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

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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 benzoyl. The Vₘₐₓ for uptake of 4-hydroxybenzoate was at least 25 nmol/min/mg of protein, and the Kₘ 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 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 Tn10. 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 KH₂PO₄, 25 mM Na₂HPO₄, 0.1% (NH₄)₂SO₄, 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 (A₅₃₀) of 0.25 (approximately 10⁸ cells/ml), cultures were induced with 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)pLysS(pHNN100) was grown for 4-HBA uptake assays in LB containing 10 mM glucose.

[³⁵S]methionine labeling of PcaK. Cells were harvested from a 1-ml culture of BL21(DE3)pLysS(pHNN100) at an A₅₃₀ of approximately 0.25. Pellets were washed twice in 0.5 ml M9 medium (5) 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
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-[35S]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) (5) on 12.5% acrylamide–0.33% bisacrylamide gels.

**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.

**TABLE 1. Bacterial strains and plasmids used**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristicsa</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td><em>P. putida</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRS29000</td>
<td>Wild type</td>
<td>34</td>
</tr>
<tr>
<td>PRS30000</td>
<td>Km′ pcaGH::Tn5 4-HBA</td>
<td>L. N. Ornst</td>
</tr>
<tr>
<td>PCH722-Gm</td>
<td>Gm′ pcaK::Tn5-B30/Gm, nonchemotactic to 4-HBA, complemented in <em>trans</em> by pcaK</td>
<td>15</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
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<tr>
<td>BL21(DE3)</td>
<td>hsdS gal (λcI857 ind-1 Sam7 nin-5 lacUV5-T7 gene 1)</td>
<td>39</td>
</tr>
<tr>
<td>TA3952</td>
<td>Tc′, donor strain for P1 transduction of ATPase mutation, hisΔ61 dhaA1 uncΔ702 asaA::Tn10</td>
<td>21</td>
</tr>
<tr>
<td>BL21(DE3)uncΔ702</td>
<td>Tc′, ATPase mutant, BL21(DE3) uncΔ702 asaA::Tn10</td>
<td>This study</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pHNN100</td>
<td>Ap′, pcaK cloned downstream of T7 promoter of pT7-5</td>
<td>15</td>
</tr>
<tr>
<td>pT7-5</td>
<td>Ap′, T7 promoter expression vector</td>
<td>40</td>
</tr>
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a 4-HBA − no growth on 4-HBA; Ap′, ampicillin resistant; Gm′, gentamicin resistant; Km′, kanamycin resistant; Tc′, 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 (NH₄)₂SO₄ was replaced with Na₂SO₄] 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 carboxyl 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.

Acicular 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 [¹⁴C]-labeled PCA. [ring-UL-¹⁴C]PCA was synthesized from [ring-UL-¹⁴C]-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 [¹⁴C]-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 the crude supernatant (24). The product was extracted into ethyl acetate, dried, and resuspended in sterile water. The specific activity of the synthesized [¹⁴C]PCA was 1 μCi/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. [¹³S]Smethionine (83 Ci/mmol), [ring-UL-¹⁴C]benzoate (13.5 μCi/mmol), and [ring-UL-¹⁴C]-4-HBA (33 μCi/mmol) were purchased from Amersham Corp. (Arlington Heights, Ill.).

RESULTS

PcaK is a membrane protein. Sonicated extracts of E. coli expressing [³⁵S]-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 to bind 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 [¹⁴C]-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 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 (pK₀ 4.48) (27). The slow-growth phenotype was more pronounced at pH 6.2 than at pH 5, in the absence of inhibitor) was 87 ± 4

<table>
<thead>
<tr>
<th>Competing substrate</th>
<th>% Inhibition of 4-HBA uptake</th>
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<tr>
<td>4-HBA</td>
<td>87 ± 4</td>
</tr>
<tr>
<td>Benzoate</td>
<td>84 ± 7</td>
</tr>
<tr>
<td>PCA</td>
<td>78 ± 8</td>
</tr>
<tr>
<td>4-Fluorobenzoate</td>
<td>73 ± 11</td>
</tr>
<tr>
<td>3-Hydroxybenzoate</td>
<td>64 ± 2</td>
</tr>
<tr>
<td>3-Fluorobenzoate</td>
<td>63 ± 13</td>
</tr>
<tr>
<td>3-Chlorobenzoate</td>
<td>62 ± 14</td>
</tr>
</tbody>
</table>

*The concentrations of 4-HBA and competing substrate were 5 and 100 μM, respectively. The rate of accumulation of 4-HBA (5 μM, in the absence of inhibitor) was 6.1 ± 0.5 mmol/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-fluorobenzoate, cis,cis-muconate, 2,6-difluorobenzoate, 3,4-difluorobenzoate, β-ketoacidipate, phenylalanine, quinate, salicylate, shikimate, succinate, p-toluic acid, tyrosine, and vanillate.

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 [³⁵S]Smethionine-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 twice the amount loaded in lane 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.
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. Intra- cellular 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 Δψ 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).
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 mphT (3-hydroxyphenylpropionate) from *Rhodococcus globus* (4) and *E. coli* (11), respectively, hpaX (4-hydroxyphenylacetate) from *E. coli* (36), and phl1 (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. The kinetic characteristics of PcaK and its ability to concentrate substrate against a concentration gradient are also consistent with the apparently broad substrate range of PcaK for chemotaxis (15). The concentration 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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