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# Repression of 4-Hydroxybenzoate Transport and Degradation by Benzoate: a New Layer of Regulatory Control in the *Pseudomonas putida* β-Ketoadipate Pathway

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***Pseudomonas putida*** **PRS2000 degrades the aromatic acids benzoate and 4-hydroxybenzoate via two parallel sequences of reactions that converge at β-ketoadipate, a derivative of which is cleaved to form tricarboxylic acid cycle intermediates. Structural genes (*pca* genes) required for the complete degradation of 4-hydroxybenzoate via the protocatechuate branch of the β-ketoadipate pathway have been characterized, and a specific transport system for 4-hydroxybenzoate has recently been described. To better understand how *P. putida* coordinates the processes of 4-hydroxybenzoate transport and metabolism to achieve complete degradation, the regulation of *pcaK*, the 4-hydroxybenzoate transport gene, and that of *pcaF*, a gene required for both benzoate and 4-hydroxybenzoate degradation, were compared. Primer extension analysis and *lacZ* fusions showed that *pcaK* and *pcaF*, which are adjacent on the chromosome, are transcribed independently. PcaR, a transcriptional activator of several genes of the β-ketoadipate pathway, is required for expression of both *pcaF* and *pcaK*, and the pathway intermediate β-ketoadipate induces both genes. In addition to these expected regulatory elements, expression of *pcaK*, but not *pcaF*, is repressed by benzoate. This previously unrecognized layer of regulatory control in the β-ketoadipate pathway appears to extend to the first two steps of 4-hydroxybenzoate degradation, since levels of 4-hydroxybenzoate hydroxylase and protocatechuate 3,4-dioxygenase activities were also depressed when cells were grown on a mixture of 4-hydroxybenzoate and benzoate. The apparent consequence of benzoate repression is that cells degrade benzoate in preference to 4-hydroxybenzoate. These findings indicate that 4-hydroxybenzoate transport is an integral feature of the β-ketoadipate pathway in *P. putida* and that transport plays a role in establishing the preferential degradation of benzoate over 4-hydroxybenzoate. These results also demonstrate that there is communication between the two branches of the β-ketoadipate pathway.**

Aromatic compounds are abundant in the environment as components of plant material and as pollutants introduced by humans. Pesticides and industrial wastes often contain aromatic or halogenated aromatic constituents, many of which are toxic. Consequently, the enzymology of many different pathways used by bacteria to degrade diverse aromatic compounds has been studied to gain an understanding of natural biodegradation and to provide models for the degradation of xenobiotics (6, 8, 17, 43). One of the best studied of these pathways is the β-ketoadipate pathway, which consists of two parallel branches for the catabolism of catechol and protocatechuate, derived from benzoate and 4-hydroxybenzoate (4-HBA), via an *ortho* ring cleavage (30, 42) (Fig. 1).

This sequence of reactions is widely distributed among bacteria and has been particularly well studied in *Pseudomonas putida*. Most of the structural genes for benzoate and 4-HBA degradation have been cloned from this organism (1, 2, 11, 18, 21, 22, 32), and several transcriptional activators required for gene expression have been described (23, 38, 39). The best-studied sets of genes are those involved in catechol (*cat* genes) and protocatechuate (*pca* genes) degradation. The catechol degradation genes *catA* and *catBC* encode catechol 1,2-dioxygenase, *cis*,*cis*-muconate lactonizing enzyme, and muconolactone isomerase, respectively. All three genes are activated by the metabolic intermediate *cis*,*cis*-muconate and the regulatory protein CatR (1, 2, 21, 39). The CatR protein has been purified, and the mechanism by which it activates transcription has been studied (36, 37, 40). The *pca* genes, required for the degradation of protocatechuate to tricarboxylic acid cycle intermediates, are arranged in four physically distinct clusters (11, 18, 22, 32) (Fig. 1) and are known from enzyme activity studies to be induced by protocatechuate in the case of *pcaHG* (encoding protocatechuate 3,4-dioxygenase) (20) and by β-ketoadipate in the case of the remaining genes (18, 29, 35). A regulatory protein, PcaR, has been implicated in the expression of *pcaBDC*, the genes encoding β-carboxymuconate lactonizing enzyme, β-ketoadipate enol-lactone hydrolase, and γ-carboxymuconolactone decarboxylase, and the expression of *pcaIJ*, the genes for the two subunits of β-ketoadipate:succinyl coenzyme A transferase (22, 34, 38). Of the *pca* genes, only *pcaIJ* operon expression has been studied at the transcriptional level (34).

Recently, it has been recognized that the microbial processes of chemotaxis and solute transport operate as early steps in benzoate and 4-HBA utilization (4, 7, 18, 19, 45) and, as such, are important adjuncts to pathway degradation enzymes. In *P. putida*, chemotactic responses to benzoate and 4-HBA are induced by β-ketoadipate (19), and transport of 4-HBA into intact cells is also a β-ketoadipate pathway-associated trait (18). A 47-kDa membrane-bound protein, PcaK, catalyzes 4-HBA transport and has also been proposed to be the chemoreceptor for 4-HBA. The gene encoding this dual-function protein, *pcaK*, is located on the chromosome between *pcaR*, the gene encoding the PcaR regulatory protein, and *pcaF*, which encodes β-ketoadipyl-coenzyme A thiolase, the last enzyme of the β-ketoadipate pathway (18) (see Fig. 2A).

To better understand how the entire process of 4-HBA deg-

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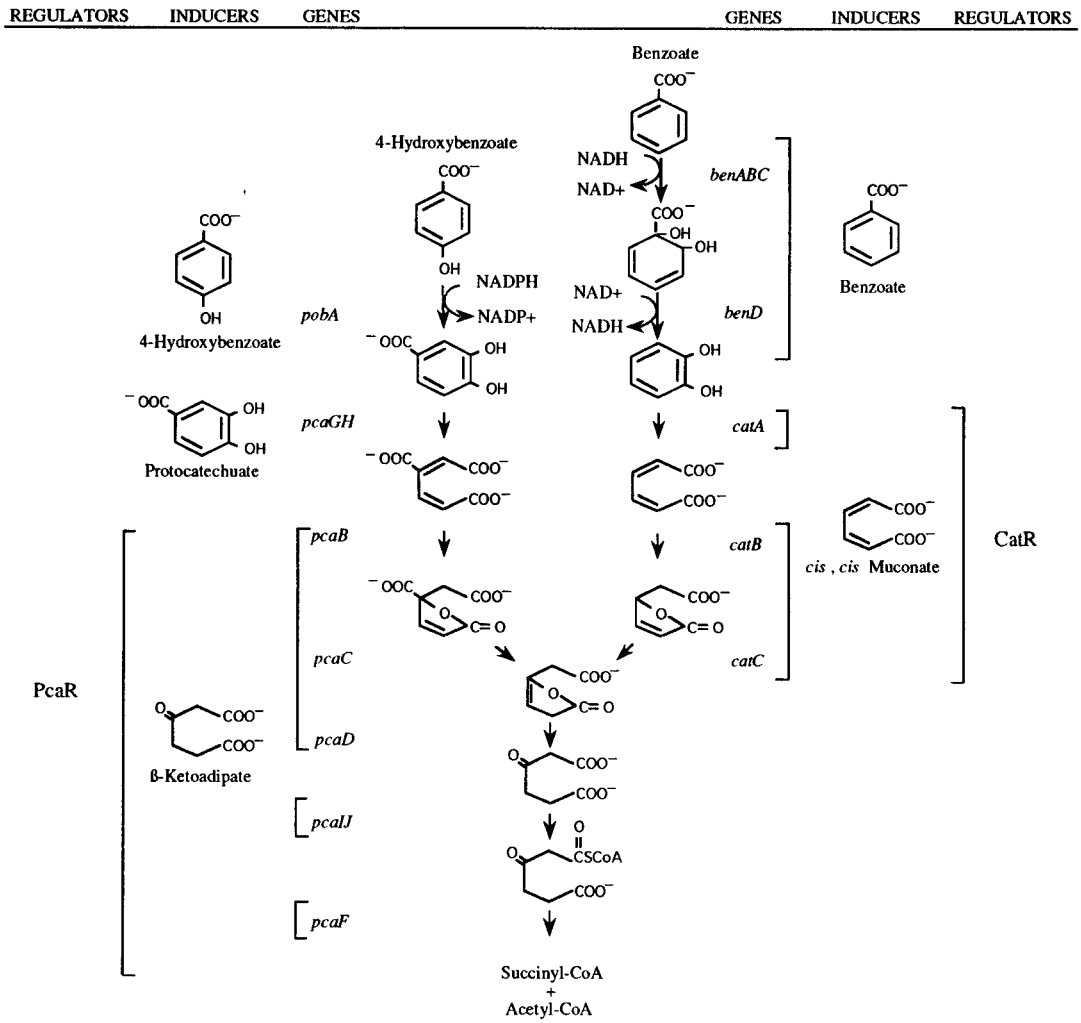


FIG. 1. The β-ketoadipate pathway and its regulation in *P. putida*.

radation by whole cells is coordinated, we examined and compared factors influencing the transcription of the chemotaxis and transport gene, *pcaK*, and the degradation gene, *pcaF*. Results presented here show that although the two genes are often expressed coordinately, expression of the *pcaK* gene is also subject to repression by benzoate. This previously unrecognized layer of regulatory control in the β-ketoadipate pathway helps to explain the observation that *P. putida* cells presented with a mixture of substrates preferentially degrade benzoate before 4-HBA.

## MATERIALS AND METHODS

**Plasmids, bacterial strains, and growth conditions.** Plasmids and bacterial strains used in this study are described in Table 1. *Escherichia coli* was cultured on Luria broth (LB) (5) at 37°C. *P. putida* was grown at 30°C in basal medium [25 mM $KH_2PO_4$, 25 mM $Na_2HPO_4$, 0.1% $(NH_4)_2SO_4$, 1% Hutner mineral base (14), (final pH 6.8L)]. Carbon sources were sterilized separately and added to media at the following final concentrations: glucose, 10 mM; succinate, 10 mM; benzoate, 4-HBA, or β-ketoadipate, 5 mM. When media were supplemented with both benzoate and 4-HBA, the concentration of each was 2.5 mM. Adipate, a nonmetabolizable compound, was added at a final concentration of 20 mM. Solid media contained 1.5% agar. Antibiotic supplementation for *E. coli* included ampicillin at 100 μg/ml, gentamicin at 5 μg/ml, and streptomycin at 25 μg/ml. For *P. putida*, supplementation included gentamicin at 5 μg/ml and streptomycin at 400 μg/ml.

**Nucleic acid manipulations.** Plasmid DNA was isolated by alkaline lysis (24). Subcloning, DNA sequencing, and PCRs were performed by standard techniques (5). Total RNA was prepared from mid-logarithmic-phase cultures of *P. putida* with TriReagent (Molecular Research Center, Inc., Cincinnati, Ohio) or by the procedure of Gosink et al. (15). *E. coli* DH5α was used for transformation of recombinant plasmids, and *E. coli* S17-1 was the donor strain in bacterial matings. Bacteria were mated by patching a mixture of donor and recipient cells on LB agar and incubating overnight at 30°C. Exconjugants were selected on basal medium containing succinate and appropriate antibiotics.

**Primer extension.** The Promega (Madison, Wis.) primer extension system was used to map transcription start sites. The antisense oligonucleotide 5′CGACGC TGTTTTGTGCTTGG3′ was used to define the *pcaK* +1 site. For *pcaF*, primers 5′GTCACAGATAAATACGTCGCGCATTAGGCTTC3′ and 5′GATCAGCG CTTTCAGCG3′ were used. The extension products were resolved on a 6% polyacrylamide gel next to a DNA sequence generated with the same primer.

**Construction of *lacZ* fusions.** A two-step cohort cloning system (33) that facilitates directional cloning of promoters into a broad-host-range *lacZ* fusion vector was used to construct *pcaK-lacZ* and *pcaF-lacZ* transcriptional fusions. For each gene, the promoter was PCR amplified from *P. putida* wild-type strain PRS2000 chromosomal DNA and cloned in a directed orientation adjacent to a streptomycin resistance determinant in the cohort vector pHRP315. These constructs were designated pHNN200 and pHNN204 (Table 1). For construction of pHNN200, upstream primer 1 (5′GACTCCGCGGCCCCCTTTCACATT TAG3′) incorporated a *Sac*II cloning site and downstream primer 2 (5′GACT GAATTCTGGACGTCGAGGCTTTTGCCG3′) incorporated an *Eco*RI site (sites indicated by underlining). The resulting 376-bp PCR product was digested with *Sac*II and *Eco*RI and ligated into corresponding sites in pHRP315. For construction of pHNN204, primer 1 was paired with primer 3 (5′GACTGAAT TCGGCTCGATCAGCGCTTTCAG3′) to incorporate an *Eco*RI site. The

TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| Strains | | |
| *P. putida* | | |
| PRS2000 | Wild type | 31 |
| PRS2015 | *catB* Ben$^-$ | 44 |
| PRS2178 | Hyperconstitutive for β-ketoadipate uptake | 35 |
| PRS3015 | *pcaR*::Tn*5* Ben$^-$ 4-HBA$^-$ | 22 |
| PRS4020 | *catR*::Gm Ben$^-$ | 34 |
| *E. coli* | | |
| DH5α | F$^-$ λ$^-$ *recA1* Δ(*lacZYA-argF*)*U169 hsdR17 thi-1 gyrA96 supE44 endA1 relA1* φ80d*lacZ*ΔM15 | Gibco-BRL |
| S17-1 | *thi pro hdsR hdsM*$^+$ *recA*, chromosomal insertion of RP4-2(Tc::Mu Km::Tn*7*) | 41 |
| Plasmids | | |
| pHRP309 | Gm$^r$; IncQ, *lacZ* transcriptional fusion vector | 33 |
| pHRP315 | Ap$^r$ Sm$^r$; cohort cloning vector for pHRP309 | 33 |
| pHNN200 | Ap$^r$ Sm$^r$; *pcaK* promoter region in pHRP315 | This study |
| pHNN201 | Gm$^r$ Sm$^r$; *pcaK-lacZ* transcriptional fusion in pHRP309 | This study |
| pHNN204 | Ap$^r$ Sm$^r$; *pcaF* promoter region in pHRP315 | This study |
| pHNN205 | Gm$^r$ Sm$^r$; *pcaF-lacZ* transcriptional fusion in pHRP309 | This study |

[a] Ben$^-$, no growth on benzoate; 4-HBA$^-$, no growth on 4-hydroxybenzoate; Ap$^r$, ampicillin resistant; Gm$^r$, gentamicin resistant; Sm$^r$, streptomycin resistant.

2,041-bp PCR product contained an internal *Kpn*I site and was digested with *Kpn*I and *Eco*RI. The resulting 1,080-bp *Kpn*I-*Eco*RI fragment, spanning the *pcaF* promoter region, was ligated into the unique *Kpn*I and *Eco*RI sites of pHRP315.

From the intermediate constructs pHNN200 and pHNN204 (containing the *pcaK* and *pcaF* promoter regions, respectively), fragments containing the vector-encoded streptomycin resistance gene plus the cloned promoter DNA were excised with *Bam*HI and *Eco*RI and then ligated into the *lacZ* transcriptional fusion vector pHRP309 to form pHNN201 (*pcaK-lacZ*) and pHNN205 (*pcaF-lacZ*) (Fig. 2). Plasmids containing inserts were identified by selection for streptomycin resistance and confirmed by restriction endonuclease digestion.

**β-Galactosidase assays.** β-Galactosidase activity in *P. putida* strains containing the *lacZ* fusion plasmids was measured as described by Miller (27). Assays were performed in triplicate on 3- to 5-ml cultures harvested in the logarithmic phase of growth. The values reported are the average of three or more assays.

**Protein determinations.** Protein concentrations were measured by the Bio-Rad (Hercules, Calif.) protein assay. Proteins from whole bacterial cells were precipitated with 0.5% trichloroacetic acid and heated at 95°C for 10 min in 0.1 M NaOH before assay. Bovine serum albumin was used as a protein standard.

**Enzyme assays.** To ensure that cells used in enzyme assays were not pre-adapted to growth on benzoate, *P. putida* cultures were inoculated from overnight cultures that had been grown with succinate or 4-HBA as the carbon source. Cultures grown on benzoate, 4-HBA, or benzoate plus 4-HBA were harvested in the early logarithmic phase, washed, and resuspended in phosphate buffer (25 mM each $KH_2PO_4$ and $Na_2HPO_4$ [pH 6.8]). The suspensions were sonicated, and cellular debris was removed by low-speed centrifugation. The supernatants, containing approximately 0.5 mg of protein per ml, were stored at −20°C. Assays were performed in duplicate with 50 or 100 μl of extract from two separate uninduced or three separate induced cultures.

4-HBA hydroxylase (EC 1.14.13.2; PobA) activity was determined by measuring the oxidation of NADPH (Sigma Chemical Co., St. Louis, Mo.), detected as a decrease in $A_{340}$ (9). A 1.0-ml reaction mixture contained 0.33 mM sodium 4-HBA, 0.23 mM NADPH, 3.3 μM flavin adenine dinucleotide, 0.33 mM sodium EDTA, and 33 mM Tris-sulfate (pH 8.0). Monooxygenase activity was determined as nanomoles of NADPH oxidized per minute per milligram of protein.

Protocatechuate 3,4-dioxygenase (EC 1.13.1.3; PcaGH) was assayed by determining the decrease in $A_{290}$ due to oxidation of protocatechuate (12). Assays were performed with a 1.0-ml total volume in 50 mM Tris-acetate buffer (pH 7.5), with 40 μM protocatechuate as the substrate. Activity was expressed as nanomoles of protocatechuate oxidized per minute per milligram protein.

**Transport assays.** Cultures for transport assays were grown as described for enzyme assays. Cells were harvested in the early logarithmic phase, washed in 0.5 volume of phosphate buffer, and resuspended in phosphate buffer to an $A_{660}$ of approximately 5. Resuspended cells were gently aerated to prevent oxygen limitation. Aliquots (0.3 ml) of cells were added to 0.3 ml of phosphate buffer containing $^{14}$C-labeled benzoate (U-$^{14}$C; specific activity, 13.3 mCi/mmol) or 4-HBA (U-$^{14}$C; specific activity, 33 mCi/mmol; both from Amersham Corp., Arlington Heights, Ill.) and 10 mM glucose or succinate as a source of energy. The final concentrations of benzoate and 4-HBA were 88 and 63.5 μM, respectively. At timed intervals, 0.1 ml was removed from the reaction mixture and filtered. Uptake of benzoate or 4-HBA into whole cells was calculated from the radioactivity retained on the filters. The rate of transport was expressed as nanomoles of substrate accumulated per minute per milligram of bacterial protein. The average rates were determined from four separate experiments.

**Measurement of aromatic acid concentration in culture supernatants.** The concentration of benzoate and 4-HBA in growth media was measured by high-performance liquid chromatography with an Ultrasphere $C_{18}$ reversed-phase column (Beckman, San Ramon, Calif.). Samples were harvested from strain PRS2000 cultures grown as described above, centrifuged briefly to remove cells, and stored at −20°C. The thawed supernatants were diluted up to fivefold with sterile water. Samples were run at a flow rate of 1 ml/min in 1% acetonitrile in 0.2 M ammonium formate (pH 5.0) for 4 min and then in a linear gradient of 1 to 99% acetonitrile for 25 min. The $A_{254}$ of the effluent was monitored. Under these conditions, 4-HBA and benzoate eluted at approximately 11.3 and 13.6 min, respectively. The concentration of aromatic acids was determined by comparing the peak areas with standard curves generated from known concentrations of benzoate and 4-HBA.

## RESULTS

***pcaK* and *pcaF* are independently transcribed.** The β-galactosidase activity in *P. putida* cells carrying the two transcriptional fusions shown in Fig. 2A indicated that *pcaK* and *pcaF* are independently transcribed. A fusion of *lacZ* to the 376-bp intergenic region between *pcaR* and *pcaK* (pHNN201) (Fig. 2B) allowed for inducible expression of β-galactosidase when cells were grown on 4-HBA (Fig. 2C). A fusion of *lacZ* to DNA spanning the 5′ end of *pcaF* (pHNN205) also gave inducible β-galactosidase activity. Cells carrying the *pcaF-lacZ* fusion expressed β-galactosidase activity at a level approximately threefold higher than that observed with the *pcaK-lacZ* fusion (Fig. 2C).

Primer extension analysis, carried out to identify the 5′ end of the *pcaK* transcript, indicated that the +1 site of *pcaK* lies either 193 or 194 bases upstream of the predicted translational start site of the gene (Fig. 3). A search of upstream DNA revealed an acceptable *E. coli* $\sigma^{70}$-type −10 region and a possible −35 region (Fig. 2B). The primer extension product was obtained from RNA isolated from cells grown on 4-HBA. Attempts to obtain a similar product from RNA isolated from succinate-grown cells were not successful. Inspection of the nucleotide sequence 5′ to the *pcaK* start site (Fig. 2B) also revealed a sequence (5′GTGCGATAAACGCAC3′) that matches 13 of 15 bases of a sequence found upstream of both *pcaIJ* (encoding β-ketoadipate:succinyl coenzyme A transferase) (34) and *pcaR* (38). A deletion analysis of the *pcaIJ* promoter has indicated that this sequence may be important for activation of transcription (34). The location of the conserved sequence in the *pcaK* promoter region is shifted upstream about 50 nucleotides relative to its location in *pcaIJ* and *pcaR*.

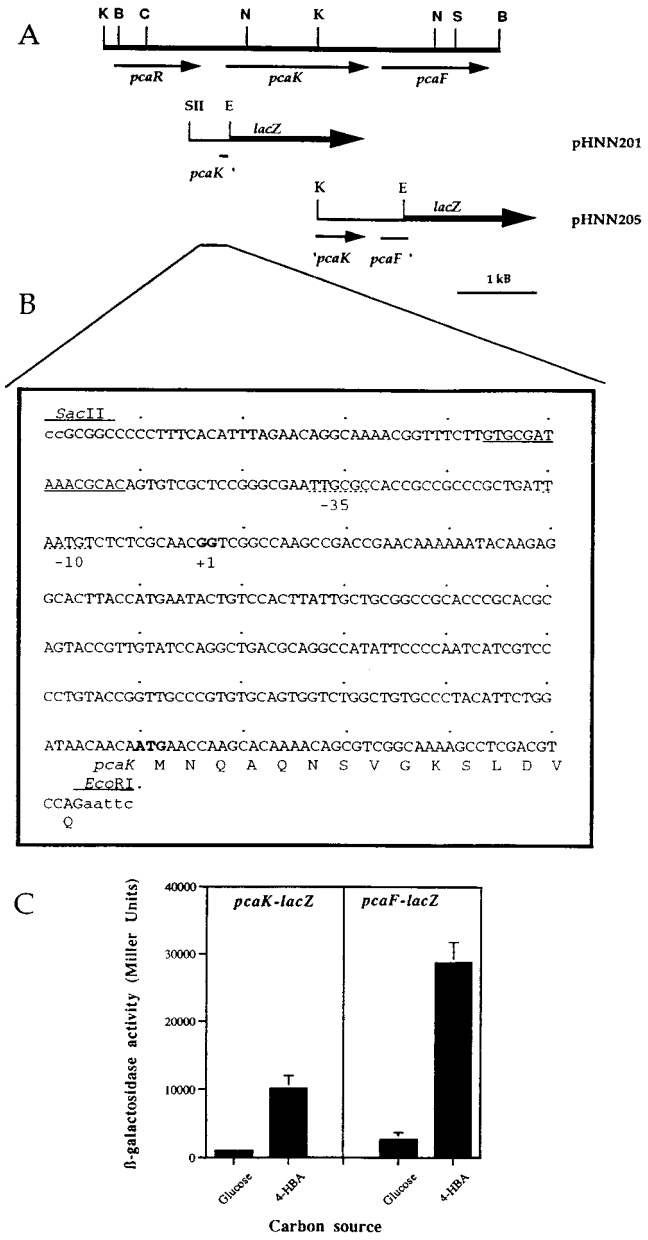


FIG. 2. (A) Physical map of the *pcaRKF* gene cluster and subcloned derivatives. Restriction site abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; N, *Nae*I; S, *Sac*I; SII, *Sac*II. The *Eco*RI and *Sac*II sites were incorporated by PCR. The DNA sequence of the region has been reported elsewhere (18, 38) and is recorded under GenBank accession numbers L33975 (*pcaR*) and U10895 (*pcaK* and *pcaF*). (B) Nucleotide sequence of the *pcaK* promoter region. The *P. putida* DNA cloned in pHNN201 is represented. The 5′ end of the *pcaK* transcript (see Fig. 3) and the predicted initiation codon of *pcaK* are shown in boldface type. The *Sac*II and *Eco*RI cloning sites incorporated by PCR are overlined, with the bases that are not derived from *P. putida* DNA shown in lowercase type. Sequences similar to the $\sigma^{70}$ −10 and −35 consensus in *E. coli* are indicated. A 13-bp sequence similar to the proposed regulatory region of *pcaIJ* and *pcaR* (see text) is underlined. (C) β-Galactosidase activity driven from the *pcaK-lacZ* and *pcaF-lacZ* fusions demonstrates that *pcaK* and *pcaF* are transcribed separately. Values shown are from induced (4-HBA) and uninduced (glucose) cultures. Error bars show standard deviations.

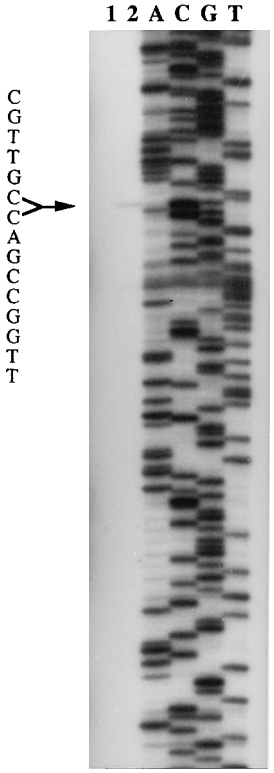


FIG. 3. Determination of the 5′ end of the *pcaK* transcript by primer extension. PRS2000 RNA was isolated from cells grown with succinate (lane 1) or 4-HBA (lane 2) as the carbon source. The DNA sequence ladder generated with the same primer is shown next to lane 2.

Despite repeated attempts with two different primers, primer extension experiments failed to identify a transcription start site for *pcaF*. This is possibly due to a large inverted-repeat hairpin structure predicted from the sequence upstream of the gene (nucleotides 1634 to 1680 described in reference 18), which may prevent in vitro extension of an annealed primer.

**β-Ketoadipate induces expression of both *pcaK* and *pcaF*.** Wild-type cells carrying the *pcaF-lacZ* fusion (pHNN205) had β-galactosidase activities that were about 10-fold higher when the cells were grown on benzoate or 4-HBA than when cells were grown on glucose or succinate. An about fivefold higher level of β-galactosidase activity was seen in cells grown on glucose with either β-ketoadipate or its nonmetabolizable analog, adipate (Fig. 4A). This level of induction corresponds well with that observed when β-ketoadipyl coenzyme A thiolase activity was assayed in *P. putida* cell extracts (18). These data indicate that β-ketoadipate, an intermediate of benzoate and 4-HBA catabolism, is the physiological inducer of *pcaF* expression.

Expression of the *pcaK-lacZ* fusion (pHNN201) was also increased about 10-fold when cells were grown on 4-HBA. Inclusion of adipate or β-ketoadipate in glucose-containing growth media resulted in about threefold higher levels of β-galactosidase activity than were seen in cells grown on glucose alone, indicating that β-ketoadipate is also the physiological inducer of *pcaK* expression (Fig. 4B).

*P. putida* has limited permeability to β-ketoadipate and adipate (35). To determine whether this would explain the relatively low levels of *pcaK* induction seen when these two compounds were supplied to wild-type cells, the *pcaK-lacZ* fusion was assayed in a mutant (PRS2178) that expresses high constitutive levels of the β-ketoadipate transport system (35). In this strain, β-ketoadipate elicited full induction of *pcaK* expression, further demonstrating that β-ketoadipate is an inducer of

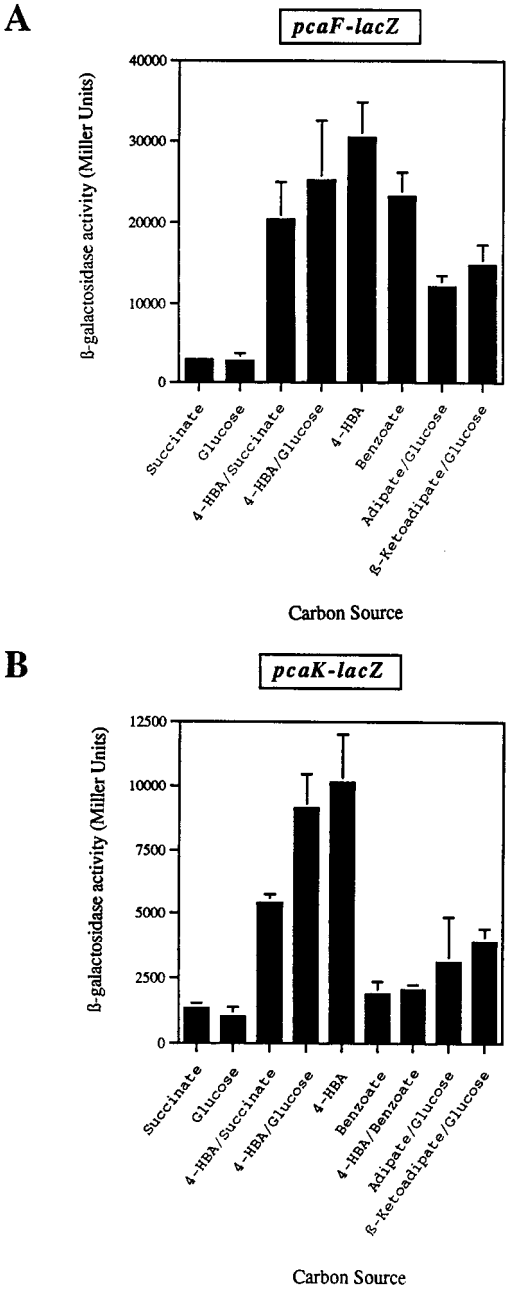


FIG. 4. (A) Induction of the *pcaF-lacZ* fusion during growth on various carbon sources. The β-galactosidase activities of pHNN205 in the wild-type strain PRS2000 are shown. The carbon sources in the growth medium are indicated. Error bars show standard deviations. (B) Induction of the *pcaK-lacZ* fusion during growth on various carbon sources. The *pcaK-lacZ* fusion in *P. putida* is induced by growth on 4-HBA and repressed by growth on benzoate. The β-galactosidase activities of pHNN201 in PRS2000 are represented. The carbon sources are indicated. Error bars show standard deviations.

*pcaK*. (Cells grown on glucose plus β-ketoadipate had 13,061 ± 4,083 Miller units of activity, compared with 12,518 ± 2,827 Miller units for cells grown on glucose plus 4-HBA and 918 ± 238 Miller units for cells grown on glucose alone.)

**Activators of *pcaK* and *pcaF*.** Expression of neither the *pcaK-lacZ* nor the *pcaF-lacZ* fusion was induced in the *pcaR* mutant strain PRS3015, indicating that the PcaR regulatory protein is required for transcriptional activation of both genes. By contrast, CatR, a transcriptional regulator specific to the catechol branch of the β-ketoadipate pathway, did not appear to be required for the activation of either *pcaK* or *pcaF* (Table 2). Although the absolute levels of β-galactosidase activity were relatively low when the *pcaK-lacZ* fusion was assayed in a *catR* mutant background, an eight- to ninefold induction of *pcaK* was nevertheless observed in both the mutant and wild-type strains grown on glucose plus 4-HBA.

**Growth on benzoate represses *pcaK* expression.** Expression of the *pcaF-lacZ* fusion was fully induced in benzoate-grown wild-type cells (Fig. 4A), as would be expected, since benzoate is metabolized to form β-ketoadipate. In view of this, we were surprised to find that benzoate did not induce expression of the *pcaK-lacZ* fusion (Fig. 4B). Moreover, only basal levels of β-galactosidase activity were seen in PRS2000(pHNN201) cells grown on a combination of benzoate and 4-HBA, suggesting that benzoate repressed *pcaK* expression. The same result was obtained when strain PRS2015, a *catB* mutant unable to metabolize benzoate past *cis,cis*-muconate, was grown on 4-HBA in the presence of benzoate (Table 3). Benzoate repression of *pcaK* expression also occurred in a *catR* background (cells grown on benzoate plus 4-HBA expressed 813 Miller units of activity).

**Transport of 4-HBA is reduced in the presence of benzoate.** A prediction that follows from the observed transcriptional repression of *pcaK* by growth on benzoate is that an effect should also be seen on 4-HBA transport since this is a *pcaK*-encoded trait. Comparisons of rates of transport by wild-type cells, grown on either 4-HBA or a combination of 4-HBA and benzoate, validated this prediction. Cells grown on 4-HBA alone accumulated 4-HBA at a rate of 14.4 nmol/min/mg of protein, whereas cells grown on both benzoate and 4-HBA transported 4-HBA at a rate of 2.3 nmol/min/mg of protein (Fig. 5). No accumulation of 4-HBA was seen with wild-type cells grown on benzoate. In parallel experiments, benzoate-grown and benzoate-plus-4-HBA-grown *P. putida* cells were found to transport benzoate at similar rates. Therefore, 4-HBA does not have a corresponding repressive effect on the benzoate branch of the β-ketoadipate pathway.

***P. putida* metabolizes benzoate in preference to 4-HBA.** The repressive effect of benzoate on 4-HBA transport suggested that *P. putida* cells given a mixture of benzoate and 4-HBA might preferentially degrade benzoate. As shown in Fig. 6, wild-type cells did, in fact, degrade benzoate at a higher rate than 4-HBA when the two compounds were provided together as growth substrates. Under these conditions, 4-HBA was utilized at 50% the rate of benzoate utilization (4-HBA and benzoate decreased at rates of 0.28 and 0.58 mM/h, respectively). When tested individually as growth substrates, 4-HBA was utilized (0.74 mM/h) at 80% of the rate of benzoate utilization (0.90 mM/h). Further experiments showed that in addition to repressing *pcaK* expression, growth on benzoate also affected the levels of activities of 4-HBA hydroxylase (encoded by *pobA*) and protocatechuate 3,4-dioxygenase (encoded by *pcaHG*), the first two enzymes in the 4-HBA branch of the β-ketoadipate pathway (Table 4). PobA activity in cells grown with benzoate plus 4-HBA was one-third of that observed in cells grown on 4-HBA alone, whereas PcaHG activity was depressed by one-half in cells grown in the presence of benzoate.

## DISCUSSION

The 4-HBA transport (*pcaK*) and degradation (*pcaF*) genes examined here are each activated by PcaR and β-ketoadipate and are coordinately expressed under some circumstances, no-

TABLE 2. β-Galactosidase activity of the *pcaK-lacZ* and *pcaF-lacZ* fusions in *P. putida* β-ketoadipate pathway regulatory mutants

| Fusion | Strain | Description | β-Galactosidase activity[a] | | |
|---|---|---|---|---|---|
| | | | Glucose | Glucose–4-HBA | Glucose–β-ketoadipate |
| *pcaK-lacZ* | PRS2000 | Wild type | 1,023 (356) | 9,122 (1,343) | 3,897 (508) |
| | PRS3015 | *pcaR* | 750 (284) | 727 (224) | 1,544 (320) |
| | PRS4020 | *catR* | 544 (141) | 4,439 (612) | 2,907 (849) |
| *pcaF-lacZ* | PRS2000 | Wild type | 2,747 (965) | 25,125 (7,410) | 14,774 (2,463) |
| | PRS3015 | *pcaR* | 2,288 (383) | 2,075 (393) | 2,783 (709) |
| | PRS4020 | *catR* | 1,970 (440) | 16,998 (3,543) | 11,718[b] (1,233) |

[a] Expressed in Miller units, with standard deviations shown in parentheses.
[b] Adipate rather than β-ketoadipate was used in this assay.

tably during growth on 4-HBA. The expression of *pcaK* differs from that of *pcaF*, however, in being repressed by growth on benzoate. This was surprising because benzoate repression of a β-ketoadipate pathway gene has not been reported previously. The finding that 4-hydroxybenzoate hydroxylase and protocatechuate dioxygenase activities were also reduced when cells were grown on 4-HBA in the presence of benzoate adds further weight to the idea that the β-ketoadipate pathway is subject to a previously unrecognized layer of regulatory control.

An analysis of the rate of disappearance of substrates from *P. putida* growth media, while not giving dramatic results, nevertheless suggests that the biological consequence of benzoate repression is to allow cells to degrade benzoate in preference to 4-HBA (Fig. 6). An explanation for this preference may lie in the energetic demands of the early steps of 4-HBA and benzoate metabolism (Fig. 1). Monooxygenation of 4-HBA to form protocatechuate requires the oxidation of NADPH (10). In contrast, the two-step formation of catechol from benzoate consumes no net reducing equivalents, since the NADH oxidized in the conversion of benzoate to 2-hydro-1,2-dihydroxybenzoate is recovered when catechol is formed in the subsequent reaction (28). This explanation is consistent with the observation that wild-type cells grow slightly faster on benzoate (doubling time, 68 ± 7 min) than on 4-HBA (doubling time, 83 ± 5 min) (18).

The β-ketoadipate pathway is widely distributed among bacteria, and one might anticipate that other species in addition to *P. putida* also degrade benzoate preferentially. Experiments using nuclear magnetic resonance to monitor the fate of aromatic acids metabolized by deuterated cells demonstrated that *Acinetobacter calcoaceticus* metabolizes benzoate in preference to 4-HBA (13). When this experimental approach was applied to *P. putida*, a pattern of preferential utilization of benzoate similar to that reported here was also observed (13). It will be interesting to see whether benzoate repression of 4-HBA degradation is a universal trait associated with the β-ketoadipate pathway.

A likely mechanism of benzoate repression is that *P. putida* cells synthesize a transcriptional regulator that functions in concert with benzoate or catechol as a repressor of *pcaK* expression. The CatR protein can be excluded as playing this role because full repression of *pcaK* expression was observed in *catR* cells provided with benzoate and 4-HBA. It is unlikely that *cis*,*cis*-muconate is responsible for corepression of *pcaK* because this metabolite does not accumulate in *catR* cells.

Although it seems likely that transcription of *pobA* and *pcaHG* is repressed by benzoate in a manner similar to *pcaK* repression, it is also possible that the reduced expression of PobA and PcaHG enzyme activity measured in benzoate-plus-4-HBA-grown cells is solely a consequence of reduced rates of 4-HBA uptake leading to reduced levels of induction. Arguing against this indirect effect is previous work showing that a *pcaI-lacZ* fusion was fully induced in a *pcaK* mutant background (18). Thus, even without a functional PcaK transport protein, sufficient 4-HBA enters and is converted by cells to β-ketoadipate to induce the lower part of the pathway.

The analysis of *pcaF* transcription indicates that this gene is regulated in the same way as the *pcaIJ* operon, which has been described previously (34). Activation of *pcaF* and *pcaIJ* requires PcaR and β-ketoadipate, and total levels of induced expression, as measured by β-galactosidase activity, are nearly the same for *pcaF* and *pcaIJ* (about 30,000 Miller units). This was not unexpected since *pcaF* and *pcaIJ* encode consecutive enzymes in the β-ketoadipate pathway. It makes sense that benzoate repression of these genes does not occur, because

TABLE 3. β-Galactosidase activity of the *pcaK-lacZ* fusion in the *catB* mutant PRS2015

| Carbon source | β-Galactosidase activity[a] |
|---|---|
| Glucose | 724 (121) |
| Glucose–4-HBA | 12,998 (1,689) |
| Glucose–benzoate | 855 (48) |
| 4-HBA | 16,818 (2,369) |
| 4-HBA–benzoate | 1,552 (674) |

[a] Expressed in Miller units, with the standard deviation shown in parentheses.

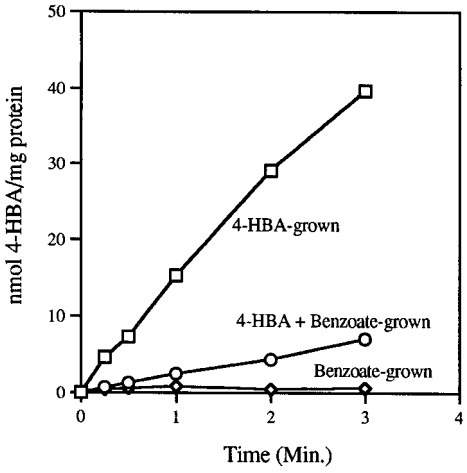


FIG. 5. Uptake of 4-HBA by PRS2000 grown on 4-HBA, benzoate, or 4-HBA plus benzoate.

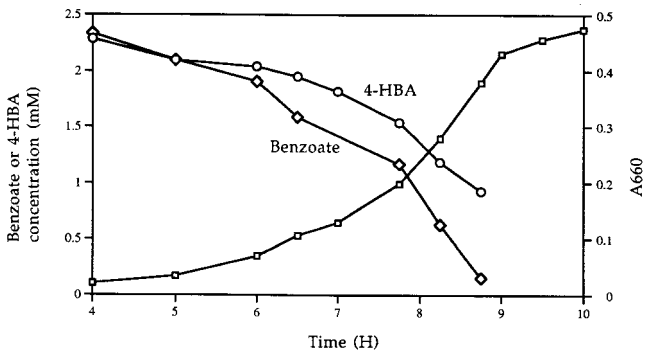


FIG. 6. Preferential utilization of benzoate by a *P. putida* culture fed both benzoate and 4-HBA. A 4-HBA-grown culture was used as the inoculum. Culture density (□) and concentrations of benzoate (◇) and 4-HBA (○) in culture supernatant are shown for one experiment, which is representative of several trials.

they encode enzymes that are required for the degradation of both benzoate and 4-HBA. Because the *pcaD* product is also required for growth on both aromatic acids, one would also not expect to see repression of the *pcaBDC* operon by benzoate.

The results reported here indicate that *P. putida* utilizes the aromatic growth substrates benzoate and 4-HBA in a preferred order and that transport, an initial step in compound recognition, plays a role in establishing this order. Wild-type cells grown with benzoate and 4-HBA had low levels of *pcaK* expression and consequently transported 4-HBA at very low rates. The physiological effects of benzoate repression of *pcaK* are consistent with a previous study showing that *pcaK* mutant cells exhibit low rates of 4-HBA transport as well as a somewhat depressed rate of growth on this compound (18). These findings underscore the importance of transport in biodegradation and are worth stressing because transport has not generally been considered a significant factor in aromatic compound catabolism. Bacterial cell membranes are permeable to aromatic acids and aromatic hydrocarbons, making it difficult to distinguish between entry of a compound into cells by active transport as opposed to passive diffusion coupled with metabolism. This may explain why, to date, only a few transport systems for aromatic compounds, other than aromatic amino acids, have been defined and studied (3, 4, 7, 16, 18, 25, 26, 45).

Studies with the *pcaK* mutant have shown that PcaK is also required for 4-HBA-grown cells to be chemotactic to a group of aromatic acids that includes benzoate and 4-HBA, and this has led to the suggestion that PcaK functions as a chemoreceptor as well as a transport protein (18). Additional data have also suggested that benzoate-grown cells synthesize a second chemoreceptor with a substrate specificity similar to that of PcaK (18). Since wild-type cells grown on benzoate or 4-HBA are equally well attracted to a range of aromatic compounds, there is no reason to think that chemotaxis influences the preferential degradation of benzoate over 4-HBA. The apparent redundancy in broad-specificity chemoreceptor systems for aromatic compounds would allow *P. putida* cells to detect and swim to the location of a particular aromatic compound before inducing the appropriate specific genes for transport and metabolism.

TABLE 4. Effect of benzoate on 4-HBA hydroxylase and protocatechuate 3,4-dioxygenase activities in *P. putida* PRS2000

| Carbon source | Activity of: | |
|---|---|---|
| | 4-HBA hydroxylase[a] (PobA) | Protocatechuate 3,4-dioxygenase[b] (PcaGH) |
| 4-HBA | 42 (7) | 3,090 (220) |
| Benzoate | 1 (3) | 90 (70) |
| 4-HBA–benzoate | 13 (3) | 1,460 (400) |

[a] Expressed as milliunits (nanomoles of NADPH oxidized per minute per milligram of protein). Standard deviations are shown in parentheses.

[b] Expressed as milliunits (nanomoles of protocatechuate oxidized per minute per milligram protein). Standard deviations are shown in parentheses.

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