JOURNAL OF BACTERIOLOGY, Sept. 1994, p. 5771–5779
0021-9193/94/$04.00+0

Vol. 176, No. 18

Characterization of the *pcaR* Regulatory Gene from *Pseudomonas putida*, Which Is Required for the Complete Degradation of *p*-Hydroxybenzoate

SANDRA ROMERO-STEINER,[1] REBECCA E. PARALES,[2] CAROLINE S. HARWOOD,[2] AND JOHN E. HOUGHTON[1]*

Department of Biology, Georgia State University, Atlanta, Georgia 30303,[1] and Department of Microbiology, University of Iowa, Iowa City, Iowa 52242[2]

Received 25 March 1994/Accepted 14 July 1994

The *pca* branch of the β-ketoadipate pathway in *Pseudomonas putida* is responsible for the complete degradation of *p*-hydroxybenzoate through *ortho* cleavage of the initial pathway metabolite, protocatechuate. The *pcaR* regulatory locus has been found to be required for both induction of all of the genes within the *pca* regulon (*pcaBDC*, *pcaIJ*, and *pcaF*) and the chemotactic response of the bacteria to aromatic compounds. Insertional inactivation mutagenesis, using Tn*5* and mini-Tn*5* transposons, was used to locate, clone, and sequence this *pcaR* regulatory gene. The *pcaR* gene product, when overexpressed in *Escherichia coli*, possessed a specific affinity for the *pcaIJ* promoter region and demonstrated that the entire PcaR protein was required for this function. The deduced amino acid sequence of the PcaR regulatory peptide bears little resemblance to its counterpart in the other branch of the pathway, CatR, but exhibits significant homology to its regulatory antecedent, PobR, which regulates the initial breakdown of *p*-hydroxybenzoate into protocatechuate. Comparisons of the *pcaIJ* and *pcaR* promoter regions revealed conservation of a 15-bp sequence centered around the −10 region in both sequences. This, together with previously defined deletional studies with the *pcaIJ* promoter region, suggests that PcaR exerts its regulatory effect through protein-DNA interactions within this region, which would be unusually close to the transcriptional start site of *pcaIJ* for a positive regulator.

The capacity to dissimilate aromatic compounds by *ortho* cleavage of the benzene ring is a characteristic common to many fluorescent soil bacteria (44) and is achieved by enzymes of the β-ketoadipate pathway. Extensive biochemical analyses of these enzymes in *Pseudomonas putida* (26, 27, 29, 31) have defined the pathway as being a convergent series of catabolic steps that degrade protocatechuate and catechol into Krebs cycle intermediates (15) (Fig. 1). Transcriptional regulation of the genes within the pathway admirably reflects their biochemical distinction into the *cat* and *pca* branches. The *catBCA* operon, which constitutes the total gene complement of the *cat* branch, is regulated by the transcriptional activator CatR (4, 15) when bound to the pathway intermediate *cis,cis*-muconate (34, 36). Organization of the *pca* genes, which are required for complete degradation of *p*-hydroxybenzoate (POB), is considerably more complex. The genes responsible for the six enzymes in this branch of the pathway are arranged within four distinct cistrons or operonic clusters, *pcaHG* (9), *pcaBDC* (16, 50), *pcaIJ* (33), and *pcaF* (Fig. 1). With the exception of *pcaHG*, which is regulated by protocatechuate (13), the remaining genes of the regulon are subject to transcriptional induction by β-ketoadipate in association with the product of a regulatory locus, *pcaR* (16, 33). This regulatory locus, first defined by its transpositional inactivation (16), has also been shown to be involved in regulation of the chemotactic response of *P. putida* to aromatic compounds (8, 12).

Although the mechanisms involved in the regulation of the *cat* branch of the pathway in different *Pseudomonas* species have received some attention (34, 36, 37), until recently (16, 33), little concern has been given to defining the regulation of the *pca* branch within the pseudomonads. In this report, we describe the use of transpositional mutagenesis to locate, clone, and sequence the *pcaR* regulatory gene from *P. putida* PRS2000, a wild-type derivative of *P. putida* type strain PRS1 (ATCC 12633) (45). We also report a definition of the gene's transcriptional initiation site, a verification of its function, and a determination of the deduced primary sequence of its gene product. Such analyses demonstrate that the *pcaR* gene encodes a DNA-binding protein that exhibits significant homology to a functionally related PobR protein from *Acinetobacter calcoaceticus* (7), confirming that these two regulatory proteins belong to an evolutionarily distinct group of bacterial regulatory proteins.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this investigation are listed in Table 1. All of the *P. putida* strains were derived from PRS2000 (45).

Media and growth conditions. *P. putida* and *Escherichia coli* cultures were grown at 30 and 37°C, respectively, in either Luria broth (21), defined minimal medium (31), or M9 minimal medium (21) supplemented with trace elements and appropriate carbon sources (31). Unless otherwise specified, aromatic compounds were supplied at a concentration of 5 mM and other sources of carbon were supplied at 10 mM. When required for selection, the antibiotics ampicillin (75 μg/ml), gentamicin (10 μg/ml), kanamycin (KM; 30 μg/ml), and tetracycline (12 μg/ml) were added.

Chemicals and reagents. All of the enzymes and chemicals used in this study were obtained from commercial sources and were of reagent grade or of the highest quality available.

Purification and in vitro manipulation of nucleic acids. Preparations of plasmid DNA were obtained by a modification of the alkali lysis method (39). Chromosomal DNA prepara-

* Corresponding author.

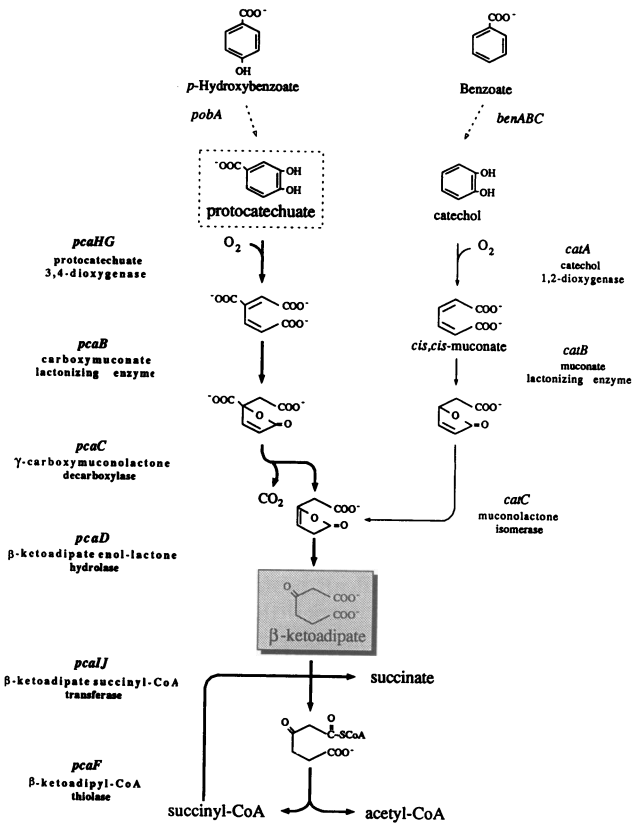

FIG. 1. β-Ketoadipate pathway in *P. putida*, which involves the catabolism of protocatechuate (heavy arrows) and catechol (light arrows) into Krebs cycle intermediates. Also shown are two of the metabolic precursors for the pathway, POB and BEN. The metabolic intermediate β-ketoadipate (shaded box) serves as the inducer molecule for the *pcaBDC*, *pcaIJ*, and *pcaF* genes. Also boxed is protocatechuate (dashed box), which induces the transcription of *pcaHG*. CoA, coenzyme A.

tions were performed by a modification of the method of Berns and Thomas (2). RNA was extracted from *P. putida* cells grown in defined medium to an to an optical density at 600 nm of 0.4, as described by Lu et al. (19).

Isolation of mini-Tn*5* insertion mutants. Mini-Tn*5* *pcaR* mutants *P. putida* were isolated in a manner similar to that defined by Hughes et al. for isolation of *pcaR*::Tn*5* mutant strain PRS3015 (16). Following overnight growth in L broth, 0.1 ml of *E. coli* S17-1λpir(pUTmini-Tn*5*Km [Table 1]) was mixed with 0.1 ml of *P. putida* PRS2000 on L broth agar and grown overnight at 30°C. The resulting cell growth was resuspended in 0.5 ml of basal medium and plated on selective medium containing Km, 1 mM succinate, and 5 mM POB, so as to give approximately 100 colonies per plate. Following prolonged incubation, small, Km-resistant colonies were screened for the inability to grow at the expense of either POB or benzoate (BEN).

DNA sequence analysis. The nucleotide sequence of the *pcaR* gene was determined by a modification of the dideoxynucleotide chain termination method of Sanger et al. (40), using enzymes and reagents supplied by United States Biochemical (except the [^{35}S]dATP radioactive label, which was purchased from Amersham, Inc.). The DNA sequence of *pcaR* and the surrounding DNA was determined on both strands (Table 1 and Fig. 2). Commercially available universal and reverse primers (USB) and a variety of additional primers that were complementary to DNA sequences within the *pcaR* gene region were used. The defined sequences were further confirmed by additional sequence analysis with deaza-dGTP (USB). Interpretation of the DNA sequence and the deduced amino acid sequence was assisted by the MacVector computer analysis program (version 4.1; IBI/Kodak).

Primer extensions. A modification of previously described procedures (14) was used to characterize the transcriptional initiation site of the *pcaR* transcript in total RNA isolated from *P. putida* PRS2000 grown under conditions that either induced or did not induce expression of *pca* genes. A synthetic oligonucleotide with the sequence 5′-CATGGCGGCCGAAGC GGGTCGCGCCGGCTC-3′ (Fig. 3) was chosen to yield quantifiable reverse transcription products from the *pcaR* transcript. This oligonucleotide was labelled with [γ^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Boehringer) in accordance with the manufacturer's specifications and used to prime the reverse transcription of cellular RNA in accordance with previously described methods (51).

PCR amplification and cloning of the *pcaR* gene. The PCR technique was used to specifically amplify the *pcaR* gene from the wild-type chromosome of *P. putida* PRS2000 by using two primers (primer 1, 5′-AAGGTACCTGACCAGTCTCTC CAA-3′; primer 2, 5′-CGGAGGCCAAGGTACCGAACAA CATGCCG-3′), the locations of which (relative to *pcaR*) are shown in Fig. 3. The amplification reaction was performed in a Perkin Elmer-Cetus thermalcycler for 35 cycles under standard PCR conditions, except that 10% dimethyl sulfoxide was used to reduce the secondary structure of the template DNA (17). The amplified PCR product (1,690 bp) was ligated into a pCR II vector (Invitrogen Co., San Diego, Calif.) by means of the T/A cloning technique, which takes advantage of the 3′ overhang of an A residue (a normal artifactual consequence of PCR amplification [20]). The presence of the insert in β-galactosidase-negative isolates was confirmed by appropriate restriction endonuclease digestion of plasmid DNA and by DNA sequence analysis.

Complementation of *pcaR* mutants. Conjugative transfers of broad-host-range vector pHS110, carrying the cloned *pcaR* gene (Table 1), between the *E. coli* donor strain (S17-1) and *P. putida* recipient strains PRS3015 and PRS4021 were performed in accordance with a previously published protocol (16). Patch matings were incubated for 4 h or overnight at 30°C on LB agar. After each series of matings, the cells were harvested and washed with M9 medium and plated onto M9 medium containing 5 mM POB, supplemented with KM and tetracycline. After 5 days of incubation at 30°C, the complemented colonies demonstrated sufficient growth to be replica plated onto the same medium with both antibiotics or Km or tetracycline alone. Only those isolates capable of utilizing POB as the sole carbon source under all three conditions were considered to have been complemented in *trans*.

Overexpression of the *pcaR* gene. To overexpress the *pcaR* gene product, the amplified PCR product was placed under the control of the late ϕ10 promoter of the RNA polymerase of phage T7 (47). An *Eco*RI fragment (~1,700 bp) containing the complete *pcaR* gene was cloned into a pT7-6 vector which had been similarly digested. This construct was designated pHS104 (Fig. 2). The orientation of the inserted DNA fragment was confirmed by restriction digest and DNA sequence analysis. The plasmid was transformed into *E. coli* K38, which already harbored plasmid pGP1-2, which is necessary for expression of the T7 RNA polymerase after heat induction of the heat-sensitive λ repressor at 42°C.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype[a] or phenotype[b]	Source or reference
P. putida		
PRS2000	Wild-type PRS1 (ATCC 12633) derivative	28, 45
PRS3015	*pcaR*::Tn*5* BEN$^-$ POB$^-$	16
PRS4041	*pcaR*::mini-Tn*5* BEN$^-$ POB$^-$	This work
E. coli		
K38	HfrC (λ)	38
DH5α	F$^-$ ϕ80 Δ*lacZ* ΔM15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* (r_K^- m_K^+) *thi-1 supE44 gyrA96 relA1*	Gibco-BRL
S17-1λpir	*thi pro hdsR hdsM*$^+$ *recA*; chromosomal insertion of RP4-2 (Tc::Mu Km::Tn*7*) lysogenized with phage λpir	42
JM101	*endA gyrA96 thi hdsR17 supE44 relA lacY* (*lac proAB*) (F′ *traD36 proAB lacI*^{q} *Z* M15)	48
Plasmids		
pT7-6	Ap^r; T7 polymerase (ϕ10); ColE1 origin	47
pUC19	Ap^r; *lac* promoter-operator; ColE1 origin	48
pUTmini-Tn*5*Km	Ap^r Km^r; ColE1 origin	6
pCR II	Ap^r Km^r; *lacZ′* (T7 promoter); ColE1 origin	Invitrogen
pGP1-2	Km^r *c*I856 (Ts); *p*L:polT7	47
pRK415	Tc^r; IncP broad-host-range vector	18
pPX42	Ap^r Km^r; pUC19 carrying 20.2-kbp *Eco*RI fragment with partial *pcaR*::Tn*5*	16
pHRP125	Ap^r; pUC18 carrying 350-bp *Eco*RI fragment encompassing promoter-operator region of *pcaI*	33
pHRP501	Ap^r Km^r; pUC18 carrying 4.8-kbp *Kpn*I fragment with complete *pcaR*::mini-Tn*5*	This work
pHS100	Ap^r Km^r; pCR II carrying 1.69-kbp PCR product containing *pcaR* from PRS2000	This work
pHS104	Ap^r Km^r; pT7-6 carrying 1.7-kbp *Eco*RI fragment from pHS100	This work
pHS105	Ap^r Km^r; pT7-6 carrying 1.45-kbp *Bam*HI-*Kpn*I fragment from pHS100	This work
pHS110	Tc^r; pRK415 carrying 1.7-kbp *Eco*RI fragment from pHS100	This work

[a] Genetic designations in the text are in parentheses.
[b] Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Tc, tetracycline.

Overexpression of the cloned *pcaR* gene was achieved by using L-[^{35}S]methionine (Amersham) in a manner similar to that defined by Tabor and Richardson (47) but with some modifications. Cells (1 ml) were grown to an optical density at 600 nm of 0.4 in Luria broth supplemented with 50 μg of ampicillin per ml and 30 μg of Km per ml. The cells were subsequently pelleted and washed with M9 medium, resuspended, and incubated for 1 h in 5 ml of M9 medium, which had been enriched with 0.4% glucose, 1 mM $MgCl_2$, 12.5 μg of ampicillin per ml, 7.5 μg of Km per ml, trace amounts of thiamine hydrochloride, and a 40-μg/ml mixture of 19 amino acids (lacking methionine). Selective expression of the *pcaR* gene was accomplished by heat induction of the cells for 30 min at 42°C (to activate T7 RNA polymerase), and following addition of 200 μg of rifampin per ml (to inhibit *E. coli* RNA polymerase activity), the cells were incubated for an additional 2 h at 30°C.

PcaR protein extracts. Crude extracts of the expressed PcaR protein were obtained after growth of *E. coli* K38(pHS104) in LB broth to an A_{600} of 0.4 at 30°C, followed by induction for 30 min at 42°C as described above. The induced cells were allowed to continue growing for an additional 12 h at 30°C, after which time they were harvested by 10 min of centrifugation at 7,600 × *g*. The pellet was resuspended in a buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–KOH (pH 7.5), 60 mM KCl, 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (22). The cells were disrupted by two passages through an Aminco French pressure cell at 12,000 lb/in^2. The lysate was centrifuged at 45,000 × *g* for 30 min, and the supernatant was recentrifuged at high speed (225,000 × *g*) in a Beckman Optima TL tabletop ultracentrifuge. The protein concentration of the resulting supernatant was determined by using the Bradford protein assay (3). A similar protocol was followed when producing crude extracts for the negative control, the pT7-6 vector, and the pcaR′ construct (pHS105) in *E. coli* K38.

DNA mobility shift assays. A 350-bp DNA fragment, derived from an *Eco*RI digest of pHRP125 (Table 1) and encompassing the operator region of the *pcaIJ* operon (33), was chosen to evaluate the capacity of the PcaR protein to bind a specific DNA sequence. This 350-bp fragment was end labelled with [γ-^{32}P]dATP (3,000 Ci/mmol; NEN, Boston, Mass.) by using polynucleotide kinase (in accordance with the manufacturer's specifications). The DNA-binding assays were performed in accordance with the protocol of Nègre and Cortay (22), with some minor modifications.

Enzyme assays. The following enzymes of the β-ketoadipate pathway were assayed in accordance with published spectrophotometric procedures: *cis,cis*-muconate-lactonizing enzyme (EC 5.5.1.1), the *catB* gene product (25); β-carboxy-*cis,cis*-muconate-lactonizing enzyme (EC 5.5.1.2), the *pcaB* gene product (24); and β-ketoadipate:succinyl coenzyme A transferase (EC 2.8.3.6), the *pcaIJ* gene product (52). For each assay, cell extracts were prepared from *P. putida* cultures that had been harvested in the exponential phase (A_{660}, 0.25 to 0.5). The cells were then washed once with minimal medium, pelleted, and stored at −20°C until just prior to disruption by sonication. Protein concentrations were determined by the method of Bradford (3).

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to GenBank and assigned accession number L33795.

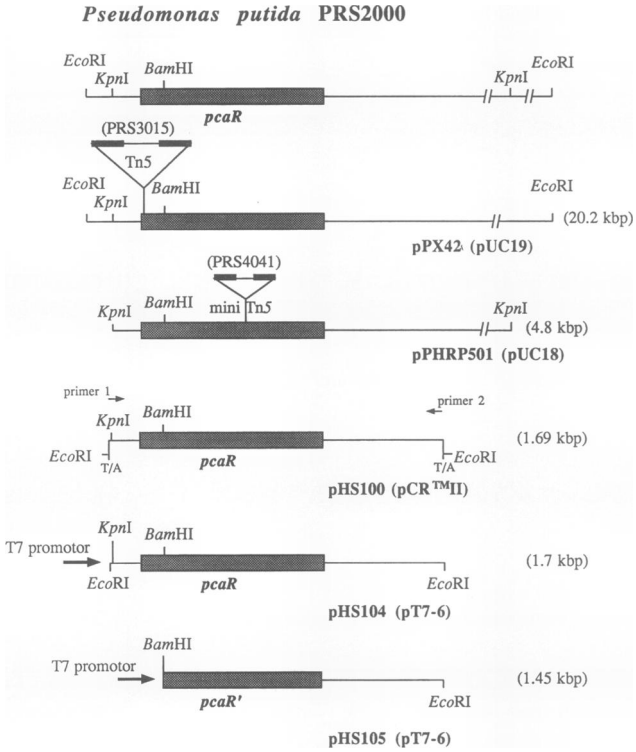

FIG. 2. Restriction map of the region of DNA around the *pcaR* gene of type strain *P. putida* PRS2000, along with sites of Tn*5* and min-Tn*5* insertions into mutant strains PRS3015 and PRS4021. Also shown are a number of the relevant cloned DNA segments within various recombinant plasmid vectors (Table 1) that were isolated in the course of the investigation. In addition, the relative locations of primers 1 and 2, which were used to amplify and clone the native *pcaR* gene from the chromosome of PRS2000, are depicted as arrows.

RESULTS

Tn*5* and mini-Tn*5* mutagenesis of *pcaR*. Transpositional mutagenesis of *P. putida* with mini-Tn*5* was employed to define insertionally inactivated mutants of *pcaR*. Isolation of such mutants was considered necessary in view of the high phenotypic reversion rates that were observed for the original *pcaR*::Tn*5* mutation within PRS3015 (16). A number of mutant strains that exhibited resistance to KM, as well as inability to grow with either BEN or POB as the sole carbon source were isolated. One of these isolates, PRS4041, expressed enzymes of the β-ketoadipate pathway at low constitutive levels when grown in the presence of β-ketoadipate or its nonmetabolizable analog adipate. Expression of muconate-lactonizing enzyme, an enzyme within the *cat* branch of the pathway (Fig. 1) that is not under the control of β-ketoadipate, was unaffected in this mutant (Table 2), suggesting that only enzymes within the *pca* branch of the pathway had been affected. Thus, PRS4041 had the phenotype expected for a *pcaR* mutant (16). Chromosomal DNA from PRS4041 was isolated and digested with *Kpn*I, an enzyme that does not restrict within the minitransposon. The digested DNA was cloned into pUC18 and transformed into *E. coli* DH5α by using resistance to ampicillin and KM to confirm the presence of both the vector and the transposon. The resulting plasmid, pHRP501, was found to contain a 4.8-kbp insert, including mini-Tn*5* (Table 1 and Fig. 2). The restriction digest map of the plasmid was analyzed, and the inserted chromosomal DNA was subsequently sequenced. Information derived from these analyses was compared with that from similar analyses of various subclones of pPX42, a *Sal*I partial digest clone of the *pcaR*::Tn*5* mutation within PRS3015. Overlapping restriction fragment and DNA sequence analyses revealed that the two transposons had been inserted approximately 500 bp away from each other.

Cloning and DNA sequence analysis of the *pcaR* gene. By using the DNA sequences derived from the cloned DNAs of the two mutant *pcaR* strains, two oligonucleotide primers that would yield a 1.69-kbp fragment of DNA encompassing the complete *pcaR* structural gene were synthesized. The resulting PCR fragment was cloned into pCRII (Invitrogen), yielding pHS100, and thereafter into pT7-6 (pHS104) and pRK415 (pHS110), as described in Table 1 and Fig. 2. That the amplified fragment encoded the entire *pcaR* structural gene was confirmed by the ability of pHS110 to complement both PRS3015 and PRS4041, allowing them to grow on POB and BEN (while still maintaining their resistance to both Km and tetracycline) at a frequency that was markedly above that observed for pseudoreversion rates (16).

The DNA sequence of the *pcaR* structural gene is given in Fig. 3. A potential open reading frame of 876 bp was defined and verified on both strands from both the wild-type strain and its transpositionally inactivated derivatives. This open reading frame would begin at an AUG codon (position +27 [Fig. 3]) and encode a polypeptide of 292 amino acids, with a deduced molecular mass of 31,808 Da and an apparent pI of 7.47. DNA encompassing this putative open reading frame has a G+C content of approximately 66%, which is consistent with that of other genes from *Pseudomonas* species (32) (GenBank, version 71), as are the codon usage and the preference for a UGA stop codon (49). The extent of the putative open reading frame, however, is not the only possible size for the PcaR polypeptide. Two additional methionine codons are present at amino acid positions 23 and 45, either of which might also serve to initiate translation, as both are preceded by DNA sequences that form respectable ribosomal binding site sequences.

Quantitative primer extension analysis. To define the 5′ end of the *pcaR* transcript, a 30-base oligonucleotide complementary to bases 66 to 95 of the sequence in Fig. 3 was hybridized to total cellular RNA derived from *P. putida* PRS2000 cells that had been grown in minimal medium supplemented with either glucose or glucose and POB. As defined in Materials and Methods, this primer was extended by using reverse transcriptase, resulting in the presence of a single major cDNA product approximately 95 bases long, corresponding to an A residue at the +1 position of the defined sequence (Fig. 3 and 4). Sequence analysis of the upstream region revealed appropriately positioned TTGcgA and gATAAT sequences that adequately correspond to consensus −35 and −10 sequences, respectively, for an *E. coli* σ^{70} RNA polymerase promoter region (35). The amount of *pcaR* transcript formed by cells was not dramatically influenced by growth in the presence of POB, which gave only a 1.47-fold increase in expression of the transcript (Fig. 4), indicating that induction of this transcript by POB or any of the residual *pca* pathway intermediates, such as β-ketoadipate, is negligible.

Identification and overexpression of the *pcaR* gene product. The 1.7-kbp *Eco*RI fragment from pHS100 was cloned into pT7-6 (Fig. 2) in an orientation that would allow expression of the *pcaR* gene from the T7 RNA polymerase promoter. The resulting plasmid (pHS104) was then transformed into a suitable host, *E. coli* DH5α(pGP1-2) (Table 1). Following T7 polymerase induction, overproduction of a single protein with an apparent molecular mass of 32,000 Da was observed (Fig. 5,

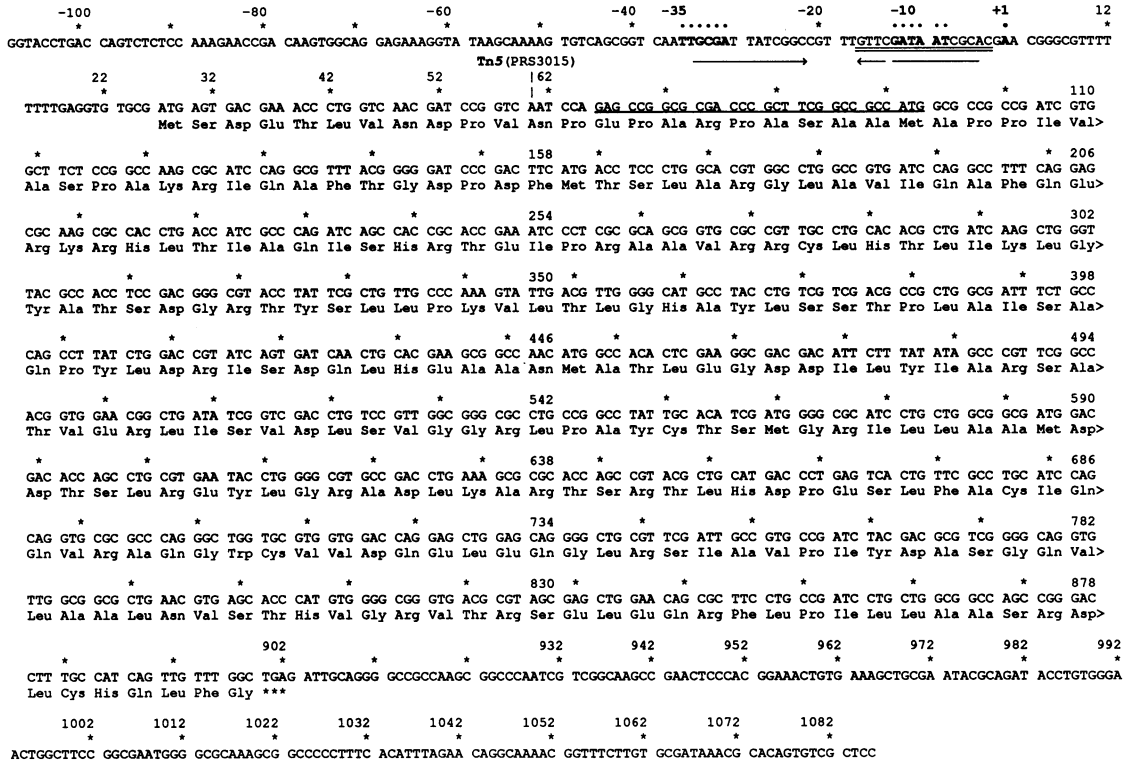

FIG. 3. Nucleotide sequence of the coding strands for the *pcaR* region from type strain *P. putida* PRS2000. Deduced amino acid sequences are shown below the corresponding DNA sequences, which are in bold print. Also in bold and defined by a dot above each base are nucleotides that make up the −35 and −10 recognition sequences and the transcriptional +1 sequence for the *pcaR* transcript (as determined by primer extension). The annotation Tn5 designates the site where insertion of the transposon has been documented within the *pcaR* mutant gene from PRS3015. The DNA sequence complementary to the oligonucleotide primer that was used for primer extension is underlined. Double underlining denotes the 15-bp sequence that is identical in both position and content to the *pcaIJ* promoter region. Convergent arrows underneath the proposed promoter sequence denotes a region of defined secondary structure (specific to the *pcaR* promoter) that involves 14 of these 15 conserved residues.

lane 5). This molecular mass was in excellent agreement with that of PcaR deduced by DNA sequence analysis, i.e., 31,808 Da.

Use of L-[^{35}S]methionine to monitor the overexpression of cloned genes was also employed to define which of the three potential methionine codons was most likely used to initiate translation of PcaR. Advantage was taken of a fortuitously positioned *Bam*HI restriction site, located at base 146 of the sequence given in Fig. 3, lying within the proposed open reading frame for the structural *pcaR* gene and 12 bases upstream of the third possible AUG start codon. In so doing, it also encodes most of an RNA sequence that would provide an adequate ribosome-binding site for this start codon. Removal of a *Bam*HI fragment from pHS104 (Fig. 2), therefore, effectively deletes the region upstream of this *Bam*HI site and results in the potential for overexpression of this foreshortened gene by the T7 promoter. As depicted in Fig. 5, lane 6, a protein with a molecular mass of approximately 28,000 Da is uniquely overexpressed after heat-induced expression of the T7 RNA polymerase. This molecular mass agrees well with

TABLE 2. Expression of *pcaIJ*, *pcaB*, and *catB* in Tn*5* derivatives of *P. putida*

Strain	Phenotype (genotype)	Growth conditions[a]	Activity (mU/mg) of [b]:		
			TR (*pcaIJ*)	CMLE (*pcaB*)	MLE[c] (*catB*)
PRS2000	Wild type	Glucose	4.3	120.0	31.0
		Glucose + adipate	39.0	1,850.0	196.0
PRS3015	BEN$^-$ POB$^-$ (*pcaR*::Tn*5*)	Glucose	6.6	170.0	47.0
		Glucose + adipate	6.2	160.0	110.0
PRS4041	BEN$^-$ POB$^-$ (*pcaR*::Tn*5*)	Glucose	3.9	140.0	20.0
		Glucose + adipate	4.5	162.0	67.0

[a] Cells were grown with either 10 mM glucose (uninduced) or 10 mM glucose plus 20 mM adipate (induced).
[b] TR, β-ketoadipate:succinyl coenzyme A transferase; CMLE, carboxymuconate-lactonizing enzyme; MLE, muconate-lactonizing enzyme.
[c] Muconate-lactonizing enzyme (encoded by *catB*) was assayed to verify that the adipate was entering the cells and still inducing the genes of the *cat* branch of the β-ketoadipate pathway.

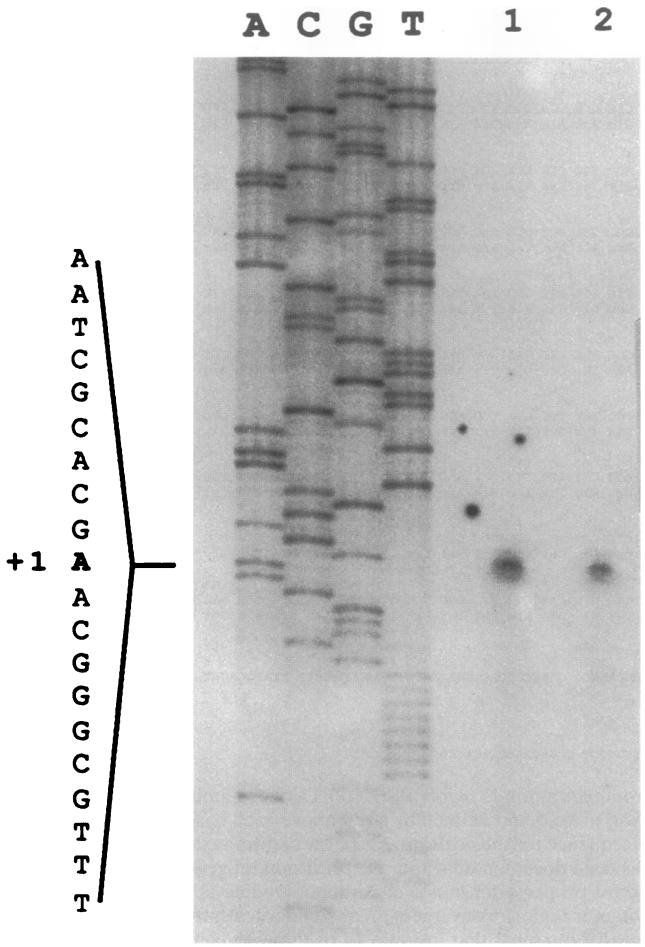

FIG. 4. Quantitative 5′ extension experiments done to define the differential transcription of *pcaR* from cells grown in the presence or absence of POB. Lanes: 1, PRS2000 in minimal medium, glucose, and POB; 2, PRS2000 in minimal medium and glucose. The DNA sequence ladder adjacent to the primer extension reaction lanes was derived by DNA sequence analysis of pHS104 with the same primer that was used in the primer extension analyses. The relative intensities of the primer extension cDNA bands (as defined by densitometry) were 343.1 (lane 1) and 234.1 (lane 2) (expressed arbitrary densitometric units).

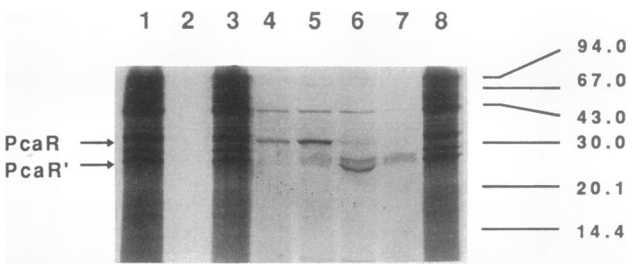

FIG. 5. Expression of the *pcaR* gene product in *E. coli*. An autoradiogram of a sodium dodecyl sulfate–12% polyacrylamide gel showing the variable expression of different proteins under a variety of uninduced and induced conditions is shown. Lanes: 1, pT7-6 (no rifampin); 2, pT7-6 (with rifampin); 3, pHS104 (no rifampin); 4, pHS104 (with rifampin); 5, pHS104 (with rifampicin and induction at 42°C for 30 min); 6, pHS105 (with rifampin and induction at 42°C for 30 min); 7, pHS105 (with rifampin); 8, pHS105 (no rifampin).

that expected for the truncated polypeptide on the basis of DNA sequence analysis i.e., 27,234 Da.

PcaR specifically binds to DNA containing the *pcaIJ* promoter. Overexpression of PcaR in *E. coli* allowed the determination of its ability to bind specifically to DNA. The DNA of choice was the promoter-operator region of the recently defined *pcaIJ* operon from *P. putida* (33). A 350-bp *Eco*RI fragment of plasmid pHRP125 (33) encompasses this region and served as the DNA template for mobility shift assays with the expressed PcaR protein. As depicted in Fig. 6A, various concentrations of PcaR protein extracted from *E. coli* (pHS104) cells were shown to bind specifically to the 350-bp fragment, resulting in distinct retardation of the electrophoresed DNA. Moreover, mobility shift assays with similar extracts of *E. coli* cells harboring either the original pT7-6 vector or plasmid pHS105 (carrying the truncated *pcaR* gene) failed to produce specific retardation of the same DNA (Fig. 6B). These findings indicate that the complete 32,000-Da *pcaR* gene product is required for full DNA-binding function.

Comparison of PcaR with other regulatory proteins. Comparison of the deduced amino acid sequence of PcaR with protein sequences within the National Biomedical Research Foundation protein database revealed only scant homology to any defined regulatory protein and was significant only in comparison to certain members of the GntR family of regulatory proteins (43), in particular, the IclR repressor protein from *Salmonella typhimurium*, which is involved in the regulation of genes required for acetate utilization (46). Comparison of PcaR with a recently defined regulatory protein, PobR from *Acinetobacter calcoaceticus* (7), yielded the most convincing amino acid sequence similarities (28% sequence identity), especially in a region of the PobR primary sequence that is proposed to encompass an α-helix–turn–α-helix structural motif (7). Moreover, such comparisons between PcaR and PobR enhanced the primary sequence alignment of PcaR with IclR, resulting in an amino acid sequence identity of up to 20%. The POB hydroxylase gene, the target of PobR regula-

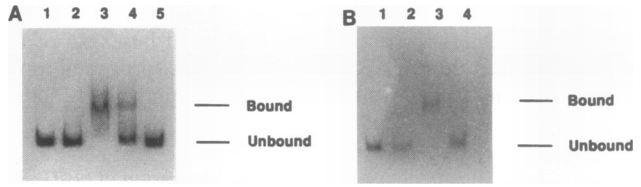

FIG. 6. (A) Mobility shift assays of a 350-bp *Eco*RI-digested DNA fragment of pHRP125 that encompasses the promoter region of the *pcaI* structural gene of *P. putida* (34). End-labelled DNA (~2 ng/1,680 cpm) was electrophoresed on a 6% nondenaturing polyacrylamide gel after incubation with various concentrations of *E. coli* cell extracts. Lanes: 1, free DNA probe; 2, 5 μg of *E. coli* DH5α(pT7-6); 3, 10 μg of *E. coli* DH5α(pHS104); 4, 1 μg of *E. coli* DH5α(pHS104); 5, 0.1 μg of *E. coli* DH5α(pHS104). (B) Mobility shift assays of a 350-bp *Eco*RI DNA fragment of pHRP125, encompassing the promoter region of the *pcaI* structural gene of *P. putida* (34). End-labelled DNA (~2 ng/800 cpm) was electrophoresed on a 6% nondenaturing polyacrylamide gel after incubation with various concentrations of cell extracts from *E. coli* that had been transformed with different vectors (as defined in Materials and Methods). Lanes: 1, free DNA probe; 2, 5 μg of *E. coli* DH5α(pT7-6); 3, 5 μg of *E. coli* DH5α(pHS104); 4, 5 μg of *E. coli* DH5α(pHS105).

tion (7), converts POB into protocatechuate (the first substrate of the β-ketoadipate pathway [Fig. 1]).

DISCUSSION

It has previously been shown that both the *pcaR* regulatory gene product and the metabolite β-ketoadipate are required for expression of five of the enzymes that catalyze POB degradation in *P. putida* (16, 33). Here, two independently isolated *pcaR* mutant strains enabled the cloning of the *pcaR* gene, which had been independently inactivated by Tn*5* and mini-Tn*5* and located by use of Km resistance as a selectable marker. DNA sequence information obtained from these clones was used to PCR amplify and clone a 1.69-kbp region of DNA that complemented both *pcaR* mutants and restored their ability to grow on POB and BEN.

DNA sequence analysis demonstrated that this cloned fragment contained the entire *pcaR* structural gene, which is 876 bp long and encodes a polypeptide of 292 amino acids (Fig. 3) (with a deduced molecular mass of 31,808 Da). Although three possible start codons lie in frame with the proposed *pcaR* gene, only a single polypeptide of the size (32 kDa) predicted for the full-length product was expressed in *E. coli* upon induction of the gene from a T7 promoter. Furthermore, a truncated gene product (28 kDa) initiated from the third possible start codon failed to retard migration of the *pcaIJ* promoter fragment in DNA mobility shift assays (Fig. 6). These results strongly suggest that translation of *pcaR* is initiated from the first methionine codon, although N-terminal amino acid sequencing of the purified PcaR protein is required to show this unequivocally.

Quantitative primer extension analyses further demonstrated that expression of the *pcaR* transcript is initiated 27 bp upstream of this proposed open reading frame for the *pcaR* structural gene (Fig. 4). This transcript would be initiated from a promoter with significant similarity to an *E. coli* consensus σ^{70} (*rpoD*) promoter sequence (Fig. 3) (35), which has been shown to behave in a manner very similar to that of *Pseudomonas* σ^{70} promoter sequences (10, 11).

Such analyses also indicate that the *pcaR* transcript itself is constitutively expressed at low levels (Fig. 4) and that its expression is also relatively insensitive to the presence of the inducer molecule β-ketoadipate. These findings are consistent with regulatory genes in a number of other inducible pathways that are involved in aromatic degradation within fluorescent soil bacteria (15). However, unlike that of a number of these regulatory elements (which characteristically share an overlapping promoter with some divergently expressed gene[s] within their regulatory system [5, 34]), the location of the *pcaR* regulatory gene within the *P. putida* chromosome is somewhat removed from those of all other defined genes within the pathway (16, 33). The *pcaBDC* and *pcaIJ* operons (known to be regulated by PcaR) are unlinked to each other and to this *pcaR* gene cluster. The *pcaR* gene must, therefore, exert its inductive effect upon these gene clusters solely in *trans*.

An independent study of chemotaxis of *P. putida* to POB (11a) has led to the identification of a cluster of three genes which, in addition to *pcaR*, includes a gene required for POB chemotaxis (*pcaK*) and *pcaF*, which encodes the last enzyme in the β-ketoadipate pathway (Fig. 1). All three of these genes are transcribed in the same direction, with *pcaR* being the first gene in the sequence. The transcriptional organization of *pcaR*, *pcaK*, and *pcaF*, however, has not been defined.

Consistent with its proposed regulatory function, the PcaR protein (when expressed in crude *E. coli* extracts) was shown to possess a specific DNA-binding affinity for the *pcaIJ* promoter ($pcaIJ_{po}$), as evidenced by a specific shift in the mobility of a 350-bp promoter-containing DNA fragment on acrylamide gels. Although it is likely that PcaR interacts with β-ketoadipate to activate transcription of the various target genes, addition of β-ketoadipate to the mobility shift assay mixture did not alter the migration of the PcaR-$pcaR_{po}$ complex (data not shown).

Deletion analysis of the *pcaIJ* promoter region has indicated that a DNA sequence proximal to the −10 region is sufficient for specific induction of these genes, implying that the binding site for transcriptional activation by PcaR lies very close to the transcriptional start site of the *pcaIJ* operon (33). While any clear-cut determination of the binding affinity of PcaR for this region awaits further experiments with the purified PcaR protein, it is worthwhile to note that comparisons between the upstream region of *pcaR* and the corresponding region of *pcaIJ* revealed 15 bp (5′-GTTCGATAATCGCAC-3′) that were conserved at precisely the same location in both promoter regions, centered around the −10 region (Fig. 3). Such sequence and position conservation suggests that some of the bases within these 15 bp may serve as a PcaR-binding site. As previously noted (33), the positioning of an operator region so close to the transcriptional start site is unusual for an inducible system and suggests a possible mechanism for induction of the *pcaIJ* gene that is similar to that for induction of the mercury resistance operon in *E. coli*. In this case, the MerR protein and RNA polymerase bind to opposite faces of the DNA strand to effect open-complex formation (1, 23).

With respect to possible *pcaR* autoregulation, binding of PcaR to the promoter operator region of its own promoter might be expected to reduce binding of RNA polymerase. Curiously, the 15-bp sequence in the *pcaR* promoter region ($pcaR_{po}$) lies within a region of strong dyad symmetry (Fig. 3). It is quite possible, therefore, that this additional twofold symmetry relates to its functionally distinct repressive autoregulation.

Sequence and function comparisons between the two analogous regulatory proteins of the β-ketoadipate pathway, PcaR and CatR, would be expected, since they share marked similarities in size, effect upon related genes within their respective branches, and specific affinity for the gratuitous inducer molecule adipate (16, 30, 33). The unusual positioning of a putative PcaR-binding site within the −10 region of *pcaIJ*, however, suggests that if the two proteins (PcaR and CatR) do share similar heritages, they would exert their respective inductive effects through two entirely different mechanisms.

Despite some regional DNA sequence similarities, comparison of the two primary sequences of PcaR and CatR from *P. putida* indicates that the two regulatory proteins bear little resemblance to each other and that if they are related, they are only distantly so. Comparisons of PcaR with other known regulatory proteins show significant homology with the recently defined PobR regulatory protein from *A. calcoaceticus* (7). These two proteins demonstrate additional primary sequence similarities to two other regulatory proteins, IclR (46) and GylR (43), which appear to form a distinct family of evolutionarily related regulatory proteins (7), the PobR family. It is curious, however, that within this family of regulatory proteins, while PobR, PcaR, and GylR serve as activator proteins for the genes in their respective pathways, IclR functions as a repressor (22, 46).

Such amino acid sequence comparisons between PcaR and its metabolically defined regulatory antecedent, PobR, are intriguing. They suggest that the regulatory proteins of the β-ketoadipate pathway within fluorescent soil bacteria exhibit more branch-specific evolutionary development than pathway

similarity. The recently defined *pobR* gene product (7) (albeit from *A. calcoaceticus*) functions in regulating expression of the POB-inducible POB hydroxylase gene required for conversion of POB into protocatechuate, the first catabolite specific to the *pca* branch of the β-ketoadipate pathway. Its significant primary sequence similarity to PcaR from *P. putida* indicates that the two proteins share an evolutionary heritage which reflects their similar roles in regulating POB catabolism. Such sequence homology (28%) is in marked contrast to that of CatR and other members of its family of activator proteins (the LysR family). These activator proteins are found in a wide variety of inducible systems within a number of gram-negative bacteria, and characteristically share exceptionally strong homology (up to 41%) with each other, especially within their N-terminal DNA-binding domains (41). The significance of these similarities and differences among the regulatory genes involved in aromatic dissimilation through *ortho* cleavage in the fluorescent pseudomonads, therefore, awaits further definition.

ACKNOWLEDGMENTS

We thank Joshua A. Appel, Timothy Brown, and Phang C. Tai for helpful and insightful comments.

This work was funded by grant GMBC94.025.E to J.E.H. and by NSF grant DMB-9103860 to C.S.H. R.E.P. was supported by NIH training grant GM08365.

REFERENCES

1. **Ansari, A., M. L. Chael, and T. V. O'Halloran.** 1992. Allosteric underwinding of DNA is a critical step in positive control of transcription by Hg-MerR. Nature (London) **355**:87–89.
2. **Berns, K. I., and C. A. Thomas, Jr.** 1965. Isolation of high molecular weight DNA from *Haemophilus influenzae*. J. Mol. Biol. **11**:476–490.
3. **Bradford, M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Brown, T. M., A. J. Appel, E. J. Hughes, and J. E. Houghton.** 1992. Preliminary characterization and DNA sequence analysis of *catR, BCA* in *P. putida*, biotype A, K-38, p. 262. 92nd Gen. Meet. Am. Soc. Microbiol. 1992. American Society for Microbiology, Washington, D.C.
5. **Coco, W. M., R. K. Rothmel, S. Henikoff, and A. M. Chakrabarty.** 1993. Nucleotide sequence and initial functional characterization of the *clcR* gene encoding a LysR family activator of the *clcABD* chlorocatechol operon in *Pseudomonas putida*. J. Bacteriol. **175**: 417–427.
6. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172**:6568–6572.
7. **DiMarco, A. A., B. Averhoff, and L. N. Ornston.** 1993. Identification of the transcriptional activator *pobR* and characterization of its role in the expression of *pobA*, the structural gene for *p*-hydroxybenzoate hydroxylase in *Acinetobacter calcoaceticus*. J. Bacteriol. **175**:4499–4506.
8. **Dispensa, M., R. E. Parales, and C. S. Harwood.** 1989. Regulation of benzoate chemotaxis in *Pseudomonas putida*, K-180, p. 275. 89th Annu. Meet. Am. Soc. Microbiol. 1989. American Society for Microbiology, Washington, D.C.
9. **Frazee, R. W., D. M. Livingston, D. C. La Porte, and J. D. Lipscomb.** 1993. Cloning, sequencing, and expression of the *Pseudomonas putida* protocatechuate 3,4-dioxygenase genes. J. Bacteriol. **175**:6194–6202.
10. **Gao, J., and G. N. Gussin.** 1991. RNA polymerases from *Pseudomonas aeruginosa* and *Pseudomonas syringae* respond to *Escherichia coli* activator proteins. J. Bacteriol. **173**:394–397.
11. **Han, C.-H., I. P. Crawford, and C. S. Harwood.** 1991. Up-promoter mutations in the *trpBA* operon of *Pseudomonas aeruginosa*. J. Bacteriol. **173**:3756–3762.

11a. **Harwood, C. S. et al.** Unpublished data.

12. **Harwood, C. S., M. Rivelli, and L. N. Ornston.** 1984. Aromatic acids are chemoattractants for *Pseudomonas putida*. J. Bacteriol. **160**:622–628.
13. **Hosokawa, K.** 1970. Regulation of synthesis of early enzymes of the *p*-hydroxybenzoate pathway in *Pseudomonas putida*. J. Biol. Chem. **245**:5304–5308.
14. **Houghton, J. E., T. M. Brown, A. J. Appel, E. J. Hughes, and L. N. Ornston.** Unpublished data.
15. **Houghton, J. E., and M. S. Shanley.** 1994. Catabolic potential of pseudomonads: a regulatory perspective, p. 11–32. *In* R. Chaudhry (ed.), Biological degradation and bioremediation of toxic chemicals. Discorides Press, Portland, Oreg.
16. **Hughes, E. J., M. J. Shapiro, J. E. Houghton, and L. N. Ornston.** 1988. Cloning and expression of *pca* genes from *Pseudomonas putida* in *Escherichia coli*. J. Gen. Microbiol. **134**:2877–2887.
17. **Innis, M. A., and D. H. Gelfand.** 1990. Optimization of PCRs, p. 3–12. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, Calif.
18. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for cloning in gram-negative bacteria. Gene **70**:191–197.
19. **Lu, C. D., M. Kilstrup, J. Neuhard, and A. Abdelal.** 1989. Pyrimidine regulation of tandem promoters for *carAB* in *Salmonella typhimurium*. J. Bacteriol. **171**:5436–5442.
20. **Mead, D. A., N. K. Pey, C. Hernstadt, R. Marcil, and L. M. Smith.** 1991. A universal method for the direct cloning of PCR amplified nucleic acid. Bio/Technology **9**:657–663.
21. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 433. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Nègre, D., and J.-C. Cortay.** 1991. Overproduction and characterization of the *iclR* gene product of *Escherichia coli* K-12 and comparison with that of *Salmonella typhimurium* LT2. Gene **97**:29–37.
23. **O'Halloran, T. V., B. Frantz, K. Shin, D. M. Ralston, and J. G. Wright.** 1989. The MerR heavy metal receptor mediates positive activation in a topologically novel transcription complex. Cell **56**:119–129.
24. **Ornston, L. N.** 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. II. Enzymes of the protocatechuate pathway. J. Biol. Chem. **241**:3787–3794.
25. **Ornston, L. N.** 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. III. Enzymes of the catechol pathway. J. Biol. Chem. **241**:3795–3799.
26. **Ornston, L. N.** 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. IV. Regulation. J. Biol. Chem. **241**:3800–3810.
27. **Ornston, L. N.** 1971. Regulation of catabolic pathways in *Pseudomonas*. Bacteriol. Rev. **35**:87–116.
28. **Ornston, L. N., and D. Parke.** 1976. Properties of an inducible uptake system for β-ketoadipate in *Pseudomonas putida*. J. Bacteriol. **125**:475–488.
29. **Ornston, L. N., and D. Parke.** 1976. Evolution of catabolic pathways in *Pseudomonas putida*. Biochem. Soc. Trans. **4**:468–473.
30. **Ornston, L. N., and D. P. Parke.** 1977. The evolution of induction mechanisms in bacteria: insights derived from study of the β-ketoadipate pathway, p. 209–262. *In* B. L. Horecker and E. R. Stadtman (ed.), Current topics in regulation. Academic Press, Inc., New York.
31. **Ornston, L. N., and R. Y. Stanier.** 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. I. Biochemistry. J. Biol. Chem. **241**:3776–3786.
32. **Palleroni, N. J.** 1984. Genus I. *Pseudomonas* Migula, p. 141–199. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
33. **Parales, R. E., and C. H. Harwood.** 1993. Regulation of the *pcaIJ* genes for aromatic degradation in *Pseudomonas putida*. J. Bacteriol. **175**:5829–5838.
34. **Parsek, M. R., D. L. Shinabarger, R. K. Rothmel, and A. M. Chakrabarty.** 1992. Roles of CatR and *cis,cis*-muconate in activation of the *catBC* operon, which is involved in benzoate degradation in *Pseudomonas putida*. J. Bacteriol. **174**:7798–7806.
35. **Raibaud, O., and M. Schwartz.** 1984. Positive control of transcrip-

tion initiation in bacteria. Annu. Rev. Genet. **18:**173–206.
36. **Rothmel, R. K., T. L. Aldrich, J. E. Houghton, W. M. Coco, L. N. Ornston, and A. M. Chakrabarty.** 1990. Nucleotide sequencing and characterization of *Pseudomonas putida catR*: a positive regulator of the *catBC* operon is a member of the LysR family. J. Bacteriol. **172:**922–931.
37. **Rothmel, R. K., D. L. Shinabarger, M. R. Parsek, T. A. Aldrich, and A. M. Chakrabarty.** 1991. Functional analysis of the *Pseudomonas putida* regulatory protein CatR: transcriptional studies and determination of the CatR DNA-binding site by hydroxyl-radical footprinting. J. Bacteriol. **173:**4717–4724.
38. **Russel, M., and P. Model.** 1984. Replacement of the *fip* gene of *Escherichia coli* by an inactive gene cloned on a plasmid. J. Bacteriol. **159:**1034–1039.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:** 5463–5467.
41. **Schell, M. A.** 1993. Molecular biology of the LysR family of transcriptional activators. Annu. Rev. Microbiol. **47:**597–627.
42. **Simon, R., U. Priefer, and A. Pühler.** 1984. A broad host range mobilization system for in vivo genetic engineering: random and site specific transposon mutagenesis in gram-negative bacteria. Biotechnology **1:**784–791.
43. **Smith, C. P., and K. F. Chater.** 1988. Structure and regulation of controlling sequences for the *Streptomyces coelicolor* glycerol operon. J. Mol. Biol. **204:**569–580.
44. **Stanier, R. Y., and L. N. Ornston.** 1973. The β-ketoadipate pathway. Adv. Microb. Physiol. **9:**89–151.
45. **Stanier, R. Y., N. J. Palleroni, and M. Douderoff.** 1966. The aerobic *Pseudomonads*: a taxonomic study. J. Gen. Microbiol. **48:**159–271.
46. **Sunnarborg, A., D. Klumpp, T. Chung, and D. C. La Porte.** 1990. Regulation of the glyoxylate bypass operon: cloning and characterization of *iclR*. J. Bacteriol. **172:**2642–2649.
47. **Tabor, S., and C. A. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
48. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13 mp7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
49. **West, S. E. H., and B. H. Iglewski.** 1988. Codon usage in *Pseudomonas aeruginosa*. Nucleic Acids Res. **16:**9323–9335.
50. **Williams, S. E., E. M. Woolridge, S. C. Ransom, J. A. Landro, P. C. Babbitt, and J. W. Kozarich.** 1992. 3-Carboxy-cis,cis-muconate lactonizing enzyme from *Pseudomonas putida* is homologous to the class II fumarase family: a new reaction in the evolution of a mechanistic motif. Biochemistry **31:**9768–9776.
51. **Wong, S. C., and A. T. Abdelal.** 1990. Unorthodox expression of an enzyme: evidence for an untranslated region within *carA* from *Pseudomonas aeruginosa*. J. Bacteriol. **172:**630–642.
52. **Yeh, W. K., and L. N. Ornston.** 1981. Evolutionary homologous $\alpha_2\beta_2$ oligomeric structures in β-ketoadipate succinyl-CoA transferases from *Acinetobacter calcoaceticus* and *Pseudomonas putida*. J. Biol. Chem. **256:**1565–1569.