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PcaK, a High-Affinity Permease for the Aromatic Compounds 4-Hydroxybenzoate and Protocatechuate from *Pseudomonas putida*

NANCY N. NICHOLS AND CAROLINE S. HARWOOD*

Department of Microbiology, University of Iowa, Iowa City, Iowa 52242

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PcaK is a transporter and chemoreceptor protein from *Pseudomonas putida* that is encoded as part of the β-ketoadipate pathway regulon for aromatic acid degradation. When expressed in *Escherichia coli*, PcaK was localized to the membrane and catalyzed the accumulation of two aromatic substrates, 4-hydroxybenzoate and protocatechuate, against a concentration gradient. Benzoate inhibited 4-hydroxybenzoate uptake but was not a substrate for PcaK-catalyzed transport. A *P. putida pcaK* mutant was defective in its ability to accumulate micromolar amounts of 4-hydroxybenzoate and protocatechuate. The mutant was also impaired in growth on millimolar concentrations of these aromatic acids. In contrast, the *pcaK* mutant grew at wild-type rates on benzoate. The V_{max} for uptake of 4-hydroxybenzoate was at least 25 nmol/min/mg of protein, and the K_m was 6 μM. PcaK-mediated transport is energized by the proton motive force. These results show that although aromatic acids in the undissociated (uncharged) form can diffuse across bacterial membranes, high-specificity active transport systems probably also contribute to the ability of bacteria to grow on the micromolar concentrations of these compounds that are typically present in soil. A variety of aromatic molecules, including naturally occurring lignin derivatives and xenobiotics, are metabolized by bacteria and may be substrates for transport proteins. The characterization of PcaK provides a foundation for understanding active transport as a critical step in the metabolism of aromatic carbon sources.

Aromatic acids, present in soil as degradation products of plant material, are used as carbon and energy sources by many microorganisms. A battery of enzymes is required for aromatic compound degradation, and it seems likely that a corresponding array of transport proteins initiates metabolism. Aromatic acids can diffuse across biological membranes (23), making transport theoretically unnecessary. However, accumulating evidence indicates that active transport of this group of compounds may be widespread among bacteria (1, 2, 14, 15, 28, 30, 38). Recently, the molecular basis for aromatic acid transport has begun to be examined.

A permease, designated PcaK, was identified in *Pseudomonas putida* as a transporter of the aromatic acid 4-hydroxybenzoate (4-HBA) (15). PcaK is a member of the major facilitator superfamily (MFS) (13, 29) of transport proteins. Like other MFS permeases, PcaK has 12 predicted membrane-spanning regions, with several conserved amino acid residues in the hydrophilic loop between the second and third membrane-spanning segments (15, 19, 20). Previous work (32) has shown that the *pcaK* gene is regulated coordinately with genes encoding enzymes of the β-ketoadipate pathway, the pathway by which *P. putida* degrades 4-HBA (Fig. 1) (16).

PcaK is unusual among MFS permeases because it is a dual-function protein. In addition to acting as a transporter, PcaK plays a role in chemotaxis to 4-HBA and other aromatic acids (15). The exact function of PcaK in chemotaxis has not yet been determined. It is not known, for example, whether transport is required for chemotaxis and if PcaK physically interacts with other cellular proteins to initiate chemosensory signal transduction. Studies of PcaK-mediated transport of aromatic acids will establish a basis for exploring the novel role of PcaK in chemotaxis. They should also provide a basis for understanding how bacteria transport structurally diverse aromatic compounds from natural or industrial sources to initiate metabolism.

Escherichia coli does not grow on most aromatic acids and does not accumulate 4-HBA. It thus provides a background in which to study 4-HBA transport separate from metabolism. Here we studied PcaK expressed in *E. coli* to define the kinetics, energy requirement, and substrate specificity of aromatic acid transport. We also examined the growth and transport characteristics of a *pcaK* mutant of *P. putida.*

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Bacterial strains and plasmids are listed in Table 1. Deletion of *unc* genes in the T7 expression strain BL21(DE3) was accomplished by phage P1 transduction (37) from the well-characterized *unc* mutant *E. coli* TA3952 (21) and selection for tetracycline resistance encoded on a closely linked Tn*10*. The constructed ATPase mutant had ATP levels upon starving, reenergizing with succinate or glucose, and treatment with uncouplers and inhibitors that were similar to values reported for the parent *unc* strain (21).

P. putida was grown at 30°C in defined mineral medium (minimal medium [25 mM KH_2PO_4, 25 mM Na_2HPO_4, 0.1% $(NH_4)_2SO_4$, 1% Hutner mineral base (12) (final pH, 6.8)]. Gentamicin and kanamycin were used at 5 and 100 μg/ml, respectively. Aromatic carbon sources were sterilized separately and added to media at a final concentration of 5 mM. *P. putida* growth studies were performed as described elsewhere (15) in minimal medium adjusted to pH 6.3, 6.8, or 8.1. *E. coli* was cultured on LB medium (3) at 37°C, supplemented when appropriate with ampicillin (100 μg/ml) and tetracycline (20 μg/ml). For expression of PcaK in *E. coli* from a T7 promoter (39, 40), an overnight culture was diluted into 50 ml of fresh medium and incubated aerobically at 37°C. At an optical density (A_{660}) of 0.25 (approximately 10^8 cells/ml), cultures were induced by addition of 100 μM isopropylthiogalactopyranoside (IPTG). Incubation was continued at 37°C until the optical density of the cultures had approximately doubled. *E. coli* BL21(DE3)*unc*Δ*702*(pHNN100) was grown for 4-HBA uptake assays in LB containing 10 mM glucose.

[^{35}S]methionine labeling of PcaK. Cells were harvested from a 1-ml culture of BL21(DE3)(pHNN100) at an A_{660} of approximately 0.25. Pellets were washed twice in 0.5 ml M9 medium (3) and suspended in 1.0 ml of M9 containing 0.02% each of 18 amino acids (no cysteine or methionine). After incubation at 37°C for

* Corresponding author. Mailing address: Department of Microbiology, University of Iowa, Iowa City, IA 52242. Phone: (319) 335-7783. Fax: (319) 335-7679. E-mail: caroline-harwood@uiowa.edu.

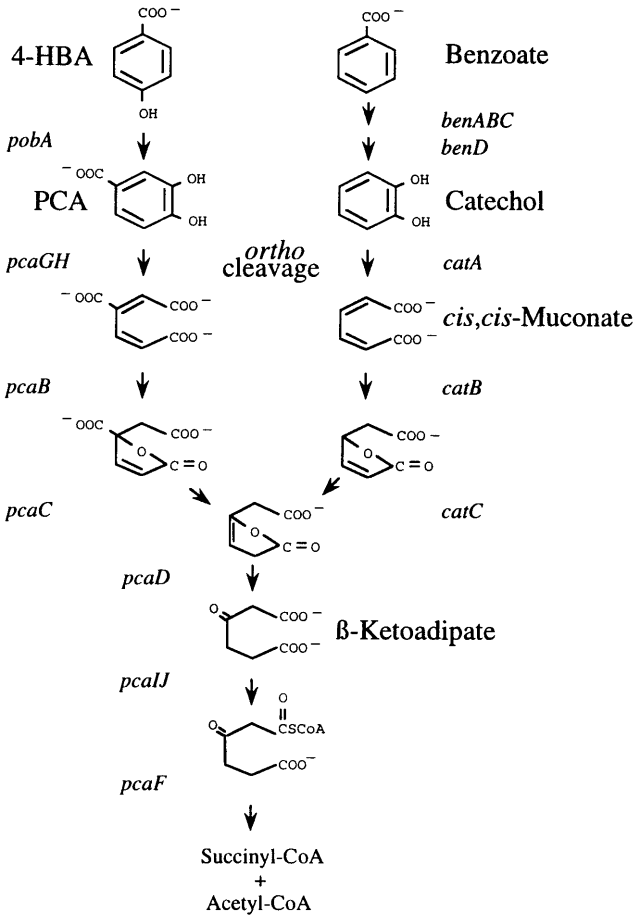

FIG. 1. *ortho* ring cleavage of 4-HBA and benzoate via the β-ketoadipate pathway in *P. putida*. The genes encoding pathway enzymes and the names of some intermediates are given.

30 min, the culture was induced with 0.4 mM IPTG and incubated for an additional 30 min. Rifampin (from a freshly prepared stock solution in methanol) was added to a final concentration of 0.5 mg/ml, and the cells were incubated for 60 min at 37°C. L-[^{35}S]methionine (10 μCi) was added, and the incubation continued for 5 min. Cells were collected by centrifugation for membrane preparation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (3) on 12.5% acrylamide–0.33% bisacrylamide gels.

Separation of membrane and cytoplasmic fractions. *E. coli* cells expressing ^{35}S-labeled PcaK were suspended in 1.0 ml of buffer containing 10 mM Tris (pH 7.4), 0.25 M NaCl, 5 mM phenylmethylsulfonyl fluoride, and 5 mM ε-aminocaproate and then disrupted by sonication. Whole cells were removed by low-speed centrifugation, and the supernatant was subjected to either ultracentrifugation or detergent phase separation.

The cell extract was ultracentrifuged for 90 min at 200,000 × *g*, and the resulting membrane pellet was stored at −20°C. The cytoplasmic supernatant fraction was mixed with 4 volumes of acetone and placed at −70°C for 30 min to precipitate proteins. The acetone-treated extracts were then centrifuged for 15 min at 15,000 × *g*, and the pellet was stored at −20°C for subsequent analysis by SDS-PAGE.

For detergent phase separation (5), Triton X-114 (0.5%) was added to 200 μl of the sonicated extract, and the mixture was placed on ice. The extract-detergent mixture was then layered over 300 μl of 6% sucrose, 10 mM Tris (pH 7.4)–150 mM NaCl–0.06% Triton X-114 in a microcentrifuge tube. The mixture was incubated for about 3 min at 30°C (until it became cloudy) and then was centrifuged for 3 min at 300 × *g* in a horizontal fixed-angle microcentrifuge. The aqueous (upper) phase that formed during centrifugation was removed from the detergent (bottom) phase and treated as described above with an additional 0.5% Triton X-114. The aqueous fraction was treated a third time with Triton X-114 (2%). Proteins remaining in the aqueous phase were precipitated with an equal volume of isopropanol. The detergent fractions from the first two Triton X-114 additions were pooled and diluted with 50 mM Tris (pH 7.4). The detergent fraction (containing membrane proteins) and aqueous fraction (containing cytoplasmic proteins) were stored at −20°C until they were analyzed by SDS-PAGE.

Uptake assays. Solute transport was assayed at 22°C in *E. coli* expressing recombinant PcaK or in mid-log-phase *P. putida* grown on 4-HBA. Cells were harvested by low-speed centrifugation, washed in an equal volume of phosphate buffer (25 mM KH_2PO_4, 25 mM Na_2HPO_4 [pH 6.8]), and resuspended in phosphate buffer at an A_{660} of 5 to 10. Cell suspensions were gently aerated until the time of assay to prevent oxygen limitation. [^{14}C]Protocatechuate (PCA) and [^{14}C]benzoate were supplied at 170 and 90 μM, respectively. [^{14}C]4-HBA was used at 5 μM in substrate competition experiments and 65 μM in other assays. Except for experiments using starved or inhibitor-treated cells, the reaction mixture for uptake assays contained 10 mM glucose and 10 mM succinate. Uptake was initiated by diluting cells into an equal volume of phosphate buffer containing the substrate at pH 6.8. At timed intervals, 0.1-ml samples were removed from the reaction and filtered through Nuclepore polycarbonate membranes (0.2-μm pore size; Costar Corp., Cambridge, Mass.). The filters were washed before and after addition of sample with 2 ml of phosphate buffer. For uptake assays performed at increased pH, cells were suspended and assayed, and the filters were washed in phosphate buffer adjusted to pH 7.5 or 7.9. Accumulated substrate was determined by scintillation counting of the cells retained on the filters.

In some experiments, after incubation with substrate, samples were centrifuged through silicone oil (24) in lieu of filtration and the subsequent aqueous wash. In a 250-μl tube, 100 μl of silicone oil [Versilube F(50); General Electric Co., Waterford, N.Y.] was layered over 10 μl of water containing 10% glycerol. Uptake was initiated by adding 50 μl of cells to 50 μl of buffer containing radioactive substrate in a discrete layer on top of the oil. After 1 min, the tubes were centrifuged for 2 min at 13,000 × *g* in a horizontal fixed-angle microcentrifuge. Accumulation of substrate was measured by scintillation counting of the cell pellet, which was collected in the aqueous drop at the bottom of the tube.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
P. putida		
PRS2000	Wild type	34
PRS3000	Km^r *pcaGH*::Tn*5* 4-HBA$^-$	L. N. Ornston
PCH722-Gm	Gm^r *pcaK*::Tn*5*-B30/Gm, nonchemotactic to 4-HBA, complemented in *trans* by *pcaK*	15
E. coli		
BL21(DE3)	*hsdS gal* (λ*c*I*ts*857 *ind-1 S*am7 *nin-5 lacUV5*-T7 gene *1*)	39
TA3952	Tc^r, donor strain for P1 transduction of ATPase mutation, *his*Δ*461 dhuA1 unc*Δ*702 asnA*::Tn*10*	21
BL21(DE3)*unc*Δ*702*	Tc^r, ATPase mutant, BL21(DE3) *unc*Δ*702 asnA*::Tn*10*	This study
Plasmids		
pHNN100	Ap^r, *pcaK* cloned downstream of T7 promoter of pT7-5	15
pT7-5	Ap^r, T7 promoter expression vector	40

[a] 4-HBA$^-$, no growth on 4-HBA; Ap^r, ampicillin resistant; Gm^r, gentamicin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant.

Cellular starvation and inhibitor treatments. Cellular energy stores were depleted by washing and suspending cells in an equal volume of minimal medium [in which $(NH_4)_2SO_4$ was replaced with Na_2SO_4] for approximately 18 h at 37°C in a shaking incubator. Starved cells were washed and suspended in phosphate buffer and incubated at 22°C with an inhibitor: 10 mM potassium cyanide (KCN) for 5 min, 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) for 1 min, or 5 mM dinitrophenol (DNP) for 1 min. CCCP and DNP stock solutions were dissolved in ethanol, and ethanol was used as a negative control. After inhibitor treatment, cells were incubated with phosphate buffer or an energy source (10 mM glucose or succinate) for an additional 15 min at 22°C. Two aliquots of cells from a starved culture were prepared for each experimental condition and assayed for either 4-HBA uptake or ATP content.

Intracellular ATP measurements. Cellular ATP was measured in parallel with 4-HBA uptake assays. Cells in phosphate buffer were added to a microcentrifuge tube containing 9 volumes of boiling 10 mM Tris (pH 7.8). The mixture was boiled in a water bath for 3 min, chilled on ice, and stored at −20°C until assay. ATP content was determined by using an ATP luminescence assay kit (Sigma, St. Louis, Mo.) and Lucy 2 automated luminometer (Anthos Labtec Instruments, Salzburg, Austria).

Synthesis of ^{14}C-labeled PCA. [*ring*-UL-^{14}C]PCA was synthesized from [*ring*-UL-^{14}C]4-HBA by *P. putida* strain PRS3000, a PCA dioxygenase mutant that converts 4-HBA to PCA but is blocked in further metabolism (Fig. 1). PRS3000 was grown to early log phase in 500 ml of minimal medium containing 10 mM glucose and 1 mM 4-HBA. Cells were harvested, washed, and resuspended in 20 ml of minimal medium containing 20 mM glucose and 10 μCi of [^{14}C]4-HBA. The 4-HBA concentration was adjusted to 0.5 mM with unlabeled 4-HBA, and the suspension was incubated for 18 h at 30°C in a shaking incubator. Conversion of 4-HBA to PCA was confirmed by high-pressure liquid chromatography analysis of culture supernatant as previously described (32). Bacterial cells were removed from the medium by centrifugation followed by filtration through a syringe filter (pore size, 0.22 μm). The filtrate was acidified to pH 1.5, and the radiolabeled product was extracted into ethyl acetate, dried, and resuspended in sterile water. The specific activity of the synthesized [^{14}C]PCA was 1 mCi/mmol.

Protein determinations. Whole cells were precipitated by the addition of 5% trichloroacetic acid and then boiled in 0.1 M NaOH for 10 min. Protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the standard.

Radiochemicals. L-[^{35}S]methionine (83 Ci/mmol), [*ring*-UL-^{14}C]benzoate (13.3 mCi/mmol), and [*ring*-UL-^{14}C]4-HBA (33 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, Ill.).

RESULTS

PcaK is a membrane protein. Sonicated extracts of *E. coli* expressing ^{35}S-labeled PcaK protein were ultracentrifuged to separate bacterial membranes from the cytoplasmic fraction. When cellular fractions were analyzed by SDS-PAGE and autoradiography, PcaK was enriched in the membrane fraction (Fig. 2). In addition, PcaK was found in the detergent phase of cell extracts treated with Triton X-114 by a method demonstrated to extract integral membrane proteins (5) (Fig. 2). The localization of PcaK to the *E. coli* membrane is consistent with the hydrophobic amino acid composition of this protein (15).

PcaK transports 4-HBA and PCA but not benzoate. A number of compounds, including aromatic amino acids as well as precursors and intermediates of the β-ketoadipate pathway, were tested for the ability to inhibit transport of radiolabeled 4-HBA by *E. coli* cells expressing PcaK. In a control experiment, a 20-fold excess of unlabeled 4-HBA inhibited the rate of transport of radiolabeled 4-HBA by 87%. The aromatic compounds benzoate and PCA, which are both metabolized by *P. putida* through the β-ketoadipate pathway (Fig. 1), inhibited transport of 4-HBA by 84 and 79%, respectively, when present in 20-fold excess. 3-Hydroxybenzoate and some halogenated benzoates also partially inhibited uptake, but catechol and the aromatic amino acids tyrosine and phenylalanine did not significantly inhibit accumulation of 4-HBA (Table 2).

When radiolabeled PCA and benzoate were used as sole substrates in transport assays, *E. coli* cells expressing PcaK accumulated ^{14}C-labeled PCA to levels similar to those observed for 4-HBA. In contrast, uptake of benzoate was not detected (Fig. 3). Thus, although benzoate can inhibit 4-HBA uptake, this aromatic acid apparently is not a substrate for PcaK-catalyzed transport. Because benzoate is less polar than

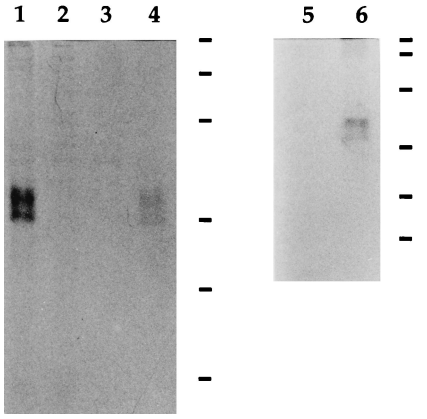

FIG. 2. PcaK expressed in *E. coli* BL21(DE3) is localized to the bacterial membrane. An autoradiograph of SDS-PAGE-separated cells and cellular fractions is shown. Lanes: 1, SDS-solubilized cells expressing [^{35}S]methionine-labeled PcaK; 2, solubilized cells carrying vector only; 3 and 4, cytoplasmic supernatant and membrane pellet, respectively, from ultracentrifuged extracts; 5 and 6, aqueous and detergent phases, respectively, from Triton X-114-fractionated extracts (see text). The amount of protein loaded in lane 1 (approximately 40 μg) was equal to the amount loaded in lane 2 and approximately threefold greater than that loaded in lanes 3 plus 4 and in lanes 5 plus 6. The positions of molecular mass standards (106, 80, 49.5, 32.5, 27.5, and 18.5 kDa) are shown to the right of each panel.

4-HBA and PCA, it seemed possible that active transport of benzoate would not be detected in *E. coli* if the substrate rapidly diffuses out of cells. To address this question, in some uptake assays, centrifugation through silicone oil replaced filtration and the subsequent aqueous wash. In this protocol, bacterial cells pass through the oil and are stripped of the surrounding medium (24). The results (not shown) were similar to those in the standard uptake assay: 4-HBA, but not benzoate, was accumulated by *E. coli* cells expressing PcaK.

When uptake was measured in *P. putida*, the *pcaK* mutant PCH722-Gm was impaired in accumulation of both 4-HBA and PCA relative to the wild-type strain PRS2000 (Fig. 4).

Growth studies. The results of growth studies with a *pcaK* mutant of *P. putida* were consistent with data from uptake assays (Table 3). The *pcaK* mutant PCH722-Gm had a longer doubling time than the wild-type strain on 4-HBA (pK_a 4.48) (27). The slow-growth phenotype was more pronounced at pH

TABLE 2. Substrate inhibition of PcaK-mediated 4-HBA uptake in *E. coli*

Competing substrate[a]	% Inhibition of 4-HBA uptake[b]
4-HBA	87 ± 4
Benzoate	84 ± 7
PCA	78 ± 8
4-Fluorobenzoate	73 ± 11
3-Hydroxybenzoate	64 ± 2
3-Fluorobenzoate	63 ± 13
3-Chlorobenzoate	62 ± 14

[a] The concentrations of 4-HBA and competing substrate were 5 and 100 μM, respectively.

[b] The rate of accumulation of 4-HBA (5 μM, in the absence of inhibitor) was 6.1 ± 0.5 nmol/min/mg of protein. Inhibition was determined by comparing the rate of 4-HBA uptake in the absence and presence of competing substrate. Values are the averages from three to six experiments ± standard deviations. The following compounds inhibited uptake less than 20%: anthranilate, benzene, catechol, 4-chlorobenzoate, *cis*,*cis*-muconate, 2,6-difluorobenzoate, 3,4-difluorobenzoate, β-ketoadipate, phenylalanine, quinate, salicylate, shikimate, succinate, *p*-toluate, tyrosine, and vanillate.

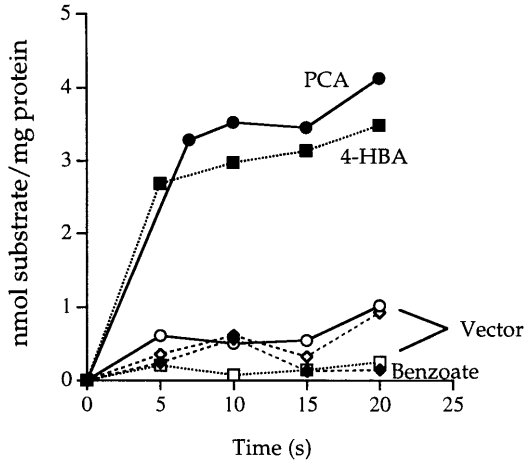

FIG. 3. PcaK transports 4-HBA (■) and PCA (●) but not benzoate (◆). Transport of the three aromatic acids was assayed in *E. coli* BL21(DE3)(pHNN100). A negative control (*E. coli* containing the cloning vector pT7-5) for each substrate is represented by the corresponding open symbol.

8.1. At this pH, less than 0.1% of the total substrate (5 mM) would be present as 4-hydroxybenzoic acid, the undissociated form that diffuses across membranes. By contrast, the *pcaK* mutant grew at wild-type rates on benzoate (pK_a 4.19) even at pH 8.1, indicating that PcaK is not important for acquisition of benzoate by *P. putida*. The *pcaK* mutant was markedly impaired in growth on PCA (pK_a 4.48), with a significantly longer doubling time on PCA, even at a slightly acidic pH (Table 3). The compromised growth of the mutant on PCA is further evidence that this compound, in addition to 4-HBA, is transported by PcaK.

Transport kinetics. The kinetics of 4-HBA transport were measured in *E. coli* cells expressing PcaK. Because uptake saturates quickly (Fig. 3), the initial rate of 4-HBA transport was difficult to determine accurately. Based on the earliest time points, the V_{max} of 4-HBA transport is at least 25 nmol/min/mg of protein, and the K_m is 6 μM. From the minimum saturating concentration of 4-HBA (10 μM) and the total accumulation of 4-HBA in whole cells (Fig. 3), we estimate that PcaK concentrates the substrate in *E. coli* 30- to 60-fold.

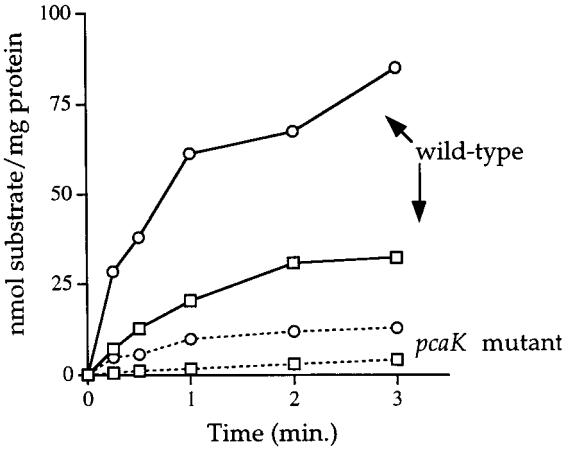

FIG. 4. Uptake of aromatic acids by *P. putida* PRS2000 (wild type) and PCH722-Gm (*pcaK* mutant). Cells grown on 4-HBA were assayed for transport of 4-HBA (□) and PCA (○).

Transport is energized by the proton motive force (PMF). The energy dependence of PcaK-mediated transport is illustrated in Fig. 5. Cells that were depleted of intracellular energy by starvation did not transport 4-HBA, whereas cells that were not starved, and starved cells resupplied with glucose, accumulated 4-HBA to high levels. Addition of the uncoupling agent DNP resulted in immediate efflux of substrate.

The source of energy for transport was determined by constructing an ATPase mutant of the T7 expression strain BL21(DE3), in which the two main cellular forms of energy, the PMF and ATP, cannot be interconverted. When given succinate, ATPase mutants generate a PMF which cannot be converted to ATP. However, a fermentable carbon source such as glucose is metabolized to generate both ATP (by substrate-level phosphorylation) and a PMF (21). Thus, in an ATPase mutant, the form of cellular energy can be manipulated by starving the cells to deplete endogenous energy reserves and then providing succinate or glucose, alone or in combination with an inhibitor.

Transport of 4-HBA did not depend on cellular ATP. Intracellular ATP measurements showed that ATPase mutant cells, induced for PcaK expression and depleted of energy reserves by starvation, could use glucose but not succinate to generate ATP (Table 4). In uptake assays, however, the same cells could use either glucose or succinate to energize 4-HBA accumulation. Cells treated with CCCP or DNP (agents that dissipate the electrochemical gradient across cell membranes) or with KCN (which inhibits electron transport) had an ATP content similar to that of untreated cells (Table 4). However, such cells did not transport 4-HBA. These results show that 4-HBA transport does not correlate with intracellular ATP levels and point to the PMF as the driving force for 4-HBA transport. *E. coli* cells expressing PcaK accumulated 4-HBA at approximately equal rates at pH 6.8, 7.5, and 7.9 (not shown). This result indicates that the $\Delta\Psi$ component of the PMF can energize transport and that a ΔpH is not required.

DISCUSSION

Although aromatic acids can enter cells by passive diffusion, active transport increases the efficiency and rate of substrate acquisition and thus may impart a growth advantage in natural environments where these compounds are present at low (micromolar) concentrations (41, 43–45). The experiments described here show that the permease PcaK is important for growth by *P. putida* on the two aromatic acids 4-HBA and PCA. Although a number of aromatic compounds inhibit transport (Table 2), PcaK does not act as a general transporter for aromatic molecules and appears to be specific to 4-HBA and PCA. Consequently, if other aromatic acids such as benzoate enter cells by active transport, *P. putida* apparently uses distinct carriers for those compounds. In contrast to the experiments described here, the *Acinetobacter calcoaceticus* PcaK homolog does transport benzoate and has overlapping but broader substrate specificity compared to the *P. putida* PcaK protein (6, 7).

Metabolism of 4-HBA and PCA in *P. putida* (Fig. 1) probably accounts for the accumulation of substrates that occurs in the *pcaK* mutant and for the higher rate of uptake in *P. putida* compared to *E. coli* (Fig. 3 and 4). It is also possible that *P. putida* has a second permease for the same substrates that is active in the mutant strain. Alternatively, aromatic substrates may cross the outer membrane into cells or efflux from cells at different rates in *P. putida* and *E. coli*. We have previously shown that *E. coli* does not modify or metabolize 4-HBA following its transport in the PcaK expression strain (15).

TABLE 3. Effects of pH on growth rates of wild-type *P. putida* cells and the *pcaK* mutant PCH722-Gm[a]

Strain	Carbon source[a]	Avg doubling time (min) ± SD at medium pH of:		
		6.3	6.8	8.1
PRS2000 (wild type)	4-HBA	86 ± 3	83 ± 5	85 ± 7
	Benzoate	70 ± 8	68 ± 7	64 ± 9
	PCA	62 ± 4	66 ± 3	79 ± 9
PCH722-Gm (*pcaK*)	4-HBA	107 ± 14	100 ± 13	141 ± 19
	Benzoate	72 ± 6	69 ± 4	72 ± 10
	PCA	128 ± 12	136 ± 12	169 ± 27

[a] The values for 4-HBA and benzoate are similar to previous results (15).

TABLE 4. 4-HBA transport and ATP content in an *E. coli* ATPase mutant[a] expressing PcaK

Starved-cell treatment[b]	% of value for unstarved cells[c]	
	4-HBA uptake	ATP content
None	18	14
Glucose	104	97
Succinate	74	30
CCCP-glucose	18	103
KCN-glucose	43	95
DNP-glucose	29	135

[a] *E. coli* BL21(DE3)*unc*Δ*702*(pHNN100) (Table 1).
[b] Described in Materials and Methods.
[c] Values for cells before starvation were considered 100%. The reference rate for 4-HBA uptake is 22.1 ± 2.4 nmol of 4-HBA accumulated per min per mg of protein; 100% ATP is 3.7 ± 1.0 nmol of ATP per mg of bacterial protein. Values are average results from three to six separate experiments, except for the KCN-glucose sample, which was assayed twice.

Physiological evidence for active transport of aromatic compounds has been found in other bacteria (1, 2, 14, 28, 30), and candidate genes have been identified as encoding probable transporters of other aromatic compounds. A *pcaK* homolog has been found in *A. calcoaceticus* (25), and *mopB* from *Burkholderia cepacia* was recently shown to encode a 4-methylphthalate transporter (38). Other genes that may encode transporters of aromatic compounds (and their probable substrate) include *benK* (benzoate) (8) and *vanK* (vanillate) (9) from *A. calcoaceticus*, *tfdK* (2,4-dichlorophenoxyacetate) from *Ralstonia eutrophus* (42), *hppK* and *mhpT* (3-hydroxyphenylpropionate) from *Rhodococcus globerulus* (4) and *E. coli* (11), respectively, *hpaX* (4-hydroxyphenylacetate) from *E. coli* (36), and *pht1* (phthalate) from *P. putida* (33). A database search and multiple sequence alignment (not shown) suggest that these proteins, with the exception of MopB, may form a new cluster of transporters in the MFS. PcaK is the first example described (15) of what now appears to be a new family of transporters for aromatic molecules, and this study defines a baseline for understanding aromatic compound transport.

Like other MFS transporters, PcaK is energized by the PMF (Table 4). The kinetic characteristics of PcaK and its ability to concentrate substrate against a concentration gradient are also comparable to those reported for other permeases (20, 26). PcaK migrates as a broad band on SDS-PAGE, with an apparent molecular mass that is considerably lower than the predicted molecular mass of 47 kDa (Fig. 2) (15). These anomalies have also been reported for the LacY polypeptide (22), another MFS transporter, and may be due to the hydrophobic nature of the proteins.

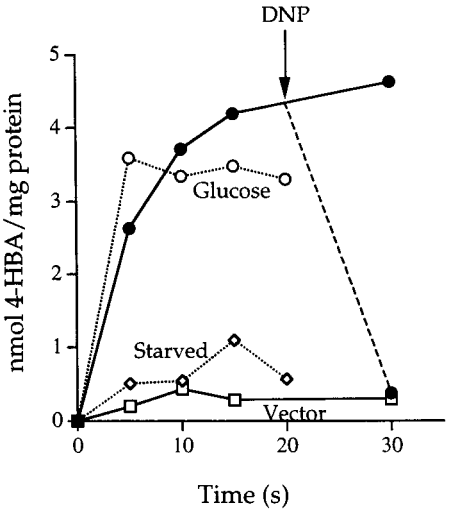

FIG. 5. PcaK-mediated transport of 4-HBA requires energy. 4-HBA uptake was tested in *E. coli* cells expressing PcaK before starvation (●), in starved cells (◇), and in starved cells reenergized with glucose (○). The addition of DNP is indicated with an arrow. Data for a vector control (□) are also shown.

A novel feature of PcaK is its function in aromatic acid chemotaxis (15). Wild-type *P. putida* is chemotactic to several aromatic acids, including benzoate, 2-, 3-, and 4-HBA, PCA, 3- and 4-chlorobenzoate, and 4-methylbenzoate (10, 17, 18). A *pcaK* mutant, grown on 4-HBA, is not attracted to any of these aromatic acids (10, 15). It is important to note that the chemotaxis phenotype of the *pcaK* mutant is evident even under conditions (pH 6.8) where the growth rate on 4-HBA is nearly that of the wild-type strain. Therefore, the nonchemotactic phenotype is not due merely to poor growth or to lack of chemoreceptor induction (15). PcaK does not resemble known chemoreceptors (31, 35), and the specific role of PcaK in chemotaxis to aromatic acids remains to be defined. In this regard, it is interesting that uptake of benzoate by PcaK was not detected (Fig. 3), because benzoate, a chemoattractant that is apparently sensed by PcaK, inhibited the PcaK-dependent uptake of 4-HBA in competition assays (Table 2). Binding of benzoate, independent of transport, might stimulate a behavioral response and would be consistent with the apparently broad substrate range of PcaK for chemotaxis (15). The conflicting results obtained with benzoate in uptake and substrate inhibition assays also serve as a reminder that results of competition assays should be interpreted with caution.

Complex lignin polymers are degraded in soil and humus to a variety of aromatic compounds, and efficient detection and transport of many different aromatic molecules may be crucial for growth of bacteria in soil. Consequently, redundancy of chemoreceptors and transporters may be required to ensure cell survival. Soil organisms such as *P. putida* may therefore elaborate a number of chemoreceptors and transport systems, with overlapping induction and substrate specificities, for aromatic compounds. Determining the roles of PcaK in chemotaxis and transport will provide a basis for understanding the behavioral response and transport as adjuncts to metabolism of aromatic compounds.

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