification of a new gene cluster designated *pcaRKF*. DNA sequencing, mutational analysis, and complementation studies revealed that *pcaR* encodes a regulatory protein required for induction of at least four of the enzymes of the β -ketoadipate pathway and that *pcaF* encodes β -ketoadipyl-coenzyme A thiolase, the last enzyme in the pathway. The third gene, *pcaK*, encodes a transporter for 4-hydroxybenzoate, and this protein is also required for chemotaxis to aromatic acids. The predicted PcaK protein is 47 kDa in size, with a deduced amino acid sequence indicative of membership in the major facilitator superfamily of transport proteins. The protein, expressed in *Escherichia coli*, catalyzed 4-hydroxybenzoate transport. In addition, whole cells of *P. putida* *pcaK* mutants accumulated 4-hydroxybenzoate at reduced rates compared with that in wild-type cells. The *pcaK* mutation did not impair growth at the expense of 4-hydroxybenzoate under most conditions; however, mutant cells grew somewhat more slowly than the wild type on 4-hydroxybenzoate at a high pH. The finding that 4-hydroxybenzoate chemotaxis can be disrupted without an accompanying effect on metabolism indicates that this chemotactic response is receptor mediated. It remains to be determined, however, whether PcaK itself is a chemoreceptor for 4-hydroxybenzoate or whether it plays an indirect role in chemotaxis. These findings indicate that aromatic acid detection and transport are integral features of aromatic degradation pathways.**

Aromatic compounds are abundant in the biosphere as components of the complex polymer lignin and as environmental pollutants. The bacterial biodegradation of structurally simple, readily degradable aromatic compounds has been studied with the expectation that this will facilitate work on more recalcitrant members of the group. As a result, much information has been obtained about the enzymology and molecular regulation of aerobic pathways of aromatic compound degradation (6, 16, 35, 53). An aspect of aromatic biology that has received almost no attention, however, is the question of how bacteria sense and respond to the presence of aromatic compounds in their environment. Chemotaxis and transport are two physiological functions that operate in this capacity, and a number of studies have shown that aromatic acids such as benzoate and 4-hydroxybenzoate are strong chemoattractants for *Pseudomonas putida*, as well as a number of other species of gram-negative bacteria including *Agrobacterium* spp. and rhizobia (19, 20, 31, 39, 40). However, very little is known about the characteristics of the presumed receptor proteins that are responsible for initial attractant recognition. Similarly, although a number of studies have inferred the existence of specific transport systems for aromatic acids and related compounds, only a few detailed studies of aromatic compound permeases have been reported (1, 2, 14, 30), and no molecular analyses of bacterial genes that encode such proteins have been presented.

The β -ketoadipate pathway for the degradation of aromatic rings is widely distributed among bacteria and consists of two parallel branches for the dissimilation of catechol and protocatechuate, derived from benzoate and 4-hydroxybenzoate, via an initial ortho-ring cleavage (Fig. 1) (48). The enzymology of this pathway has been particularly well studied in *P. putida* PRS2000, and many of the structural genes for aromatic acid degradation have been cloned from this organism (35, 37, 56). We have determined that strain PRS2000 has a strong chemotactic attraction to 4-hydroxybenzoate and benzoate and that biodegradation and aromatic acid chemotaxis are coordinately regulated (10, 19, 20). In this study, we identified a mutant that is specifically nonchemotactic to benzoate and 4-hydroxybenzoate. A molecular analysis of this mutant led to the identification of a new gene cluster, designated *pcaRKF*, which encodes a regulatory protein required for 4-hydroxybenzoate degradation; β -ketoadipyl-CoA thiolase, the last enzyme of the β -ketoadipate pathway; and a 4-hydroxybenzoate transport protein. The transport protein, PcaK, is also required for 4-hydroxybenzoate-grown cells to exhibit wild-type chemotaxis to aromatic acids.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. Cultures of *P. putida* were routinely grown at 30°C in a defined mineral medium (minimal medium) which contained 25 mM KH_2PO_4 , 25 mM Na_2HPO_4 , 0.1% $(\text{NH}_4)_2\text{SO}_4$, and 1% Hutner mineral base (final pH, 6.8) (12). Minimal medium containing tetracycline was made with

* Corresponding author. Mailing address: Department of Microbiology, University of Iowa, Iowa City, IA 52242. Phone: (319) 355-7783. Fax: (319) 335-9006. Electronic mail address: caroline-harwood@uiowa.edu.

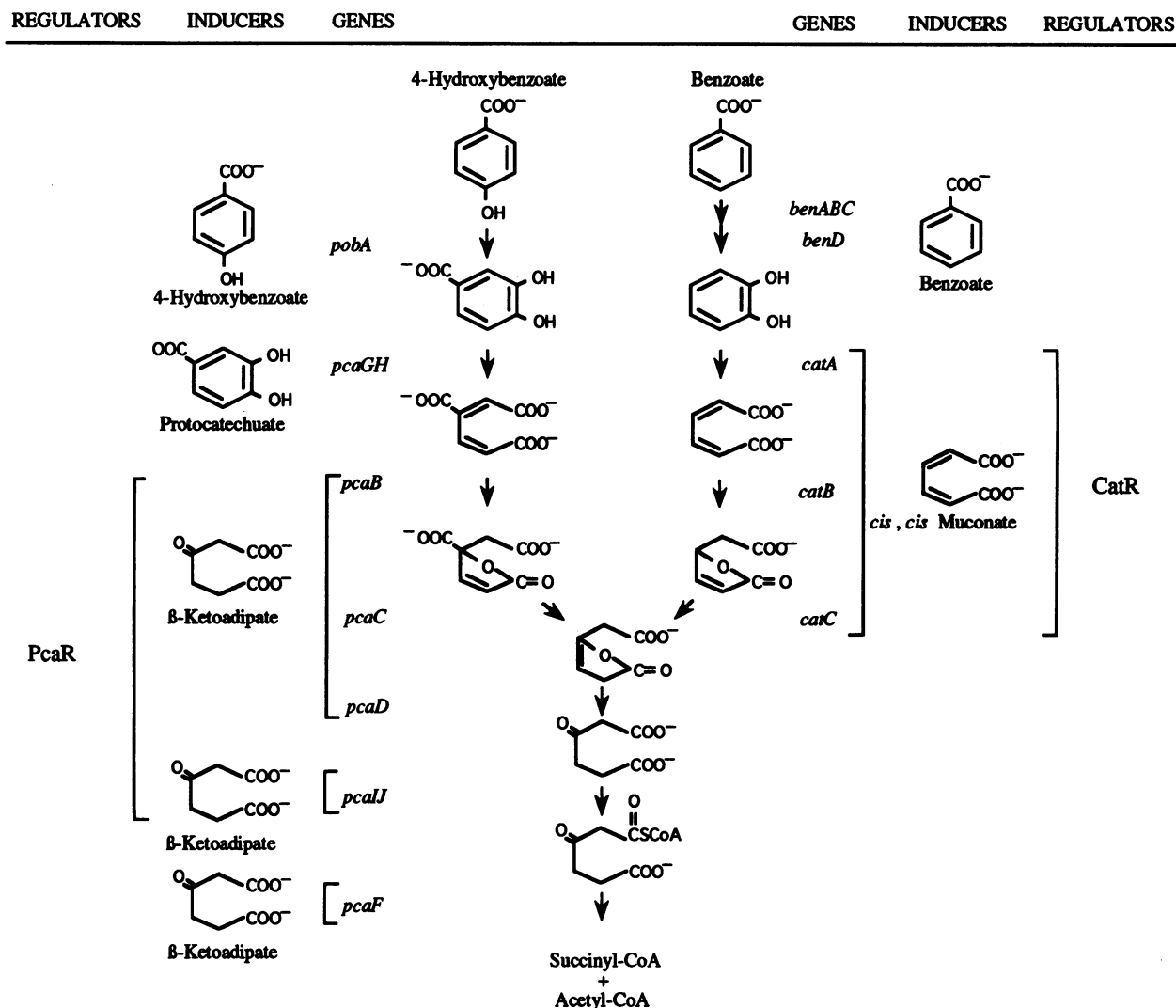


FIG. 1. The β -ketoadipate pathway and its regulation in *P. putida*.

0.1% rather than 1.0% mineral base. Carbon sources were sterilized separately and added at the time of inoculation (final concentrations [unless otherwise noted]: benzoate and 4-hydroxybenzoate, 5 mM; succinate, 10 mM; and glucose, 10 mM). *Escherichia coli* strains were grown in Luria broth (LB) (7) at 37°C. For *P. putida*, antibiotics were used at the following final concentrations, unless stated otherwise: gentamicin, 20 µg/ml; kanamycin, 100 µg/ml; and tetracycline, 100 µg/ml. For *E. coli*, antibiotics were used at the following concentrations: ampicillin, 100 µg/ml; gentamicin, 20 µg/ml, and kanamycin, 100 µg/ml.

Growth studies. For some studies, minimal medium was modified to be buffered at three different pHs. All media formulations contained 0.1% (final concentration) $(\text{NH}_4)_2\text{SO}_4$ and 0.1% Hutner mineral base (12). A minimal medium of pH 6.3 was prepared by the further addition of 18.75 mM KH_2PO_4 , 6.25 mM Na_2HPO_4 , 18.75 mM NaCl, and 6.25 mM KCl. Medium with a final pH of 6.8 contained 12.5 mM KH_2PO_4 , 12.5 mM Na_2HPO_4 , 12.5 mM NaCl, and 12.5 mM KCl. A pH 8.1 minimal medium contained 25 mM Na_2HPO_4 and 25 mM KCl. For growth studies, 10-ml cultures were

grown in 50-ml Erlenmeyer flasks. Incubation was at 30°C on a rotary shaker, and growth was monitored by measuring A_{660} .

Bacterial transformations and conjugations. *E. coli* was transformed with plasmid DNA by the method of Hanahan (15). Plasmids were mobilized from *E. coli* S17-1 into *P. putida* by patch matings on LB agar plates with incubation overnight at 30°C.

DNA manipulations and sequencing. *P. putida* chromosomal DNA was purified as previously described (13). Plasmid DNA to be used for cloning and sequencing was purified by the method of Lee and Rasheed (29) or Hattori and Sakaki (21). Restriction endonuclease digestions were according to the manufacturer's instructions (New England Biolabs, Beverly, Mass.). Dephosphorylation reactions with calf intestinal phosphatase (Boehringer Mannheim, Indianapolis, Ind.) were carried out by the method of Ausubel et al. (3). Ligation reactions and agarose gel electrophoresis methods were as described previously (45). DNA fragments for subcloning were purified from gel slices by using GeneClean (Bio 101, La Jolla, Calif.). Vent polymerase (Promega, Madison, Wis.) was used to amplify DNA in PCRs. DNA sequences were determined by

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>P. putida</i>		
PRS2000	Wild type	36
PRS3015	<i>pcaR</i> ::Tn5; Ben ⁻ , 4-OHBen ⁻	25
PRS4061	<i>pcaF</i> ::Gm ^r ; Ben ⁻ ; 4-OHBen ⁻	This study
PCH722	<i>pcaK</i> ::Tn5-B30; 4-OHBen taxis ⁻	This study
PCH722-Gm	<i>pcaK</i> ::Tn5-B30/Gm; 4-OHBen taxis ⁻	This study
<i>E. coli</i>		
S17-1	<i>thi pro hdsR hdsM⁺ recA</i> , chromosomal insertion of RP4-2 (Tc::Mu Km::Tn7)	46
BL21(DE3)	<i>hsdS gal</i> (λ CLs857 <i>ind-1 Sam7 nin-5 lacUV5-T7 gene 1</i>)	50
JM109(DE3)	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi</i> Δ (<i>lac⁻ proAB</i>) F'(<i>traD36 proAB⁺ lacI^a</i> <i>lacZ</i> Δ M15) (λ CLs857 <i>ind-1 Sam7 nin-5 lacUV5-T7 gene 1</i>)	50
Plasmids		
pHG165	Ap ^r ; ColE1 replicon; controlled copy number (<i>rop⁺</i>) cloning vector	49
pK19	Km ^r ; pUC19 multiple cloning site	43
pRK415	Tc ^r ; IncP; broad-host-range cloning vector	27
pSUP102-Gm::Tn5-B30	Gm ^r ; vector pSUP102-Gm loaded with Tn5-B30, encoding Tc ^r and promoterless Km ^r	47
pT7-5	Ap ^r ; T7 promoter expression vector	51
pUC8	Ap ^r ; ColE1 replicon cloning vector	54
pHJD100	Tet ^r ; <i>pcaK</i> with native promoter cloned in pRK415	This study
pHNN100	Ap ^r ; <i>pcaK</i> cloned downstream of T7 promoter of pT7-5	This study
pHRP124	Ap ^r ; <i>pcaI</i> cloned downstream of T7 promoter of pT7-5	37
pHRP150	Gm ^r ; pHRP309 with <i>pcaI</i> promoter fused to <i>lacZ</i>	38
pHRP302	Ap ^r Gm ^r genes in pUC1318	37
pHRP401	Ap ^r ; pHG165 carrying 4-kb <i>Hind</i> III fragment of <i>P. putida</i> DNA from pMKK722 downstream of Tn5-B30 insertion	This study
pHRP402	Ap ^r ; pUC8 carrying 7-kb <i>Bam</i> HI- <i>Hind</i> III fragment from pMKK722; includes Tn5-B30 insertion	This study
pHRP403	Ap ^r ; pMKK722 digested with <i>Xho</i> I to delete Tc ^r , and Gm ^r from pHRP302 inserted as a <i>Sac</i> I fragment	This study
pHRP404	Tc ^r ; 9-kb <i>Bam</i> HI fragment from pHRP403 inserted in pRK415; used to construct PCH722-Gm	This study
pHRP410	Ap ^r ; pHRP401 with Gm ^r gene inserted into <i>Sac</i> I site	This study
pHRP411	Tc ^r ; <i>Hind</i> III fragment from pHRP410 inserted in pRK415; used to construct PRS4061	This study
pHRP414	Km ^r ; <i>Cla</i> I fragment from PRS4061 inserted in <i>Acc</i> I site of pK19; carries intact <i>pcaK</i> gene	This study
pMKK722	Ap ^r Tc ^r ; pUC8 carrying a 10-kb <i>Bam</i> HI fragment from PCH722	This study

^a Ben⁻, no growth on benzoate; 4-OHBen⁻, no growth on 4-hydroxybenzoate; 4-OHBen taxis⁻, no chemotaxis in 4-hydroxybenzoate swarm plates; Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

the dideoxy chain termination method with the Sequenase kit from United States Biochemical Corp. (Cleveland, Ohio) and the Promega *fmol* sequencing kit. [α -³⁵S]dATP and [γ -³²P]dATP were from Amersham Corp. (Arlington Heights, Ill.). Sequencing of the *pcaRKF* region was done by using universal forward and reverse primers, primers to the IS50 element of Tn5, specific synthetic oligonucleotide primers, and a combination of DNA templates including pHRP401, pHRP402, pHRP411, pHRP414, and pMKK722 (Fig. 2). The sequence was determined for both strands of DNA. Oligonucleotide primers were synthesized by the DNA facility at the University of Iowa, and some sequencing was done at this facility with an Applied Biosystems 373A automated DNA sequencer. DNA and protein sequence data were analyzed with DNA Inspector IIe, version 3.15 (Textco, Inc., W. Lebanon, N.H.) and the University of Wisconsin Genetics Computer Group software package, version 7.0 (8). The FASTA program was used to search for similar sequences (41). Pairwise comparisons and multiple sequences analysis were carried out with the GAP and PileUp programs (33).

Cloning of *pcaK*. For expression in *E. coli*, *pcaK* was cloned in pT7-5 under the control of the T7 promoter. A 1,462-bp segment of DNA encompassing the *pcaK* gene was amplified from pHRP401 by using PCR (3). The upstream primer (5'G

ACTGAATTC^{CCCCAATCATCGTCCCCTGTA3'}) incorporated an *Eco*RI site, and the downstream primer (5'CAGTG GATCCTTGTGCTGCGAATGGTCTCAAG3') incorporated a *Bam*HI site (underlined). Amplified product was restricted with *Eco*RI and *Bam*HI and ligated into pT7-5, forming pHNN100 (Fig. 2).

For complementation of PCH722, the broad-host-range vector pRK415 was used to clone *pcaK* in a construct designated pHJD100 (Fig. 2). A 1,723-bp segment of DNA, spanning *pcaK* and its promoter region, was amplified from pHRP 414 by PCR. The upstream primer (5'GCACGTGAAGCT TGTGCGATAAACGCACAGTGTGCG3') incorporated a *Hind*III cloning site. The downstream primer was the same as that used in construction of pHNN100.

Chemotaxis assays. Soft agar swarm plates for qualitative measurement of chemotaxis consisted of minimal medium containing 0.1% rather than 1.0% Hutner mineral base, 0.3% Noble agar (Difco, Detroit, Mich.) and the carbon source (the chemoattractant) at a final concentration of 1 mM. Behavioral responses of cells were also measured by using computer-assisted motion analysis to analyze changes in the swimming behavior of cells that occurred in response to the addition of a chemical (17). Behavior was quantitated in terms of the number of changes of direction of swimming per second for a

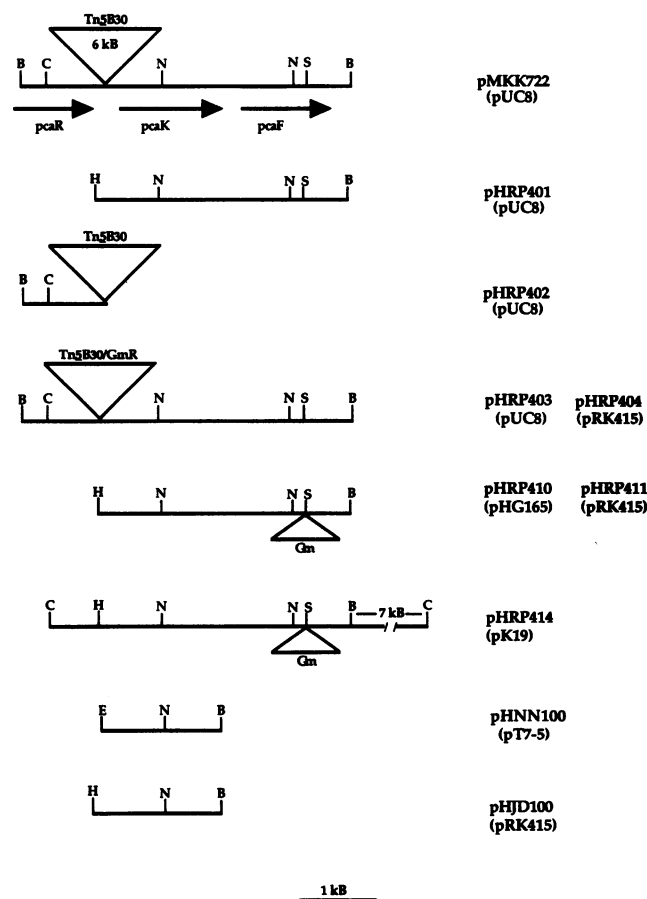


FIG. 2. Restriction map of the *pcaRK* gene cluster and plasmid derivatives. The cloning vector for each construct is indicated in parentheses. Restriction sites are as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; N, *Nae*I; and S, *Sac*I. The transposon Tn5-B30 is present in pMKK722 and pHRP402. Tn5-B30/Gm^r indicates the replacement of the tetracycline resistance gene of Tn5-B30 with a gentamicin resistance gene in pHRP403 and pHRP404. The insertion of a gentamicin resistance gene in *pcaF* is shown in pHRP410, pHRP411, and pHRP414. The *Hind*III site in pHRP401, pHRP410, and pHRP411 is derived from the IS50 element of Tn5-B30. The *Eco*RI and *Bam*HI sites of pHNN100 and *Hind*III and *Bam*HI sites of pHJD100 were introduced by PCR.

population of cells. Behavioral responses were assayed within 30 s after the addition of attractant. For *P. putida*, a value of greater than 0.4 changes of direction per second reflects random swimming behavior, whereas a value of less than 0.3 changes of direction per second corresponds to chemotactic stimulation (17).

Expression of PcaK in *E. coli*. The PcaK protein was expressed from pHNN100 in an *E. coli* strain carrying an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible gene for T7 RNA polymerase (3). Cultures (100 ml) of *E. coli* cells carrying the cloned *pcaK* gene were induced with 100 μ M IPTG when they reached an A_{660} of 0.25 and were harvested for use in uptake assays after an additional 30 min of incubation at 37°C with shaking. By this time, the cultures had generally reached an A_{660} of 0.5.

Enzyme assays. Development of an assay to measure β -ketoadipyl-coenzyme A (CoA) thiolase activity depended on the use of *E. coli* extracts expressing β -ketoadipate:succinyl-CoA

transferase to provide a source of β -ketoadipyl-CoA, the substrate of the thiolase reaction. The cloned *pcaIJ* genes, encoding the transferase, were expressed according to the protocol described above for *pcaK* expression, and cell extracts were prepared as described previously (37). Cell extract prepared from IPTG-induced cultures of JM109(DE3)(pHRP124) was allowed to catalyze the formation of β -ketoadipyl-CoA in a 2-ml reaction mixture as described previously (37). After the reaction had proceeded for 25 min, *P. putida* cell extract was added to 1 ml of the reaction mixture and β -ketoadipyl-CoA thiolase activity was assayed spectrophotometrically as the decrease in A_{305} due to the disappearance of β -ketoadipyl-CoA-Mg²⁺ ion complex. The other 1 ml of the transferase reaction mixture was used as the blank for the thiolase reaction.

β -Galactosidase activities were assayed as described previously (38).

Uptake assays. Cells to be used in uptake assays were harvested in mid-exponential growth phase, washed, and resuspended in 20 mM Tris, pH 7.0, to an A_{660} of 5 to 10. Air was bubbled gently through the cell suspension to ensure that it did not become oxygen limited. Uptake was initiated by the addition of a portion of the cell suspension to an equal volume of 20 mM Tris buffer containing the labeled substrate. Samples (0.1 ml) were taken at timed intervals and filtered through polycarbonate membranes (0.2 μ m pore diameter; Nucleopore Corp., Pleasanton, Calif.). The filters were washed before and after the sample addition with 2 ml of the same 20 mM Tris buffer used for cell suspension. Some experiments with *P. putida* were carried out at pH 8.0. In this case, cells were washed and resuspended in 20 mM Tris, pH 8.0, and uptake assays were carried out with 20 mM Tris buffer, pH 8.0. In one uptake experiment, radiolabeled material which had accumulated intracellularly in *E. coli* cells provided with ¹⁴C-labeled 4-hydroxybenzoate was extracted and analyzed by thin-layer chromatography and autoradiography as described previously (42).

Transposon mutagenesis. *E. coli* S17-1 carrying pSUP102-Gm loaded with the Tn5 derivative Tn5-B30 was mixed and plated with *P. putida* PRS2000 on LB plates and incubated overnight at 30°C. The mating mixture, suspended in minimal medium, was plated on minimal medium plates containing 1 mM succinate, 5 mM 4-hydroxybenzoate, and 100 μ g of kanamycin per ml. Colonies from these plates were then replica plated to minimal medium plates containing 200 μ g of kanamycin per ml plus 10 mM succinate, 200 μ g of kanamycin per ml plus 1 mM succinate plus 5 mM 4-hydroxybenzoate, and 200 μ g of kanamycin per ml plus 1 mM succinate plus 5 mM benzoate to identify mutants with aromatic acid-inducible kanamycin resistance that were relatively unimpaired in growth on benzoate and 4-hydroxybenzoate.

Protein determinations. Protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the standard. Whole cells were precipitated with trichloroacetic acid, and the precipitate was heated at 95°C for 10 min in 0.1 M NaOH before protein was assayed.

Chemicals. [ring-U-¹⁴C]4-Hydroxybenzoic acid (specific activity, 33 mCi/mmol) was from Amersham Corp. (Arlington Heights, Ill.). [ring-U-¹⁴C]Benzoic acid (specific activity, 13 mCi/mmol) was from Sigma Corp. (St. Louis, Mo.).

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to GenBank and assigned accession number U10895.

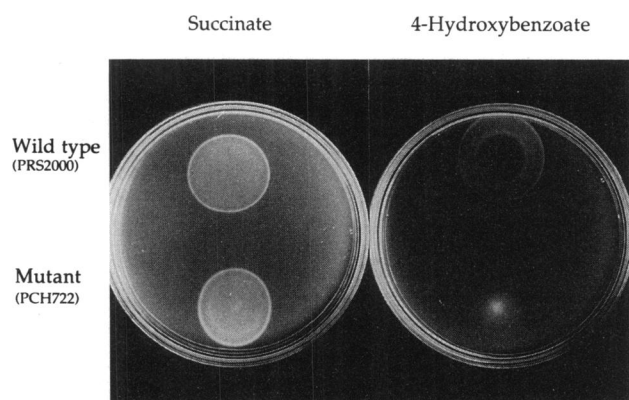


FIG. 3. Chemotactic swarms formed by wild-type *P. putida* and the mutant strain PCH722 on soft agar plates containing 1 mM succinate or 1 mM 4-hydroxybenzoate. Plates were inoculated with motile cells at a point corresponding to the center of the swarm ring and incubated for 17 h at 30°C. A wild-type response is indicated by a sharp rapidly moving ring that is formed in response to a gradient of attractant that is created as cells metabolize the carbon source. A fuzzy, slowly moving ring reflects random movement of motile cells that are nonchemotactic to the carbon source provided.

RESULTS

Identification and characterization of a mutant defective in chemotaxis to aromatic acids. Aromatic acid chemotaxis by *P. putida* is induced by growth on benzoate or 4-hydroxybenzoate (20). *P. putida* cells mutagenized with a Tn5 derivative carrying a promoterless Km^r gene were therefore screened to identify strains that were Km^r when grown in the presence of benzoate or 4-hydroxybenzoate, but Km^s when grown on succinate alone. From among these, three strains that failed to form chemotactic rings on soft agar swarm plates containing 4-hydroxybenzoate as the sole carbon source were identified (Fig. 3). These strains had wild-type swarm patterns on succinate (Fig. 3) and benzoate soft agar plates (not shown). One strain, PCH722, selected for further study, failed to respond to the chemoattractants benzoate and 4-hydroxybenzoate in quantitative computer-assisted assays of chemotaxis after growth on 4-hydroxybenzoate (Table 2). Benzoate-grown cells did respond to these compounds, however, and PCH722 cells grown on either benzoate or 4-hydroxybenzoate responded normally to Casamino Acids (Table 2) and succinate (data not shown).

TABLE 2. Behavioral responses of wild-type and *pcaK* mutant strains to benzoate and 4-hydroxybenzoate

Chemical stimulus ^a	Changes of swimming direction/s ^b			
	PRS2000 ^c		PCH722	
	4OHB grown	Ben grown	4OHB grown	Ben grown
Buffer	0.85	0.51	0.67	0.44
Benzoate	0.07	0.18	0.63	0.10
4-Hydroxybenzoate	0.11	0.18	0.54	0.06
Casamino Acids	0.13	0.08	0.17	0.09

^a Aromatic acids were tested at a final concentration of 500 μ M; Casamino Acids were at a final concentration of 0.01%.

^b Behavior was analyzed by computer-assisted motion analysis. Each value was determined from at least 60 s of analyzed behavior. For *P. putida*, >0.4 changes per s indicates random swimming behavior, and <0.3 changes per s reflects chemotactic stimulation. Growth substrates for cultures are indicated. 4OHB, 4-hydroxybenzoate; Ben, benzoate.

^c PRS2000 is the wild-type *P. putida* strain; PCH722 is a *pcaK* mutant.

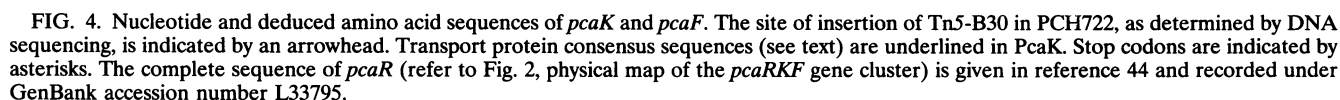
Strain PCH722 was therefore specifically nonchemotactic to aromatic acids, but only under certain growth conditions. This mutation was designated *pcaK*.

Cloning and sequencing of the *pcaRKF* gene cluster. A 10-kb *Bam*HI fragment encoding Tc^r was cloned from PCH722 to give the recombinant plasmid pMKK722. To verify that the cloned DNA carried a Tn5-B30-disrupted *pcaK* gene, the mutation was reconstructed on the *P. putida* chromosome by homologous recombination. The Tc^r gene was deleted from pMKK722 and replaced with the Gm^r gene from pHRP302. The 9-kb *Bam*HI fragment from the clone (pHRP403) thus formed was inserted into pRK415, forming pHRP404 (Fig. 2). This plasmid was mobilized into wild-type *P. putida*, and a Gm^r Tc^s isolate was identified. This strain, designated PCH722- Gm , has the same chemotaxis phenotype as PCH722, and its kanamycin resistance is also inducible by growth on aromatic acids.

The 4-kb region of *P. putida* DNA from pMKK722 was sequenced (Fig. 4), and computer analysis revealed three open reading frames (ORFs) oriented in the same direction (Fig. 2). The deduced amino acid sequence of the first ORF demonstrated homology to a recently defined family of transcriptional regulator proteins which includes PobR, an activator of 4-hydroxybenzoate hydroxylase from *Acinetobacter calcoaceticus*; IclR, a repressor of genes involved in acetyl-CoA utilization via the glyoxylate shunt in enteric bacteria; and GylR, an activator of genes required for glycerol utilization by *Streptomyces coelicolor* (9). For reasons noted below, this gene has been designated *pcaR*. The second ORF was designated *pcaK* and encodes a predicted hydrophobic protein of 47 kDa (Fig. 5) with homology (20 to 25% amino acid identity) to a large number of membrane-bound transport proteins, including the glucose transporter of *Synechocystis* spp. (58), and the bicyclic mycin resistance determinant of *E. coli* (5). The third ORF encodes a 42.7-kDa predicted protein with high amino acid identity (30 to 50%) to β -ketoacyl-CoA thiolase enzymes from a variety of prokaryotic and eukaryotic organisms. This gene was subsequently shown to be *pcaF*, encoding β -ketoacyl-CoA thiolase (see below).

***PcaR*, a positive regulator of *pca* genes for 4-hydroxybenzoate-degradation.** A regulatory locus, *pcaR*, has been previously defined by mutational analysis and shown to be required for expression of the *pcaBDC* and *pcaIJ* gene clusters, encoding four of the enzymes of the β -ketoadipate pathway (25, 38). We determined that the *pcaR* mutant, PRS3015, was complemented by pHRP404, indicating that we had cloned the *pcaR* gene. Additional experiments have shown that pHRP404 carries a slightly truncated version of *pcaR* (44). The full-length gene encodes a protein with a predicted molecular mass of 31.8 kDa. The complete sequence of *pcaR* and experiments showing that its product functions together with the effector β -ketoacyl-CoA thiolase to regulate transcriptional expression of *pca* structural genes are the subjects of a separate communication (44).

Construction and analysis of a *pcaF* mutant, defective in β -ketoacyl-CoA thiolase. Strain PRS4061, carrying an inactivated *pcaF* gene, was constructed by mobilizing pHRP411 (Fig. 2) into wild-type *P. putida* and screening for colonies that were Tc^s Gm^r . The *pcaF* mutant was unable to grow with either benzoate or 4-hydroxybenzoate as sole carbon and energy sources. PRS4061 was also defective in the synthesis of β -ketoacyl-CoA thiolase, confirming the suggestion, on the basis of DNA sequence analysis, that *pcaF* encodes the final enzyme in the β -ketoadipate pathway. PRS2000 and PRS4061 were grown in minimal medium with 10 mM glucose (thiolase uninduced) or 10 mM glucose plus 20 mM adipate (induced) (adipate is a nonmetabolizable analog of the inducer β -keto-



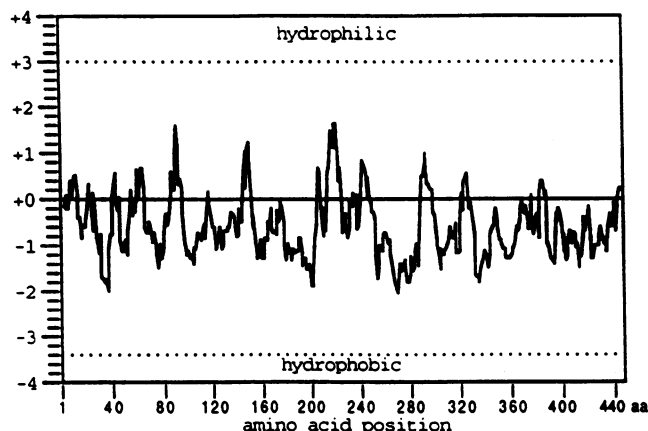


FIG. 5. Hydrophilicity plot of the deduced amino acid sequence for PcaK from *P. putida*. The algorithm of Hopp and Woods (24) was used, with a span of six amino acids.

dipate). Thiolase activity for PRS2000 was 16.1 and 111.0 nmol of β -ketoacyl-CoA disappeared per min per mg of protein, respectively, for the two culture conditions, while PRS4061 thiolase activity was 13.9 and 19.5 nmol/min/mg of protein, respectively.

Effect of the *pcaK* mutation on *P. putida* aromatic acid uptake and growth. In initial studies, the *pcaK* mutation had no obvious effect on growth of PCH722-Gm cells on plates or in liquid minimal medium supplied with benzoate or 4-hydroxybenzoate as sole growth substrates. However, since the deduced amino acid sequence of the PcaK protein indicated homology to membrane-bound transporters, we were prompted to carefully reexamine growth rates on aromatic acids and to investigate the possibility that the PcaK protein might function as a permease for these compounds. Since aromatic acids in their undissociated, but not dissociated, forms diffuse across biological membranes (26, 34), an effect of medium pH on growth might be expected if entry into cells was not catalyzed by active transport but instead depended on diffusion of free benzoic acid (pK_a 4.19) or free 4-hydroxybenzoic acid (pK_a 4.48) across the membrane (55). We therefore compared growth rates of wild-type and *pcaK* mutant cells on succinate, benzoate, and 4-hydroxybenzoate in minimal media at three different pHs. As shown in Fig. 6, wild-type and mutant cells grew equally well on succinate and benzoate at pHs ranging from 6.3 to 8.1. The *pcaK* mutant did not grow as rapidly as the wild type on 4-hydroxybenzoate at pH 8.1, however. At this pH, less than 0.05% of the available substrate would be present in the membrane-diffusible (4-hydroxybenzoic acid) form.

Washed suspensions of 4-hydroxybenzoate-grown wild-type cells accumulated ^{14}C -labeled 4-hydroxybenzoate at a linear rate for 30 s. After this time, the rate of uptake plateaued, apparently as the system became saturated with labeled substrate. *PcaK* mutant cells, by contrast, accumulated 4-hydroxybenzoate at a much lower rate, and the rate of uptake remained linear over the assay period of 3 min. In addition, rates of 4-hydroxybenzoate uptake by *pcaK* cells were slightly depressed when experiments were carried out at pH 8.0 compared with at pH 7.0. (Fig. 7).

PcaK, expressed in *E. coli*, catalyzes 4-hydroxybenzoate transport. The low rates of 4-hydroxybenzoate uptake by *pcaK* mutant cells suggested that the PcaK protein might function as a transporter. The low-level uptake that was seen could be

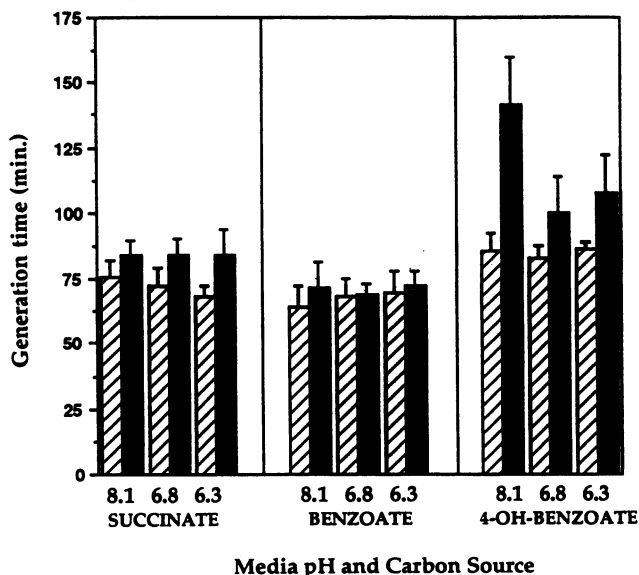


FIG. 6. Loss of PcaK has a subtle effect on growth of *P. putida*. Growth of the wild-type (PRS2000) (▨) and *pcaK* mutant (PCH722-Gm) (■) strains was determined in minimal medium with succinate, benzoate, or 4-hydroxybenzoate as the carbon source at pH 6.3, 6.8, or 8.1. Error bars are standard deviations.

accounted for by diffusion of 4-hydroxybenzoic acid across cell membranes followed by metabolism. If this is the case, then a *P. putida* double mutant blocked in the first step of 4-hydroxybenzoate metabolism and also defective in the synthesis of PcaK should have no measurable ability to accumulate 4-hydroxybenzoate. Since such a mutant was not available, we opted instead to test the function of PcaK in *E. coli*, a bacterial species that lacks the ability to grow on 4-hydroxybenzoate, benzoate, or most other aromatic compounds. As shown in Fig. 8, *E. coli* cells expressing the PcaK protein accumulated

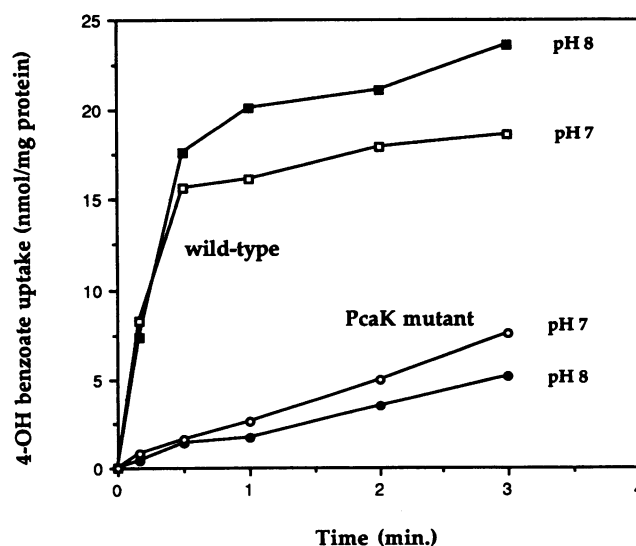


FIG. 7. Cells of the *P. putida* *pcaK* mutant strain PCH722-Gm accumulated 4-hydroxybenzoate at a substantially lower rate than wild-type cells. Results of uptake assays performed at pH 7.0 and 8.0 are shown.

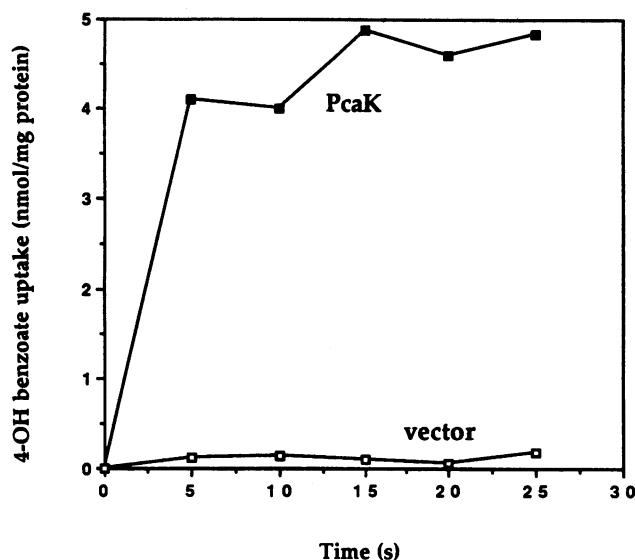


FIG. 8. The PcaK protein, when expressed in *E. coli*, catalyzes transport of 4-hydroxybenzoate. Uptake of 4-hydroxybenzoate by *E. coli* BL21(DE3)(pHNN100) is compared with uptake by *E. coli* BL21(DE3)(pT7-5).

4-hydroxybenzoate at a substantial rate. Cells that were heated at 80°C for 5 min failed to accumulate label, indicating that 4-hydroxybenzoate was not simply sticking to the surfaces of cells. In addition, labeled 4-hydroxybenzoate, extracted from whole cells and analyzed by thin-layer chromatography, was found to be unmodified, confirming that *E. coli* did not metabolize 4-hydroxybenzoate (data not shown). *E. coli* cells expressing PcaK did not accumulate benzoate over time, suggesting that the PcaK transport protein has a narrow substrate range.

Role of PcaK in aromatic acid chemotaxis. Since the *pcaK* mutant grows at wild-type rates on 4-hydroxybenzoate at neutral pH, it seemed unlikely that its chemotaxis phenotype was due to a lack of induction of the aromatic acid taxis system. To further rule out this possibility, however, we assayed expression of a *pcaI-lacZ* fusion (on plasmid pHRP150) in PCH722 and found that cells expressed similar, fully induced levels of β -galactosidase activity regardless of whether they were grown on benzoate (22,300 Miller units) or 4-hydroxybenzoate (22,600 Miller units). This indicates that the *pcaK* mutant metabolized benzoate and 4-hydroxybenzoate equally well, since β -ketoadipate, a common intermediate of benzoate and 4-hydroxybenzoate catabolism, is the inducer of *pcaI* expression (Fig. 1).

Two experiments indicate that the chemotaxis phenotype of PCH722 was due to a disruption of *pcaK* and not to a polar effect on a downstream gene. First, the *pcaF* mutant PRS4061, which has an insertion in the gene immediately downstream of *pcaK*, had a wild-type chemotaxis phenotype. When grown on glucose in the presence of 4-hydroxybenzoate, PRS4061 exhibited wild-type behavioral responses to benzoate (0.17 changes of direction per s) and 4-hydroxybenzoate (0.16 changes of direction per s) in computer-assisted chemotaxis assays. In addition, the *pcaK* gene (supplied on pHJD100) complemented PCH722-Gm *in trans* to give a wild-type chemotaxis phenotype as determined by growth in 4-hydroxybenzoate swarm plates (not shown). Therefore, PcaK, in addition to its function in transport of 4-hydroxybenzoate, has a role in chemotaxis to this compound.

DISCUSSION

With the identification of *pcaR* and *pcaF*, the full complement of genes known to regulate and encode enzymes of the protocatechuate branch of the β -ketoadipate pathway in *P. putida* has now been reported. The *pca* genes are arranged in four physically distinct clusters: *pcaHG* (11), *pcaBDC* (56), *pcaIJ* (37), and the new cluster reported here, *pcaRKF*. PcaR is required for expression of *pcaBDC* and *pcaIJ*, genes known to be inducible by the pathway intermediate, β -ketoadipate (25, 38). We do not yet know whether PcaR also regulates expression of *pcaF* and *pcaK*. With the exception of *pcaR*, all of the *pca* genes have also been cloned and sequenced from *Acinetobacter calcoaceticus*, in which they are arranged in a single *pcaIJFBDKCHG* gene cluster that is inducible by protocatechuate (28). The β -ketoadipyl-CoA thiolases (*pcaF* gene products) from *P. putida* and *A. calcoaceticus* are 62% identical over their entire lengths, and the predicted PcaK proteins from the two organisms are 57% identical at the amino acid level. The discovery of the *pcaK* genes was unexpected for both organisms. Although PcaK is presumed to be involved in aromatic compound transport in *A. calcoaceticus*, its function in this bacterium has not yet been explored.

Several lines of evidence, the most compelling of which is its functional expression in *E. coli*, indicate that the PcaK protein from *P. putida* is a transporter for 4-hydroxybenzoate. Since aromatic acids are membrane permeable, it has been argued that specific carriers for this class of compounds need not necessarily be evoked (18). However, the existence of transporters for several aromatic acids, including 4-chlorobenzoate, 4-hydroxybenzoate, and mandelate, has been inferred from physiological studies (2, 14, 23). In addition, at least three groups have described aromatic acid permease mutants. A mutant blocked in benzoate uptake has been reported for *P. putida* PRS2000 (52), a 4-hydroxyphenylacetic acid transport mutant has been described for *Klebsiella pneumoniae* (1), and mutants blocked in 4-hydroxybenzoate transport have been identified in two biovars of *Rhizobium leguminosarum* (57). All of these mutants were completely blocked in growth on the aromatic acids that the permease recognized. The *P. putida* *pcaK* mutant, by contrast, was not significantly impaired in growth on 4-hydroxybenzoate at neutral pH. It is possible that this phenotype reflects the presence of more than one transporter, or it could be that the pH gradient across the cell membrane is sufficiently steep at an external pH of 6.8 to allow diffusion of 4-hydroxybenzoic acid into cells at rates sufficient to support good growth of the *pcaK* strain. The latter interpretation is supported by the finding that a change in the pH gradient between cells and the external medium (incubation at pH 8.1) had a marked effect on growth rates. It is possible that a subtle growth phenotype, such as that seen with the *pcaK* strain, is a general property of aromatic acid transport mutants, and this may explain why relatively few transport systems for members of this class of compounds have been identified. We anticipate that an intact 4-hydroxybenzoate transport system is important for optimal growth of *P. putida* at the very low external 4-hydroxybenzoate concentrations that would be expected to occur in natural environments.

The deduced amino acid sequence of PcaK is similar to a large group of transport proteins termed the major facilitator superfamily (32). The superfamily, primarily made up of prokaryotic and eukaryotic transporters of sugars and drugs, was identified on the bases of function and a conserved secondary structure predicted to have 12 membrane-spanning α -helices. The helices are hydrophobic or amphipathic and believed to form a channel for transport through the cell

membrane (4). Specific regions of conserved amino acids were also identified in the primary sequence of the transporters (22). The Hopp-Woods hydrophilicity profile of *P. putida* PcaK (Fig. 5) indicates that it is a hydrophobic polypeptide that may indeed span the membrane 12 or more times. In addition, PcaK possesses the hallmark amino acid sequences of the superfamily (Fig. 4). Amino acids 89 to 94 (DRFGRK) match perfectly the consensus (N/D)(R/K)XGR(R/K) predicted to occur in the cytoplasmic linker between membrane-spanning helices two and three. Two additional versions of the less stringent sequence (R/K)XXX(R/K) are found in PcaK at residues 25 to 29 and 216 to 220, and the PESPR sequence of sugar transporters (22) is present as PESAR at amino acids 205 to 209. Within the superfamily of transport proteins, five clusters have been identified (32). PileUp alignment of the PcaK sequence with members from each cluster of the major facilitator superfamily placed it in cluster 1, the drug resistance protein family. It will be interesting to see whether other transporters of aromatic compounds cluster in the same group. To date, however, no other genes encoding transporters of aromatic acids or aromatic hydrocarbons have been identified.

The *pcaK* mutant is specifically nonchemotactic to 4-hydroxybenzoate and benzoate (Fig. 3); this was the basis for its initial identification. The most straightforward interpretation for this chemotaxis phenotype is that the PcaK protein is an aromatic acid chemoreceptor as well as a 4-hydroxybenzoate transporter. Two problems with this interpretation are (i) that PcaK is not homologous to known bacterial chemoreceptors and (ii) the *pcaK* mutant is, in fact, chemotactic to aromatic compounds under certain growth conditions, notably after growth on benzoate. The latter observation might be accounted for by the synthesis of two chemoreceptors for aromatic acids by *P. putida*, and it is possible that PcaK represents a new, previously unrecognized, type of chemoreceptor protein. It is also possible, however, that the PcaK protein, although intimately involved in aromatic acid chemotaxis, does not mediate direct transmission of sensory information to the central cellular chemotaxis machinery. PcaK may instead function indirectly, possibly in concert with a chemoreceptor, to influence aromatic acid-stimulated transduction of sensory information.

ACKNOWLEDGMENT

This work was supported by grant MCB 93-16257 from the National Science Foundation.

REFERENCES

- Allende, J. L., A. Gibello, M. Martin, and A. Garrido-Pertierra. 1992. Transport of 4-hydroxyphenylacetic acid in *Klebsiella pneumoniae*. Arch. Biochem. Biophys. **292**:583-588.
- Allende, J. L., M. Suarez, M. Gallego, and A. Garrido-Pertierra. 1993. 4-Hydroxybenzoate uptake in *Klebsiella pneumoniae* is driven by electric potential. Arch. Biochem. Biophys. **300**:142-147.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1990. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
- Baldwin, S. A. 1992. Mechanisms of active and passive transport in a family of homologous sugar transporters found in both eukaryotes and prokaryotes, p. 169-217. In J. J. H. M. de Pont (ed.), Molecular aspects of transport proteins. Elsevier Science Publishers, BV, Amsterdam.
- Bentley, J., L. S. Hyatt, K. Ainley, J. H. Parish, R. B. Herbert, and G. R. White. 1993. Cloning and sequence analysis of an *Escherichia coli* gene conferring bicyclomycin resistance. Gene **127**:117-120.
- Dagley, S. 1986. Biochemistry of aromatic hydrocarbon degradation in pseudomonads, p. 527-556. In J. R. Sokatch (ed.), The bacteria, vol. 10. The biology of *Pseudomonas*. Academic Press, Inc., Orlando.
- Davis, R. W., D. Botstein, and J. R. Roth. 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387-395.
- DiMarco, A. A., B. Averhoff, and L. N. Ornston. 1993. Identification of the transcriptional activator *pobR* and characterization of its role in the expression of *pobA*, the structural gene for *p*-hydroxybenzoate hydroxylase in *Acinetobacter calcoaceticus*. J. Bacteriol. **175**:4499-4506.
- Dispensa, M., R. E. Parales, and C. S. Harwood. 1989. Regulation of benzoate chemotaxis in *Pseudomonas putida*, K180. Abstr. 89th Annu. Meet. Am. Soc. Microbiol. 1989. American Society for Microbiology, Washington, D.C.
- Frazee, R. W., D. M. Livingston, D. C. LaPorte, and J. D. Lipscomb. 1993. Cloning, sequencing, and expression of the *Pseudomonas putida* protocatechuate 3,4-dioxygenase genes. J. Bacteriol. **175**:6194-6202.
- Gerhardt, P., R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.). 1981. Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
- Gray, K. M., and E. P. Greenberg. 1992. Physical and functional maps of the luminescence gene cluster in an autoinducer-deficient *Vibrio fischeri* strain isolated from a squid light organ. J. Bacteriol. **174**:4384-4390.
- Groenewegen, P. E. J., A. J. M. Driessen, W. N. Konings, and J. A. M. de Bont. 1990. Energy-dependent uptake of 4-chlorobenzoate in the coryneform bacterium NTB-1. J. Bacteriol. **172**:419-423.
- Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557-580.
- Harayama, S., M. Kok, and E. L. Neidle. 1992. Functional and evolutionary relationships among diverse oxygenases. Annu. Rev. Microbiol. **46**:565-602.
- Harwood, C. S., K. Fosnaugh, and M. Dispensa. 1989. Flagellation of *Pseudomonas putida* and analysis of its motile behavior. J. Bacteriol. **171**:4063-4066.
- Harwood, C. S., and J. Gibson. 1986. Uptake of benzoate by *Rhodospseudomonas palustris* grown anaerobically in light. J. Bacteriol. **165**:504-509.
- Harwood, C. S., R. E. Parales, and M. Dispensa. 1990. Chemotaxis of *Pseudomonas putida* toward chlorinated benzoates. Appl. Environ. Microbiol. **56**:1501-1503.
- Harwood, C. S., M. Rivelli, and L. N. Ornston. 1984. Aromatic acids are chemoattractants for *Pseudomonas putida*. J. Bacteriol. **160**:622-628.
- Hattori, M., and Y. Sakaki. 1986. Dideoxy sequencing method using denatured plasmid templates. Anal. Biochem. **152**:232-238.
- Henderson, P. J. F. 1990. The homologous glucose transport proteins of prokaryotes and eukaryotes. Res. Microbiol. **141**:316-328.
- Higgins, S. J., and J. Mandelstam. 1972. Evidence for induced synthesis of an active transport factor for mandelate in *Pseudomonas putida*. Biochem. J. **126**:917-922.
- Hopp, T. P., and K. R. Woods. 1981. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA **78**:3824-3828.
- Hughes, E. J., M. K. Shapiro, J. E. Houghton, and L. N. Ornston. 1988. Cloning and expression of *pca* genes from *Pseudomonas putida* in *Escherichia coli*. J. Gen. Microbiol. **134**:2877-2887.
- Kashket, E. R. 1982. Stoichiometry of the H⁺-ATPase of growing and resting, aerobic *Escherichia coli*. Biochemistry **21**:5534-5538.
- Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger. 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70**:191-197.
- Kowalchuk, G. A., G. B. Hartnett, A. Benson, J. E. Houghton, K.-L. Ngai, and L. N. Ornston. 1994. Contrasting patterns of evolutionary divergence within the *Acinetobacter calcoaceticus* *pca* operon. Gene **146**:23-30.
- Lee, S.-Y., and S. Rasheed. 1990. A simple procedure for maximum yield of high-quality plasmid DNA. BioTechniques **9**:676-679.

30. Locher, H. H., B. Poolman, A. M. Cook, and W. N. Konings. 1993. Uptake of 4-toluene sulfonate by *Comamonas testosteroni* T-2. *J. Bacteriol.* **175**:1075–1080.
31. Lopez-de-Victoria, G., and C. R. Lovell. 1993. Chemotaxis of *Azospirillum* species to aromatic compounds. *Appl. Environ. Microbiol.* **59**:2951–2955.
32. Marger, M. D., and M. H. Saier, Jr. 1993. A major superfamily of transmembrane facilitators that catalyze uniport, symport, and antiport. *Trends Biochem. Sci.* **18**:13–19.
33. Needleman, S. B., and C. D. Wunsch. 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. *J. Mol. Biol.* **48**:443–452.
34. Nicholls, D. G. 1982. *Bioenergetics*. Academic Press, Inc., London.
35. Ornston, L. N., J. Houghton, E. L. Neidle, and L. A. Gregg. 1990. Subtle selection and novel mutation during evolutionary divergence of the β -ketoadipate pathway, p. 207–225. In S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
36. Ornston, L. N., and D. Parke. 1976. Properties of an inducible uptake system for β -ketoadipate in *Pseudomonas putida*. *J. Bacteriol.* **125**:475–488.
37. Parales, R. E., and C. S. Harwood. 1992. Characterization of the genes encoding β -ketoadipate:succinyl-coenzyme A transferase in *Pseudomonas putida*. *J. Bacteriol.* **174**:4657–4666.
38. Parales, R. E., and C. S. Harwood. 1993. Regulation of the *pcaII* genes for aromatic acid degradation in *Pseudomonas putida*. *J. Bacteriol.* **175**:5829–5838.
39. Parke, D., L. N. Ornston, and E. W. Nester. 1987. Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti plasmid in *Agrobacterium tumefaciens*. *J. Bacteriol.* **169**:5336–5338.
40. Parke, D., M. L. Rivelli, and L. N. Ornston. 1985. Chemotaxis to aromatic and hydroaromatic acids: comparison of *Bradyrhizobium japonicum* and *Rhizobium trifolii*. *J. Bacteriol.* **163**:417–422.
41. Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* **85**:2444–2448.
42. Perrotta, J. A., and C. S. Harwood. 1994. Anaerobic metabolism of cyclohex-1-ene-1-carboxylate, a proposed intermediate of benzoate degradation, by *Rhodopseudomonas palustris*. *Appl. Environ. Microbiol.* **60**:1775–1782.
43. Pridmore, R. D. 1987. New and versatile cloning vectors with kanamycin-resistance marker. *Gene* **56**:301–312.
44. Romero-Steiner, S., R. E. Parales, C. S. Harwood, and J. E. Houghton. 1994. Characterization of the *pcaR* regulatory gene from *Pseudomonas putida*, which is required for the complete degradation of *p*-hydroxybenzoate. *J. Bacteriol.* **176**:5771–5779.
45. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
46. Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technology* **1**:784–789.
47. Simon, R., J. Quandt, and W. Klipp. 1989. New derivatives of transposon Tn5 suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. *Gene* **80**:161–169.
48. Stanier, R. Y., and L. N. Ornston. 1973. The β -ketoadipate pathway. *Adv. Microbiol. Physiol.* **9**:89–151.
49. Stewart, G. S. A. B., S. Lubinsky-Mink, C. G. Jackson, A. Cassel, and J. Kuhn. 1986. pHG165: a pBR322 copy number derivative of pUC8 for cloning and expression. *Plasmid* **15**:172–181.
50. Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**:60–89.
51. Tabor, S., and C. C. Richardson. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Gene* **82**:1074–1078.
52. Thayer, J. R., and M. L. Wheelis. 1976. Characterization of a benzoate permease mutant of *Pseudomonas putida*. *Arch. Microbiol.* **110**:37–42.
53. van der Meer, J. R., W. M. deVos, S. Harayama, and A. J. B. Zehender. 1992. Molecular mechanisms of genetic adaptation to xenobiotic compounds. *Microbiol. Rev.* **56**:677–694.
54. Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertional mutagenesis and sequencing with synthetic oligonucleotides. *Gene* **19**:259–268.
55. Weast, R. C. (ed.). 1988. *Handbook of chemistry and physics*, 1st student ed., p. D101–D102. CRC Press, Inc., Boca Raton, Fla.
56. Williams, S. E., E. M. Woolridge, S. C. Ransom, J. A. Landro, P. C. Babbitt, and J. W. Kozarich. 1992. 3-Carboxy-cis,cis-muconate lactonizing enzyme from *Pseudomonas putida* is homologous to the class II fumarase family: a new reaction in the evolution of a mechanistic motif. *Biochemistry* **31**:9768–9776.
57. Wong, C. M., M. J. Dilworth, and A. R. Glenn. 1991. Evidence for two uptake systems in *Rhizobium leguminosarum* for hydroxyaromatic compounds metabolized by the 3-oxoadipate pathway. *Arch. Microbiol.* **156**:385–391.
58. Zhang, C.-C., M.-C. Durand, R. Jeanjean, and F. Joset. 1989. Molecular and genetical analysis of the fructose-glucose transport system in the cyanobacterium *Synechocystis* PCC6803. *Mol. Microbiol.* **3**:1221–1229.