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BenR, a XylS Homologue, Regulates Three Different Pathways of Aromatic Acid Degradation in *Pseudomonas putida*

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Pseudomonas putida **converts benzoate to catechol using two enzymes that are encoded on the chromosome and whose expression is induced by benzoate. Benzoate also binds to the regulator XylS to induce expression of the TOL (toluene degradation) plasmid-encoded *meta* pathway operon for benzoate and methylbenzoate degradation. Finally, benzoate represses the ability of *P. putida* to transport 4-hydroxybenzoate (4-HBA) by preventing transcription of *pcaK*, the gene encoding the 4-HBA permease. Here we identified a gene, *benR*, as a regulator of benzoate, methylbenzoate, and 4-HBA degradation genes. A *benR* mutant isolated by random transposon mutagenesis was unable to grow on benzoate. The deduced amino acid sequence of BenR showed high similarity (62% identity) to the sequence of XylS, a member of the AraC family of regulators. An additional seven genes located adjacent to *benR* were inferred to be involved in benzoate degradation based on their deduced amino acid sequences. The *benABC* genes likely encode benzoate dioxygenase, and *benD* likely encodes 2-hydro-1,2-dihydroxybenzoate dehydrogenase. *benK* and *benF* were assigned functions as a benzoate permease and porin, respectively. The possible function of a final gene, *benE*, is not known. *benR* activated expression of a *benA-lacZ* reporter fusion in response to benzoate. It also activated expression of a *meta* cleavage operon promoter-*lacZ* fusion inserted in an *E. coli* chromosome. Third, *benR* was required for benzoate-mediated repression of *pcaK-lacZ* fusion expression. The *benA* promoter region contains a direct repeat sequence that matches the XylS binding site previously defined for the *meta* cleavage operon promoter. It is likely that BenR binds to the promoter region of chromosomal benzoate degradation genes and plasmid-encoded methylbenzoate degradation genes to activate gene expression in response to benzoate. The action of BenR in repressing 4-HBA uptake is probably indirect.**

Pseudomonas putida converts a variety of environmental pollutants and plant phenolic compounds to a small number of structurally simple aromatic compounds that are the starting points for pathways of aromatic ring fission (20). Ring fission is termed *ortho* cleavage when it occurs between two adjacent hydroxyl groups and *meta* cleavage when it occurs adjacent to a single hydroxyl group. *P. putida* can degrade the aromatic acid benzoate, after converting it to catechol, by either a *meta* ring cleavage pathway or an *ortho* ring cleavage pathway (20). The aromatic acid 4-hydroxybenzoate (4-HBA) is degraded by an *ortho* ring cleavage pathway after conversion to protocatechuate (Fig. 1).

The TOL (toluene degradation) catabolic plasmid (pWW0) from *P. putida* carries genes for the degradation of toluene and xylenes (34). On the plasmid are found two operons involved in aromatic compound degradation. One of the operons encodes enzymes for the conversion of toluene and xylenes to benzoate and methylated benzoates (the upper pathway), and the other encodes enzymes that catalyze a *meta* ring cleavage and subsequent reactions leading to the formation of tricarboxylic acid cycle intermediates (the *meta* pathway). Transcription of the *meta* pathway genes is regulated by XylS, a protein also encoded on the TOL catabolic plasmid (34). *P. putida* will also convert benzoate to catechol using chromosomally encoded enzymes. The genes for these enzymes have not yet been sequenced or fully characterized (25). Catechol, but not methylated catechols, is then further degraded to trichloroacetic acid cycle intermediates by an *ortho* ring cleavage pathway (Fig. 1) (20).

Benzoate induces the synthesis of the TOL plasmid-encoded enzymes of the *meta* fission pathway (11, 22) as well as synthesis of the chromosomally encoded enzymes that convert benzoate to catechol. Additionally, a surprising recent finding is that benzoate represses the utilization of 4-HBA (40). When *P. putida* is given a mixture of benzoate and 4-HBA, it degrades benzoate in preference to 4-HBA. Presumably, this is a reflection of the fact that benzoate supports a slightly higher rate of growth than 4-HBA. Benzoate was found to depress the levels of 4-HBA hydroxylase and protocatechuate dioxygenase activity, as well as the level of 4-HBA transport in cells grown on benzoate plus 4-HBA. Benzoate represses 4-HBA transport by preventing transcription of *pcaK*, the gene encoding the 4-HBA permease (40).

Here we described a chromosomally encoded cluster of eight genes from *P. putida* that is involved in conversion of benzoate to catechol and that includes a new regulatory gene, *benR*. We determined that BenR activates expression of benzoate dioxygenase genes in response to benzoate. It is also necessary for benzoate-dependent repression of 4-HBA transport gene expression. In addition, we demonstrate the likeness of BenR to XylS by showing that BenR activates expression of the *meta* cleavage pathway operon of the TOL catabolic plasmid. BenR thus has roles as an activator of benzoate degradation via *ortho* ring fission, as an activator of benzoate and methylbenzoate degradation via *meta* ring fission, and in repression of 4-HBA degradation.

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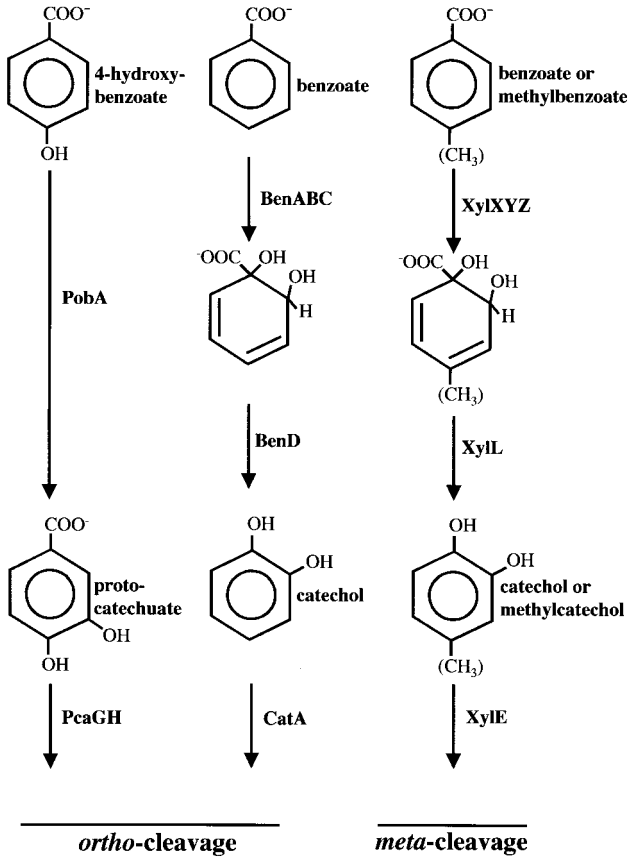

FIG. 1. Initial steps for the *ortho* and *meta* cleavage pathways used by *P. putida* to degrade 4-HBA, benzoate, and methylbenzoates. The *meta* cleavage pathway is encoded by the TOL catabolic plasmid. The methyl group can be present in either the 3 or 4 position of the ring.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The plasmids and bacterial strains used in this study are listed in Table 1. *P. putida* was grown at 30°C in basal mineral (BM) medium [25 mM KH_2PO_4, 25 mM Na_2HPO_4, 0.1% $(NH_4)_2SO_4$, 1% Hutner mineral base (14) (final pH 6.8)] containing an appropriate carbon and energy source. Carbon sources were sterilized separately and added to final concentrations of 5 mM (benzoate or 4-HBA) and 10 mM (glucose or succinate). Plasmids were mobilized from *E. coli* DH5α into *P. putida* via a triparental mating system using *E. coli* HB101(pRK2013) as the mobilizing strain. The mating mixtures were incubated overnight on solid Luria-Bertani medium at 30°C. Unless specified otherwise, *E. coli* strains were grown at 37°C in Luria broth (LB) (7). The TOL plasmid (pWW0) was transferred from *P. putida* PaW1 to the *P. putida benR* mutant (strain 4157) by direct mating with selection on solid BM medium containing 5 mM 3-methylbenzoate plus kanamycin. Antibiotics were added to the following final concentrations (in micrograms per milliliter): ampicillin, 100; gentamicin, 5; kanamycin, 100; spectinomycin, 100; and tetracycline, 25. Solid media contained 1.5% agar.

Mutagenesis and screening for benzoate-nondegrading mutants. *P. putida* cells were randomly mutagenized with the transposon mini-Tn*5* by mating *P. putida* PRS2000 cells with *E. coli* S17-1λpir (pUTminiTn*5*-Km) at 30°C overnight on LB plates with a donor-to-recipient cell ratio of 1:5. Mating mixtures were suspended in BM and plated onto BM plates containing 5 mM benzoate, a low concentration of succinate (1 mM), and kanamycin. Small colonies were patched onto plates containing kanamycin and either benzoate, 4-HBA, or succinate as the sole carbon source to identify mutants that could not utilize benzoate as a sole carbon source.

Arbitrary PCR amplifications. The region of DNA flanking the transposon insertion in strain PRS4157 was amplified by arbitrary PCR (3) using arbitrary primers as described elsewhere (45). During the first round of amplification, a primer specific to the 5′ end of the transposon and an arbitrary primer were used to amplify sequences flanking the upstream end of the inserted transposon. In a similar manner, a primer specific to the 3′ end and an arbitrary primer were used to amplify sequences flanking the downstream end of the transposon. Product from the first-round reactions was used as template in second-round reactions with primers annealing to the 5′ end of the arbitrary primers and the respective transposon-specific primers from the first-round reactions. PCR products were sequenced using the transposon-specific primers as sequencing primers.

Colony hybridization. A 335-bp probe specific to the region flanking the transposon insertion in strain PRS4157 was labeled with [^{32}P]dCTP using Ready To Go DNA labeling beads (−dCTP) and purified with a ProbeQuant G-50 Micro column (Pharmacia Biotech, Piscataway, N.J.). *E. coli* colonies carrying *Eco*RI-generated fragments of *P. putida* genomic DNA in pUC19 were screened by colony hybridization (48) to identify wild-type DNA corresponding to the region that was indicated by transposon mutagenesis to be required for benzoate degradation. The probe hybridized to an approximately 12-kb fragment of DNA that was designated pCNN100. A 1.3-kb *Eco*RI-*Xma*I fragment containing the portion of the *benF* reading frame not contained on pCNN100 was subsequently cloned by inverse PCR (41). This clone was designated pCCH108.

DNA sequencing and analysis. DNA sequencing was performed by the University of Iowa DNA Sequencing Facility (Iowa City). Sequence assembly and analysis were done with GENE Inspector, version 1.0.1 (Textco Inc., West Lebanon, N.H.). The amino acid sequences of open reading frames were submitted to the National Center for Biotechnology Information (Bethesda, Md.) and analyzed using the BLASTp 2.0.9 algorithm (1). The sequence alignment was constructed using the CLUSTAL W multiple-sequence alignment program at the Baylor College of Medicine Human Genome Center (52), and the program BOXSHADE (version 3.21) was used to shade aligned sequences.

Cloning and DNA manipulations. Standard protocols were used for cloning and transformations. Restriction digests and ligations were performed using standard techniques. Plasmid DNA was prepared by using a QIAprep Spin Miniprep kit, and DNA restriction fragments were isolated from agarose gels using the QIAquick gel extraction kit (Qiagen Inc., Santa Clarita, Calif.).

Total RNA was isolated from PRS2000 cells grown on either glucose or glucose plus benzoate (2.5 mM) using the SV total RNA isolation system as instructed by the manufacturer (Promega Corp., Madison, Wis.). The transcription start site of *benA* was determined by primer extension analysis using the Promega AMV-RT (Avian myeloblastosis virus reverse transcriptase) primer extension system. The primer was complementary to bases 45 to 28 of *benA*. Primer extension products were analyzed on a 6% polyacrylamide gel next to a sequence ladder generated with the same primer. The Access reverse transcription-PCR system (Promega) was used to determine the transcriptional organization of the *benA*, *-B*, and *-C* genes. In each case, a reverse transcriptase-free control was included to ensure that reaction mixtures did not contain contaminating DNA.

Reporter plasmids pHNN216 and pCCH101 were constructed using a two-step cloning procedure described previously (46). The promoter regions of *benA* and *pcaK* were amplified by PCR and then directionally inserted adjacent to a Ω Sp^r/Sm^r cassette in either pHRP315 or pHRP317. Fragments containing the ΩSp^r/Sm^r cassette and promoter region were then inserted upstream of the promoterless *lacZ* gene of pHRP309 to create pCCH101 (*benA-lacZ*) or pHNN216 (*pcaK-lacZ*). The fusions of the promoter-containing fragments and *lacZ* were confirmed by sequencing.

A 1,189-bp PCR product containing the *benR* open reading frame and suspected promoter region was cloned into the *Eco*RI/*Pst*I sites of pRK415 to generate pCCH107. A 989-bp PCR product containing only the *benR* open reading frame was cloned into the *Nde*I/*Pst*I sites of pT7-7 downstream of the T7 promoter to construct the BenR expression plasmid, pCCH106.

β-Galactosidase assays. β-Galactosidase activities of *P. putida* cells carrying *benA-lacZ* or *pcaK-lacZ* transcriptional fusion plasmids were assayed according to Miller (36). For analysis of *P. putida* strains carrying pCCH101, cultures were grown to an A_{660} of 0.1 with succinate as the sole carbon source, at which time compounds to be tested for the ability to induce gene expression were added to a final concentration of 1 mM. For analysis of *P. putida* strains carrying pHNN216, cultures were grown with the indicated concentrations of compounds. β-Galactosidase activities were determined for cells harvested at an A_{660} of 0.2. Six to eight independently grown cultures were assayed in triplicate, and the values were averaged. For analysis of gene expression in *E. coli*, cells were grown in LB at 30°C to an A_{660} of 0.25, at which time benzoate and catechol, if added, were added to a final concentration of 1 mM. *E. coli* cells were harvested at a final A_{660} of 0.5.

4-HBA uptake assays. Cells (50 ml) grown in BM with either 4-HBA or a mixture of benzoate (2.5 mM) and 4-HBA (2.5 mM) as carbon sources were harvested at mid-logarithmic phase, washed in 25 ml of phosphate buffer (25 mM KH_2PO_4, 25 mM Na_2HPO_4), and resuspended in 2 ml of phosphate buffer. Resuspended cells (300 μl) were added to an equal volume of reaction mixture (25 mM KH_2PO_4, 25 mM Na_2HPO_4, 4.0 mM succinate, 4.0 mM glucose, 127 μM ^{14}C-labeled 4-HBA) to start the assay. At timed intervals, 100-μl samples were removed, filtered through Nucleopore polycarbonate membranes (0.2-μm pore size; Costar Corp., Cambridge, Mass.), and washed with 1.8 ml of phosphate buffer. Accumulated substrate was determined by scintillation counting of the cells retained on the filters.

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

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
E. coli strains		
BL21(DE3)	Carries T7 RNA polymerase under the control of *lacUV5* promoter	51
CC118 *Pm-lacZ*	Tc^r; CC118 with chromosomal mini-Tn*5*::*Pm*::*lacZ* insertion	31
DH5α	F$^-$ λ$^-$ *recA1* Δ(*lacZYA-argF*)*U169 hsdR17 thi-1 gyrA96 supE44 endA1 relA1* ϕ80d*lacZ*ΔM15	GIBCO-BRL
HB101	F$^-$ λ$^-$ *recA1* Δ(*lacZYA-argF*)*U169 hsdR17 thi-1 gyrA96 supE55 endA1 relA1*	48
S17-1λpir	*thi pro hdsR hdsM*$^+$ *recA*, chromosomal insertion of RP4-2(Tc::Mu Km::Tn*7*)	50
P. putida strains		
PRS2000	Wild type	44
PRS4157	Km^r; mini-Tn*5*Km insertion in *benR*	This work
Plasmids		
pHRP309	Gm^r; IncQ, *lacZ* transcriptional fusion vector	46
pHRP311	Gm^r, Sm^r, Sp^r; negative control plasmid (Ω cassette from cohort vector inserted into pHRP309)	46
pHRP315	Ap^r, Sm^r, Sp^r; cohort cloning vector for use with pHRP309	46
pHRP317	Km^r, Sm^r, Sp^r; cohort cloning vector for use with pHRP309	46
pRK415	Tc^r; IncP1, mobilizable broad-host-range cloning vector	29
pRK2013	Km^r; ori (ColE1), RP4 mobilization functions	10
pT7-7	Ap^r; ori (ColE1), expression vector with T7 promoter	51
pUC19	Ap^r; high-copy-number cloning vector	55
pUTminiTn*5*-Km	Ap^r, Km^r; delivery plasmid for mini-Tn*5*-Km	8
pCCH100	Km^r, Sm^r, Sp^r; *benA* promoter region in pHRP317	This work
pCCH101	Gm^r, Sm^r, Sp^r; *benA-lacZ* transcriptional fusion in pHRP309	This work
pCCH106	Ap^r; pT7-7 with the *benR* open reading frame inserted in the *Nde*I/*Pst*I sites	This work
pCCH107	Tc^r; pRK415 with *benR* inserted in the *Eco*RI/*Pst*I sites	This work
pCCH108	Ap^r; 1.3-kb inverse-PCR-generated *Eco*RI-*Xma*I fragment cloned in pUC19	This work
pCNN100	Ap^r; 12.2-kb *Eco*RI PRS2000 genomic fragment in pUC19	This work
pHNN211	Ap^r, Sm^r, Sp^r; *pcaK* promoter region in pHRP315	This work
pHNN216	Gm^r, Sm^r, Sp^r; *pcaK-lacZ* transcriptional fusion in pHRP309	This work

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Gm^r, gentamicin resistance; Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance; Tc^r, tetracycline resistance.

Radiochemicals. [^{14}C]uniformly-ring-labeled 4-HBA (33 Ci/mmol) and [^{32}P]dCTP were obtained from Amersham Corp. (Arlington Heights, Ill.).

Nucleotide accession number. The nucleotide sequence has been assigned GenBank accession number AF218267.

RESULTS

Identification of *P. putida* genes involved in benzoate degradation. To identify genes involved in benzoate degradation, we isolated a transposon mutant, PRS4157, that was unable to utilize benzoate as a sole carbon source but that grew at wild-type rates on succinate and 4-HBA. Sequencing of the region of DNA flanking the transposon revealed that the transposon was inserted in a gene predicted to encode a regulator with high similarity to XylS, a TOL plasmid-encoded activator of benzoate and methylbenzoate degradation (Fig. 2) (17). We named this gene *benR*. A 13.3-kb segment of *P. putida* DNA was subsequently cloned and sequenced and was found to include eight genes that can be inferred to be involved in benzoate degradation based on their sequence similarity to known genes (Fig. 3; Table 2).

Downstream of *benR* are three genes, *benA*, *-B*, and *-C*, that have very high deduced amino acid sequence identity to the

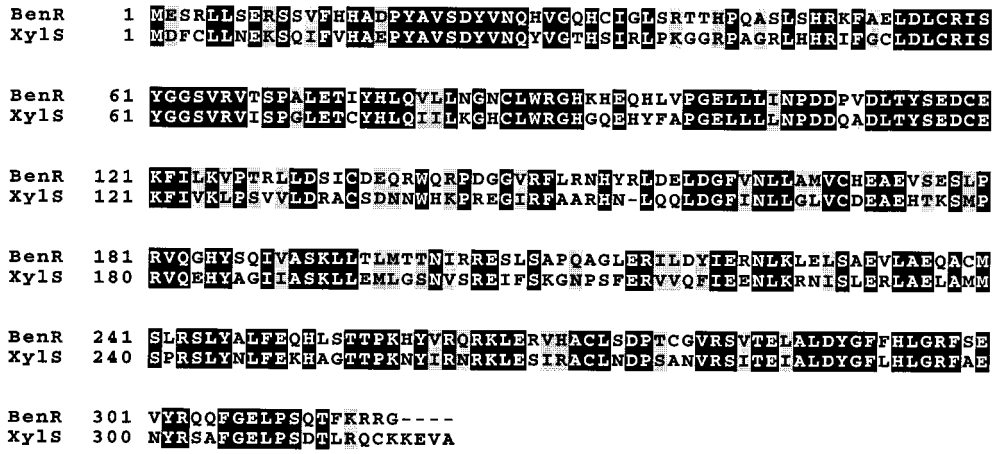

FIG. 2. Amino acid sequence alignment of BenR and XylS proteins. Identical residues are outlined in black. Similar residues are shaded gray.

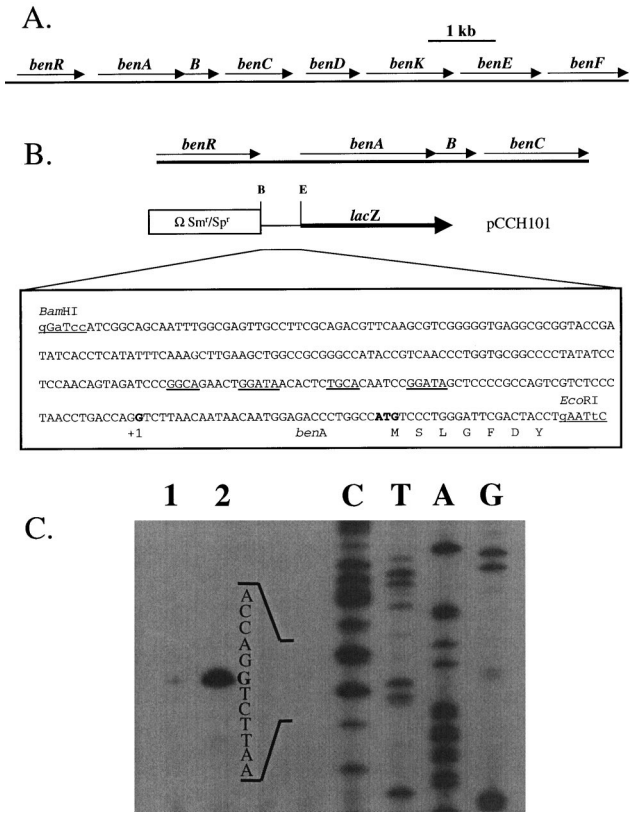

FIG. 3. (A) Map of chromosomally encoded benzoate degradation genes from *P. putida*. (B) Map of the *benA* promoter region, diagram of the region incorporated to construct the *benA-lacZ* fusion plasmid, pCCH101, and nucleotide sequence of the *benA* promoter region. The putative BenR binding sites are heavily underlined. Introduced restriction sites are lightly underlined. The transcriptional and translational start sites are in bold. (C) Determination of the 5′ end of the *benA* transcript by primer extension. RNA was isolated from glucose-grown cells (lane 1) and glucose-benzoate-grown cells (lane 2) as described in the text. A sequence ladder was generated with the same primer (lanes C, T, A, and G). The first nucleotide in the transcript is shown in bold.

terminal oxygenase and reductase components of methylbenzoate and benzoate dioxygenases. BenD is similar to *cis*-diol dehydrogenases involved in the conversion of 2-hydro-1,2-dihydroxybenzoates (the products of benzoate dioxygenase reactions) to catechols (Fig. 1). Following *benABCD* are three genes that may encode proteins involved in benzoate uptake. The *benK* and *benF* genes almost certainly encode a benzoate transporter and a porin, respectively. The function of *benE*, predicted to encode a membrane protein, is unknown. Each *ben* gene has a guanine-plus-cytosine content of between 60 and 67%.

BenR activates the expression of *benABC* in response to benzoate. The *benR* mutant (strain PRS4157) was able to grow on benzoate when a plasmid-borne copy of *benR* was supplied in *trans* on pCCH107. The complemented mutant grew on benzoate with a generation time of 2.4 h, compared to about 1.8 h for the wild-type strain. The sequence of the *benR* gene and the growth phenotype of the *benR* mutant suggested that the BenR protein is probably involved in regulating expression of the *benA*, *benB*, and *benC* genes, predicted to encode benzoate 1,2-dioxygenase. Reverse transcription-PCR amplification of the regions between *benA* and *benB* and between *benB* and *benC* showed that these three genes are cotranscribed in benzoate-grown cells (results not shown). Primer extension analysis indicated that the 5′ end of *benA* lies 30 bp upstream from its predicted translational start site (Fig. 3).

To test whether BenR regulates *benA* expression, we constructed a reporter plasmid that has the *benA* promoter fused to a promoterless *lacZ* gene (pCCH101). *P. putida* wild-type cells carrying the *benA-lacZ* fusion expressed β-galactosidase activity at levels that were 15-fold higher in cells grown on succinate in the presence of benzoate compared to succinate-grown cells (Fig. 4A). The presence of catechol, the product of *cis*-diol dehydrogenase, did not induce expression of the *benA-lacZ* fusion. Expression of the *benA-lacZ* fusion was not induced by benzoate in *benR* mutant cells. Overexpression of BenR from a T7 promoter (plasmid pCCH106) in *E. coli* BL21(DE3) cells carrying the *benA-lacZ* fusion plasmid resulted in a 25-fold increase in β-galactosidase expression over the levels seen in the absence of *benR* (Fig. 4B). This result shows that BenR directly activates the *benA* promoter. In this system, addition of benzoate did not influence the levels of β-galactosidase expression.

BenR activates expression of the TOL plasmid-encoded *meta*-cleavage pathway operon. *Pm*, the promoter of the *meta*-cleavage operon from the TOL catabolic plasmid, is activated by the TOL plasmid-encoded regulator XylS when benzoate or methylbenzoates are present. A long-standing observation in studies of TOL plasmid gene regulation is that *Pm* can also be activated in the presence of benzoate by a chromosomally encoded regulator. The regulatory gene responsible for this activation was identified genetically in 1988 and given the name *benR* (6), but this gene was never sequenced. To determine whether the *benR* gene described here might be the regulatory gene that is responsible for XylS-independent activation of *Pm*, we expressed the BenR protein from pCCH107 in *E. coli* CC118 *Pm-lacZ*, a strain that has a *Pm*::*lacZ* fusion inserted in its chromosome. When BenR was present, 13,000 Miller units of β-galactosidase was expressed from *Pm* over an undetectable background. A slight increase in β-galactosidase production (17,000 Miller units) was seen when benzoate was included in the growth medium. The addition of catechol had no effect.

XylS responds to benzoate, but not 3-methylbenzoate, to modulate expression of *benA*. To determine whether XylS can restore benzoate-dependent regulation of the *benA* promoter in the absence of BenR, the TOL catabolic plasmid was introduced from *P. putida* PaW1 into the *benR* mutant (PRS4157) containing the *benA-lacZ* fusion plasmid (pCCH101). When the TOL plasmid was present, the observed level of *benA-lacZ* expression was fivefold higher when benzoate was included in the growth medium together with succinate than when cells were grown on succinate alone or on succinate plus 4-HBA or 3-methylbenzoate (Fig. 5). These results indicate that XylS, encoded on the TOL plasmid, can partially complement BenR function.

BenR is required for benzoate-mediated repression of 4-HBA degradation. When *P. putida* cells that are growing on 4-HBA as a sole carbon source are transferred to a medium that includes equal amounts of 4-HBA and benzoate, their rate of 4-HBA degradation decreases and they start to degrade benzoate at a high rate. Once all of the benzoate is depleted, rapid degradation of 4-HBA resumes (40). This preferred usage of benzoate over 4-HBA by *P. putida* can be partially explained by the observation that benzoate represses expression of *pcaK*, a gene that encodes a 4-HBA permease. To determine if BenR plays a role in this repression, we compared the levels of β-galactosidase produced by wild-type and *benR* mutant strains carrying a *pcaK-lacZ* transcriptional fusion (Fig. 6). As was previously demonstrated (40), wild-type *P. putida*

TABLE 2. Benzoate degradation genes of *P. putida*

Gene designation	Proposed function of gene product	Size of gene product in:		Most similar gene product (species) (% amino acid identity/% amino acid similarity) (accession no.) known function of gene product (reference)
		Residues	kDa	
benR	Regulatory protein	318	36.4	XylS (*P. putida*) (59/73) (P07859) *meta*-cleavage benzoate degradation operon transcriptional activator (23) EutR (*Salmonella typhimurium*) (27/44) (Q9ZFU7) ethanolamine degradation operon transcriptional activator (32) RhaS (*S. typhimurium*) (25/47) (P09377) L-rhamnose degradation operon transcriptional activator (53) ArgR (*P. aeruginosa*) (26/47) (AAC45653) arginine biosynthesis operon transcriptional activator (47)
benA	Benzoate dioxygenase	452	51.5	XylX (*P. putida*) (73/86) (P23099) toluate 1,2-dioxygenase α subunit (18) BenA (*Acinetobacter* sp.) (68/80) (P07769) benzoate 1,2-dioxygenase α subunit (38) CbdA (*Burkholderia cepacia*) (59/76) (CAA55681) 2-halobenzoate 1,2-dioxygenase α subunit (16) AntA (*Acinetobacter* sp.) (47/65) (AAC34813) anthranilate dioxygenase α-subunit (2)
benB	Benzoate dioxygenase	161	19.1	XylY (*P. putida*) (76/89) (P23100) toluate 1,2-dioxygenase β subunit (18) BenB (*Acinetobacter* sp.) (68/79) (P07770) benzoate 1,2-dioxygenase β subunit (38) CbdB (*B. cepacia*) (57/73) (CAA55682) 2-halobenzoate 1,2-dioxygenase β subunit (16) AntB (*Acinetobacter* sp.) (34/56) (AAC34814) anthranilate dioxygenase β subunit (2)
benC	Ferredoxin reductase	336	36.4	XylZ (*P. putida*) (74/86) (P23101) methylcatechol 1,2-dioxygenase reductase component (18) BenC (*Acinetobacter* sp.) (51/67) (S23479) benzoate 1,2-dioxygenase reductase component (37) CbdC (*B. cepacia*) (46/64) (CAA55683) 2-halobenzoate 1,2-dioxygenase reductase component (16) AntC (*Acinetobacter* sp.) (38/57) (AAC34815) anthranilate 1,2-dioxygenase reductase component (2)
benD	*cis*-Diol dehydrogenase	253	27.1	XylL (*P. putida*) (78/86) (P23102) 2-hydro-1,2-dihydroxymethylbenzoate dehydrogenase (37) BenD (*Acinetobacter* sp.) (58/71) (P07772) 2-hydro-1,2-dihydroxybenzoate dehydrogenase (37) CmtC (*P. putida*) (34/49) (AAB62287) 2,3-dihydroxy-2,3-dihydro-*p*-cumate dehydrogenase (9) TsaC (*Comamonas testosteroni*) (32/46) (AAB62287) *p*-sulfobenzyl alcohol dehydrogenase (27)
benK	Benzoate transporter	443	47.1	BenK (*Acinetobacter* sp.) (41/58) (AAC46425) benzoate transporter (5) PcaK (*Acinetobacter* sp.) (29/45) (Q43975) 4-hydroxybenzoate transporter (33) PcaK (*P. putida*) (29/44) (Q51955) 4-hydroxybenzoate transporter (19) VanK (*Acinetobacter* sp.) (24/41) (AAC27108) vanillate transporter (49)
benE	Unknown	399	41.3	BenE (*Acinetobacter* sp.) (39/58) (P07775) membrane protein of unknown function
benF	Porin	398	43.2	PhaK (*P. putida*) (44/57) (AAC24339) porin-like phenylacetate-specific-channel-forming protein (43) OprE (*P. aeruginosa*) (38/55) (S34969) anaerobically induced porin (54) OprD (*P. aeruginosa*) (37/51) (P32722) imipenem-specific-channel-forming protein (21) OprE3 (*P. aeruginosa*) (33/49) (BAA22267) porin-like outer membrane protein (42)

cells grown on a mixture of benzoate and 4-HBA expressed β-galactosidase from the *pcaK* promoter to levels that were fivefold lower than those seen in cells grown on 4-HBA only. This repressive effect of benzoate on 4-HBA-induced *pcaK* expression was not seen when the *pcaK-lacZ* fusion was present in a *benR* mutant (Fig. 6). This indicates that BenR is involved in benzoate-mediated repression of *pcaK* expression. To determine whether BenR directly regulates *pcaK*, we examined the expression of the *pcaK-lacZ* fusion in *E. coli* BL21 cells in the presence and absence of BenR and benzoate. Low

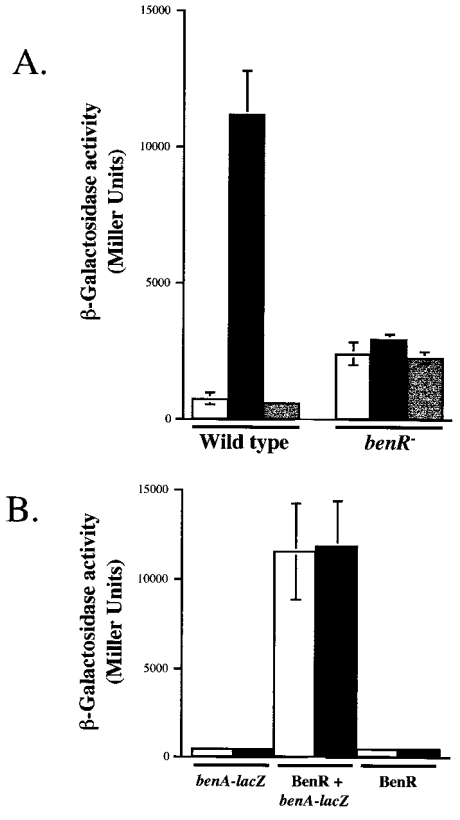

FIG. 4. (A) β-Galactosidase activities of the *benA-lacZ* fusion (pCCH101) in wild-type (PRS2000) and *benR* mutant (PRS4157) cells. Cells were grown on succinate (white), succinate plus benzoate (black), or succinate plus catechol (gray) and assayed as described in Materials and Methods. (B) β-Galactosidase activities of *E. coli* cells harboring the *benA-lacZ* fusion (pCCH101) and/or the BenR expression construct (pCCH106) in the absence (white) or presence (black) of 1 mM benzoate. β-Galactosidase activity was assayed as described in Materials and Methods.

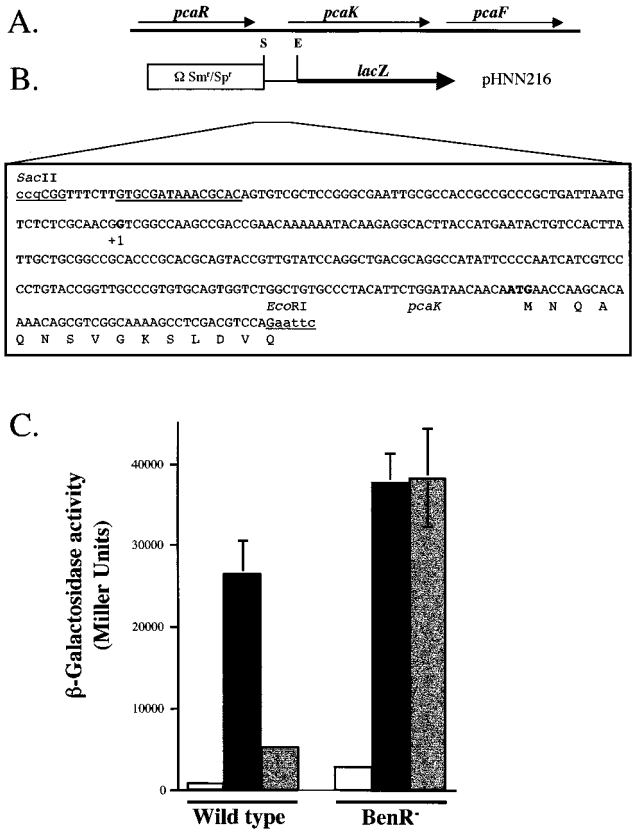

FIG. 6. (A) Map of the chromosomally encoded *pcaRKF* cluster of 4-HBA degradation genes. (B) Map of the *pcaK-lacZ* fusion, pHNN216. The putative PcaR binding site is heavily underlined. Restriction sites introduced by cloning are lightly underlined. The transcriptional and translational start sites are in bold (36). (C) β-Galactosidase activities of the *pcaK-lacZ* fusion in wild-type (PRS2000) and *benR* mutant (PRS4157) cells. Cells were grown on succinate (white), 4-HBA (black), or 4-HBA plus benzoate (gray), and β-galactosidase activity was assayed as described in Materials and Methods.

levels of *pcaK-lacZ* expression that were measured in *E. coli* were not influenced by BenR or benzoate (data not shown).

To show that the observed repressive effects of BenR on *pcaK* transcription have physiological significance, we measured rates of 4-HBA uptake in wild-type and *benR* mutant cells grown on 4-HBA only or on a mixture of 4-HBA and benzoate. When grown on 4-HBA alone, the wild type and the *benR* mutant accumulated 4-HBA intracellularly from the external medium at nearly identical rates of about 25 nmol per min per mg of protein. After growth on a mixture of benzoate and 4-HBA, wild-type cells accumulated 4-HBA very poorly at a 10-fold lower rate, whereas *benR* mutant cells took up 4-HBA from the medium at rates equivalent to those for cells grown on 4-HBA alone (Fig. 7).

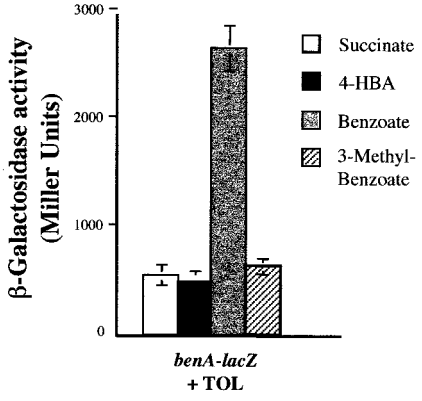

FIG. 5. β-Galactosidase activities of *benR* mutant (PRS4157) cells carrying the *benA-lacZ* fusion plasmid (pCCH101) and the TOL plasmid. Cells were grown on succinate, succinate plus 4-hydroxybenzoate, succinate plus benzoate, or succinate plus 3-methylbenzoate, as indicated, and assayed as described in Materials and Methods.

DISCUSSION

A cluster of genes similar to the *P. putida ben* genes described here is also present in the gram-negative, nonmotile soil bacterium *Acinetobacter* sp. strain ADP1. This microbe has genes in the order *benP benK benM benABDE* (4, 26). Biochemical evidence indicates that the *Acinetobacter benA*, *-B*, and *-C* genes encode benzoate 1,2-dioxygenase and the *benD* gene encodes 2-hydro-1,2-dihydroxybenzoate dehydrogenase (39). The *Acinetobacter benK* gene encodes a benzoate transporter (5). We have assigned these functions to the homologous *benABCD* and *-K* genes from *P. putida* (Fig. 3). The *benE* genes of *Acinetobacter* and *P. putida* are homologous but do not resemble any known genes in the databases. *benF* from *P.*

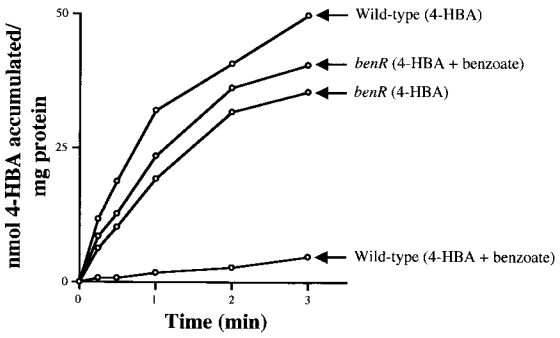

FIG. 7. Representative 4-HBA uptake assay of wild-type (PRS2000) and *benR* mutant (PRS4157) cells grown on 4-HBA or 4-HBA plus benzoate as indicated. Assays were performed as described in Materials and Methods.

putida and *benP* from *Acinetobacter* each resemble porins in deduced amino acid sequence, although they do not resemble each other very much. The proposed porin function has yet to be demonstrated for either organism. A major difference between the two *ben* gene clusters is that they are controlled by members of two different families of regulatory proteins. Whereas expression of the *Acinetobacter ben* genes is controlled by BenM, a member of the LysR family of regulatory proteins (4), *P. putida ben* gene expression is regulated by BenR, an AraC/XylS family member.

BenR not only activates expression of the *benABC* genes in response to benzoate but also represses the 4-HBA-inducible expression of the 4-HBA transport protein, PcaK. Thus, BenR has the effect of shutting down the ability of cells to take up 4-HBA from their environment when benzoate is present. This provides a mechanism for the preferential degradation of benzoate by *P. putida* cells given a mixture of benzoate and 4-HBA. Regional regulation of aromatic compound degradation is not unique to *P. putida*; *Acinetobacter* sp. strain ADP1 also degrades benzoate in preference to 4-HBA (12). There is some evidence that BenM plays a role in mediating this preference (4).

Genes termed *benR* that regulated expression of chromosomal *benABCD* genes and that also activated *Pm* from the TOL plasmid were described for *P. putida* and *P. aeruginosa* some years ago (6, 25) but were not sequenced. Subsequently, Kessler et al. (30) concluded that very similar, if not identical, *Pm* sequence elements were recognized by the chromosomally encoded regulator BenR and the TOL plasmid-encoded regulator XylS. This prompted speculation that *benR* should be homologous to *xylS*; however, no hybridization between *xylS* and *benR* DNAs was detected in Southern hybridization experiments (25). This left open the possibility that two different types of regulators might be able to activate *Pm*. Results presented here show that the chromosomally encoded *P. putida benR* gene that activates *Pm* is, in fact, homologous to *xylS*.

Members of the AraC/XylS family of regulators are found widely distributed among bacteria. The family is characterized by a consensus sequence in the C-terminal 100 amino acids that includes two helix-turn-helix motifs that are proposed to mediate binding of the regulator to DNA (13). BenR has this consensus sequence. The activity of BenR and the *benA* promoter sequence have characteristics that match those of XylS activation of *Pm* transcription. The XylS protein is thought to bind to *Pm* as a dimer to a recognition sequence, $TGCAN_6GGNTA$, that is repeated between nucleotides −70 and −56 and between nucleotides −49 and −35 (15). The *benA* promoter contains a direct repeat sequence between nucleotides −68 and −34 that matches the experimentally determined XylS binding site almost exactly (Fig. 3). In interacting with the downstream binding site that overlaps the −35 binding site for RNA polymerase, BenR may compete with RNA polymerase for binding to DNA. This is consistent with our observation of a threefold-higher basal level of β-galactosidase expression from the *benA* promoter under nonactivating conditions in the *benR* mutant compared to the wild type (Fig. 4A). Kaldalu et al. (28) have suggested that the N-terminal region of XylS interacts with its C-terminal domain to cause intramolecular repression of XylS function. On binding the benzoate effector, the N-terminal domain is proposed to undergo a change in conformation that then allows the C-terminal domain of XylS to function to allow initiation of transcription. The N-terminal regions of BenR and XylS share about 65% amino acid identity (Fig. 2), and BenR, like XylS, responds to benzoate as an effector. We found that BenR activates *Pm* expression to high levels in the absence of the benzoate effector when it is overexpressed in an *E. coli* background. A similar observation has been made with XylS (24, 28, 35). In the latter case, it has been proposed that a small amount of XylS is always present in a conformation that is active and able to stimulate transcription. When XylS is produced in large amounts, there is enough of the active form available to induce high levels of transcription in the absence of the effector (28, 35).

The effects of BenR in repressing transcription from the *pcaK* promoter may be indirect. We were unable to demonstrate an effect of BenR on *pcaK* expression in an *E. coli* background. Also, there are no detailed reports of an AraC/XylS family member responding to an effector molecule to mediate repression of gene transcription. Moreover, there is no recognizable XylS/BenR binding site in the *pcaK* promoter region. Clearly, much more study is required to determine the exact role of BenR in repressing *pcaK* transcription in response to benzoate.

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