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Regulation of the *pcaIJ* Genes for Aromatic Acid Degradation in *Pseudomonas putida*

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Six of the genes encoding enzymes of the β-ketoadipate pathway for benzoate and 4-hydroxybenzoate degradation in *Pseudomonas putida* are organized into at least three separate transcriptional units. As an initial step to defining this *pca* regulon at the molecular level, *lacZ* fusions were made with the *pcaI* and *pcaJ* genes, which encode the two subunits of β-ketoadipate:succinyl-coenzyme A transferase, the enzyme catalyzing the next-to-last step in the β-ketoadipate pathway. Fusion analyses showed that *pcaI* and *pcaJ* constitute an operon which requires β-ketoadipate or its nonmetabolizable analog, adipate, as well as the *pcaR* regulatory gene for induction. The *pcaIJ* promoter is likely to be a σ^{70}-type promoter; it has a σ^{70}-type consensus sequence and did not require the alternative σ factor, RpoN, for induction. Deletion analysis of the promoter region of a *pcaI-lacZ* transcriptional fusion indicated that no specific DNA sequences upstream of the −35 region were required for full induction. This implies that the binding site for the activator protein, PcaR, is unusually close to the transcriptional start site of *pcaIJ*.

In many aerobic organisms, aromatic compounds used as growth substrates are catabolized via the β-ketoadipate pathway. This pathway (Fig. 1) funnels compounds through either protocatechuate or catechol to the common intermediate, β-ketoadipate, which is then broken down to components of the tricarboxylic acid cycle.

Most of the studies of β-ketoadipate pathway regulation have focused on the catechol branch of the pathway. In *Pseudomonas putida*, catechol degradation requires the induction of the *catBC* genes by the inducer, *cis,cis*-muconate, and a positive regulatory element, *catR* (37). The CatR protein has been purified, and its mechanism of *catBC* operon activation has been investigated (34, 38). Sequence analysis of *catR* has revealed that the encoded protein is a member of the LysR family of transcriptional regulators (37). A closely related subgroup of this family includes a number of proteins (CatR [37], ClcR [2], CatM [25], TfdS [17], and TcbR [43]) that are involved in the degradation of catechol, chlorocatechols, or chlorinated phenols.

In contrast to the recent contributions to the understanding of the regulation of catechol degradation, the study of protocatechuate catabolic gene regulation has received little attention. In *P. putida*, five enzymes are required to degrade protocatechuate to tricarboxylic acid cycle intermediates. The six genes (*pca* genes) encoding these enzymes are induced by β-ketoadipate or its nonmetabolized analog, adipate. A regulatory gene, *pcaR*, is required for induction of *pca* structural gene expression (15). The *pcaR* gene has recently been cloned and sequenced (14), and its product is not a member of the LysR family but belongs to a new family of regulatory proteins which includes PobR, an activator of *p*-hydroxybenzoate hydroxylase gene expression in *Acinetobacter calcoaceticus*; IclR, the acetate operon repressor from *Escherichia coli*; and others (5).

This report describes an initial analysis of the *P. putida* *pca* regulon, focusing on the genes (*pcaI* and *pcaJ*) encoding the two subunits of the second-to-last enzyme of the pathway, β-ketoadipate:succinyl-coenzyme A (β-ketoadipate: succinyl-CoA) transferase. The activity of the *pcaI* promoter was studied in wild-type and regulatory mutant strains of *P. putida* as well as in *E. coli* and *Pseudomonas aeruginosa* by using a new *lacZ* transcriptional fusion vector. A deletion analysis was also performed to determine boundaries of the *pcaI* operator region.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively.

Media and growth conditions. Cultures of *P. putida* were grown at 30°C in defined mineral medium as described previously (30), except that media containing tetracycline were made with 1% rather than 10% mineral-salts solution. Carbon sources were sterilized separately and added at the time of inoculation (final concentrations: benzoate and 4-hydroxybenzoate, 5 mM; succinate, 10 mM; glucose, 10 mM). *P. aeruginosa* PAO1 was grown in the same medium but at 37°C. *P. aeruginosa* PAK-SR and N1 were grown in a similar manner but with the addition of 0.01% glutamine, since the *rpoN* mutant has this requirement (16). *E. coli* strains were grown in Luria broth at 37°C, except that cultures carrying fusion plasmids for β-galactosidase assays were grown in mineral medium with 10 mM glucose and 0.05% yeast extract. For *P. putida* and *P. aeruginosa*, antibiotics were used at the following final concentrations: tetracycline, 50 μg/ml; kanamycin, 100 μg/ml; gentamicin, 20 μg/ml; and streptomycin, 400 μg/ml. For *E. coli*, the following antibiotics were used: ampicillin, 100 μg/ml; tetracycline, 25 μg/ml; gentamicin, 20 μg/ml; streptomycin, 25 μg/ml; and kanamycin, 100 μg/ml. For Lac$^+$ colony screening, isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were added to final concentrations of 0.1 mM and 40 μg/ml, respectively. Solid media contained 1.5% agar.

Bacterial transformations and conjugations. *E. coli* was

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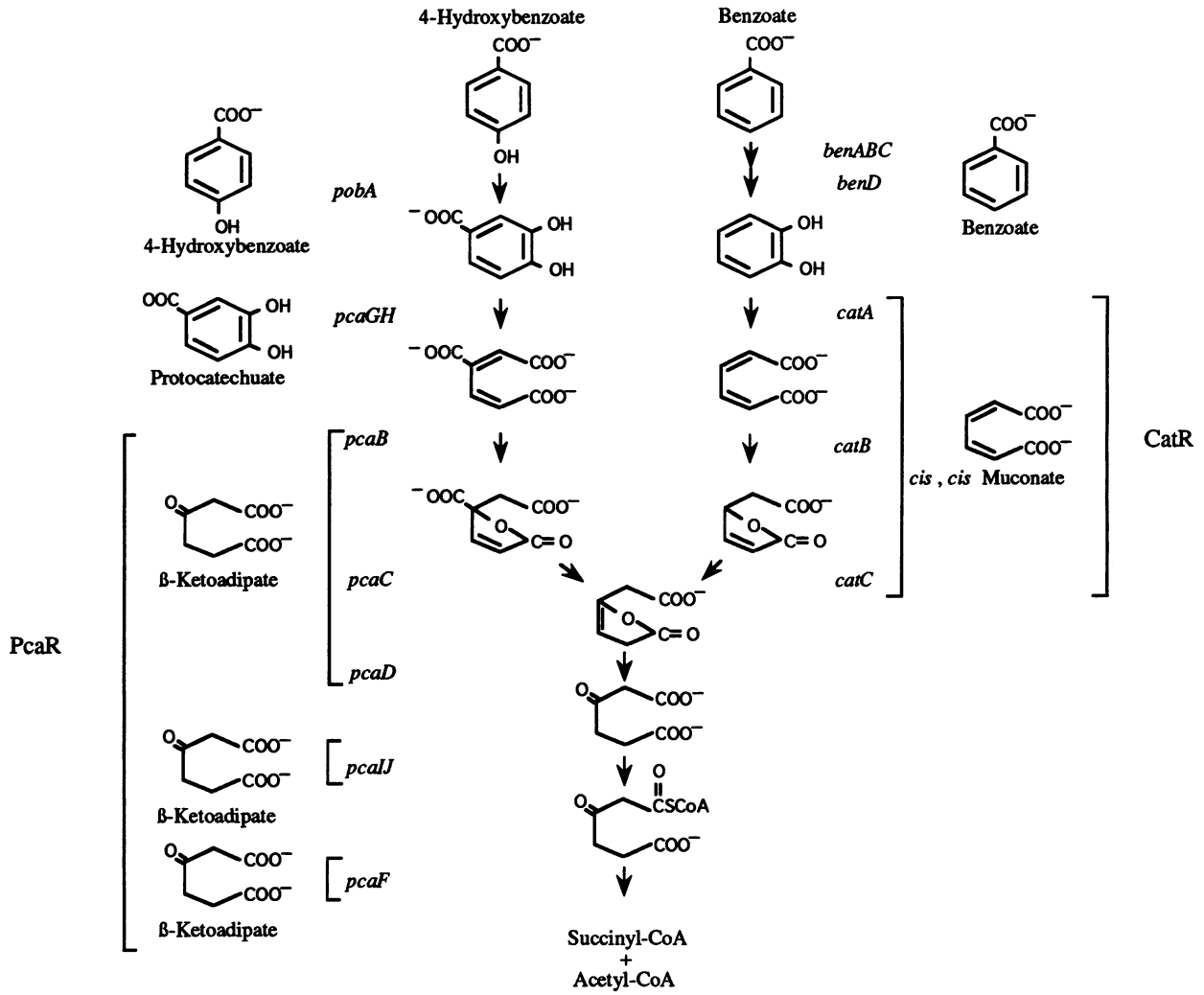


FIG. 1. The β-ketoadipate pathway and its regulation in fluorescent pseudomonads.

transformed with plasmid DNA according to the method of Hanahan (11). Plasmids were mobilized from *E. coli* S17-1 into *P. putida* by patch matings on Luria broth agar plates that were incubated overnight at 30°C. Triparental matings with HB101 carrying plasmid pRK2013 as the mobilizing strain were also done.

DNA manipulations. *P. putida* chromosomal DNA was purified from 50-ml cultures grown overnight in Luria broth as previously described (9). Plasmid DNA was purified by the method of Lee and Rasheed (23). Restriction endonuclease digestions were performed according to the instructions of the manufacturer (New England Biolabs, Beverly, Mass.). Dephosphorylation reactions with calf intestinal phosphatase (Boehringer Mannheim, Indianapolis, Ind.) were carried out as described previously (1). Ligation reactions and agarose gel electrophoresis methods were as described previously (39). DNA fragments for subcloning were purified from gel slices by using GeneClean (Bio 101, LaJolla, Calif.).

Measurement of enzyme activities. Two- or four-milliliter cultures were grown to mid-exponential phase for β-galactosidase assays. Gentamicin (20 μg/ml) was included in all growth media for strains carrying derivatives of plasmid pHRP309. Fusion plasmids were maintained in gentamicin-resistant strains with streptomycin (resistance encoded on the Ω cassette). β-Galactosidase assays were performed as described by Miller (24), using 2 drops of chloroform and 1 drop of 0.1% sodium dodecyl sulfate to permeabilize cells. All reported enzyme activities are the averages of several replicate assays of at least three independently grown cultures. Error bars in graphic representations of β-galactosidase activities are standard deviations.

Construction of vectors carrying a promoterless *lacZ* cassette. Plasmid pHRP314 was constructed by inserting the *Eco*RI fragment carrying the promoterless *lacZ* gene and kanamycin resistance gene from pUTmini-Tn*5lacZ1* into pUC1813 in both orientations. This new plasmid has several restriction sites flanking the *lacZ* and kanamycin resistance genes for easy excision and cloning.

Construction of gentamicin resistance gene cassettes for use

TABLE 1. Bacterial strains

Strain	Relevant characteristics[a]	Reference or source
P. putida		
PRS2000	Wild type	29
PRS3015	*pcaR*::Tn*5* Ben$^-$ 4-OH-Ben$^-$	15
PRS4020	*catR*::Gm Ben$^-$	This work
PRS4021	*catR*::Gm *pcaR*::Tn*5* Ben$^-$ 4-OH-Ben$^-$	This work
PRS4050	*pcaI-lacZ* chromosomal fusion	This work
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
HB101	F$^-$ *hsdS20 recA13 ara-14 proA2 lacY1 supE44 galK2 rpsL20 xyl-5 mtl-1*	39
P. aeruginosa		
PAO1	Wild type	13
PAK-SR	Streptomycin resistant	16
N1	*rpoN*::Tc	16

[a] Ben$^-$, no growth with benzoate; 4-OH-Ben$^-$, no growth with 4-hydroxybenzoate.

in insertional mutagenesis. The 3.4-kb *Pst*I fragment carrying the gentamicin resistance gene from pML7 (22) was inserted into the *Pst*I site of pUC7, forming pHRP300. By using flanking *Eco*RI sites, the gentamicin resistance gene from pHRP300 was subcloned into the *Eco*RI site of pUC1318 in both orientations, forming pHRP302 and pHRP306. These vectors have a variety of restriction sites flanking the gentamicin resistance gene and can be used for insertional inactivation of cloned genes.

Construction of a suicide plasmid carrying the *pcaI-lacZ* fusion and flanking *P. putida* DNA for use in the generation of a chromosomal insertion of the *lacZ* fusion. Plasmid pHRP123 is identical to pHRP122, except that the insert carrying the *pcaI* and *pcaJ* genes is inserted in the opposite orientation in the vector pHG165 (42). The promoterless *lacZ* gene was excised from pHRP314 on an *Sph*I-*Not*I fragment and inserted into *Sph*I-*Eag*I-cleaved pHRP123. This deleted the end of *pcaI* and the beginning of *pcaJ* and inserted the *lacZ* gene into the site of the deletion, between the *pcaI* and *pcaJ* genes and in the same orientation. This plasmid, pHRP188, was cleaved with *Xmn*I, which cuts twice in the pHG165 vector. The fragment carrying the *lacZ* gene and flanking *P. putida* DNA was inserted into the *Sca*I site of the suicide delivery vector pSUP102, forming pHRP191 (Fig. 2).

Promoter deletion constructions. Plasmid pHRP125 was digested with *Kpn*I, cutting at the 5′ end of the 350-bp *pcaI* promoter fragment. Batches of the cut plasmid were treated with *Bal* 31 (Boehringer Mannheim) for various lengths of time, and samples were run on agarose gels to determine the extent of digestion. Batches of plasmid that had been reduced in size by 100 to 500 bp were ethanol precipitated, cut with *Eco*RI, and run on agarose gels. DNA fragments in the 100- to 300-bp size range were purified from gels slices by using GeneClean and ligated into pK19 that had been cut with *Eco*RI and *Sma*I (*Eco*RI cuts in the pHRP125 vector at the opposite end of the insert from the site of Bal 31 digestion, and a reasonable proportion of the Bal 31-treated ends should be blunt and therefore compatible with *Sma*I). DH5α was transformed, and plasmid DNA was purified from white, kanamycin-resistant colonies. Insert sizes were determined on 1.5% agarose gels after cleavage with *Bam*HI and *Eco*RI. The exact deletion endpoints of the inserts from several plasmids were determined by dideoxy chain termination sequencing using the universal forward and reverse primers, a commercial kit from United States Biochemical Corp. (Cleveland, Ohio), and ^{35}S-dATP purchased from Amersham (Arlington Heights, Ill.). Four different deletion fragments were chosen and cloned into the *lacZ* fusion vector pHRP309 as follows. First, the ΩSm/Sp cassette was excised from pHRP310 with *Bam*HI and cloned into the *Bam*HI site upstream of each deletion promoter, forming pHRP160, -161, -162, and -170. Each Ω-promoter fragment was then excised with *Eco*RI and *Sal*I and cloned into pHRP309 that had been digested with the same enzymes. Plasmids pHRP163, -164, -165, and -171 were constructed in this way.

Two other promoter deletions were constructed by using the polymerase chain reaction (1). Two oligonucleotide primers, PCR2 and PCR3, were made (PCR2, 5′ CGGGATCCACCAGAACTGCTCGCA 3′; PCR3, 5′ CCGGATCCAAATTCCGCTAAACCC 3′), each with a *Bam*HI site (underlined) added to the 5′ end to simplify cloning of the amplified product. In separate experiments, each of these primers was used with the opposing primer PCR1 (32) and plasmid pHRP123 as the template. Amplified products were partially purified with 30,000-molecular-weight pore size Ultrafree-MC filters (Millipore Corp., Bedford, Mass.), cut with *Bam*HI and *Eco*RI, and cloned into pK18, forming pHRP166 and pHRP173. The ΩSm/Sp cassette was excised from pHRP315 with *Bam*HI and inserted into the *Bam*HI sites of pHRP166 and pHRP173, forming pHRP167 and pHRP174. The Ω-promoter fragments from these plasmids were inserted into pHRP309 with *Xba*I-*Eco*RI ends, finally forming pHRP168 and pHRP175.

Construction of pHRP154 and pHRP153. Two *pcaIJ′*::*lacZ* fusions were constructed to determine whether *pcaJ* is expressed from its own promoter or from the *pcaI* promoter. One fusion includes the *pcaI* upstream region (pHRP154), and the other has this region deleted (pHRP153; see Fig. 3A). The *pcaI* and *pcaJ* genes were excised from plasmid pHRP100 with *Eco*RV and *Bam*HI and inserted into pHRP317 that had been cut with *Sma*I and *Bam*HI, forming pHRP189. Plasmid pHRP154 was constructed by digesting pHRP189 with *Xba*I, partially digesting with *Eco*RI, and inserting the fragment carrying the Ω cassette, the *pcaI* promoter, *pcaI*, and part of *pcaJ* into pHRP309. To form a similar fusion with the entire *pcaI* promoter region deleted (pHRP153), first the 300-bp region upstream of *pcaI* was deleted from pHRP189 (up to the Shine-Dalgarno site of *pcaI*) by cleaving with *Sac*I and religating, forming

TABLE 2. Plasmids

Plasmid	Relevant properties[a]	Reference
pRK2013	Km^r, ColE1 origin, RP4 mobilization functions	7
pK18	Km^r, pUC18 polylinker	35
pK19	Km^r, pUC19 polylinker	35
pRK415	Tc^r, IncP, mobilizible cloning vector	19
pUC1318	Ap^r, combined pUC13 and pUC18 polylinker	18
pUC1813	Ap^r, combined pUC18 and pUC13 polylinker	18
pUC7	Ap^r, ColE1 replicon	44
pUC18	Ap^r, ColE1 replicon	45
pUT-mini-Tn*5lacZ1*	Ap^r Km^r, promoterless *lacZ* gene	4
pSUP102	Tc^r Cm^r, p15A replicon, *mob*$^+$	40
pHRP100	Tc^r, pRK415 with 8.5-kb *Eco*RI fragment carrying the *pcaIJ* genes	31
pHRP122	Ap^r, pHG165 with 3.3-kb *Pst*I fragment from PRS2000 carrying *pcaIJ* and upstream DNA	31
pHRP123	Ap^r, pHG165 with 3.3-kb insert from pHRP122 in the opposite orientation	This work, Fig. 3A
pHRP125	pUC1813 with the 350-bp *pcaI* upstream PCR product	32
pHRP150	Gm^r Sm^r, pHRP309 with Ω cassette and 350-bp *pcaI* promoter fragment fused to *lacZ*	32 and Fig. 3A
pHRP153	Gm^r Sm^r, pHRP309 with Ω cassette, promoterless *pcaI*, and part of *pcaJ* fused to *lacZ*	This work, Fig. 3A
pHRP154	Gm^r Sm^r, pHRP309 with Ω cassette, *pcaI* promoter, *pcaI*, and part of *pcaJ* fused to *lacZ*	This work, Fig. 3A
pHRP163	Gm^r Sm^r, pHRP309 with deleted *pcaI* promoter region fused to *lacZ*	This work, Fig. 5B
pHRP164	Gm^r Sm^r, pHRP309 with deleted *pcaI* promoter region fused to *lacZ*	This work, Fig. 5B
pHRP165	Gm^r Sm^r, pHRP309 with deleted *pcaI* promoter region fused to *lacZ*	This work, Fig. 5B
pHRP168	Gm^r Sm^r, pHRP309 with deleted *pcaI* promoter region fused to *lacZ*	This work, Fig. 5B
pHRP171	Gm^r Sm^r, pHRP309 with deleted *pcaI* promoter region fused to *lacZ*	This work, Fig. 5B
pHRP175	Gm^r Sm^r, pHRP309 with deleted *pcaI* promoter region fused to *lacZ*	This work, Fig. 5B
pHRP188	Ap^r, pHRP123 with promoterless *lacZ* gene inserted between *pcaI'* and *'pcaJ*	This work
pHRP191	Tc^r, pSUP102 with *lacZ* gene and flanking *P. putida* DNA from pHRP188	This work, Fig. 2
pHRP202	pHG165 with 1-kb *Eco*RV-*Hin*dIII fragment from pHRP200 carrying *catR* gene	This work
pHRP210	pHRP202 with 3.4-kb *Sac*I fragment with the Gm^r gene from pHRP302 in the *catR* gene	This work
pHRP211	pHRP210 *Hin*dIII-*Eco*RI fragment in pRK415	This work
pHRP302	Ap^r; Gm^r gene from pML7 (22) in pUC1318	This work
pHRP306	Ap^r; Gm^r gene from pML7 (22) in pUC1318 (opposite orientation to pHRP302)	This work
pHRP309	Gm^r, IncQ, *lacZ* transcriptional fusion vector	32
pHRP310	Km^r Sm^r, pK19 with ΩSm/Sp cassette in *Bam*HI site	32
pHRP314	Ap^r Km^r, promoterless *lacZ* gene and kanamycin resistance gene from pUT-miniTn*5lacZ1* in pUC1813	This work
pHRP315	Ap^r Sm^r, pUCBM20 with ΩSm/Sp cassette in the *Bam*HI site	32
pHRP317	Km^r Sm^r, ΩSm/Sp cassette from pHRP316 (*Msc*I-*Eco*RI fragment) in *Hin*cII-*Eco*RI of pK19	32

[a] Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance; PCR, polymerase chain reaction.

pHRP190. Plasmid pHRP190 was then cleaved with *Xba*I and *Eco*RI, and the Ω-promoterless *pcaI-pcaJ* fragment was inserted into pHRP309, forming pHRP153.

Construction of the *P. putida catR* and *pcaR catR* mutants by gene replacement. Plasmid pHRP200 carries a 5.5-kb *Kpn*I fragment from the PRS2000 chromosome on the vector pUC18. The presence of the *catR* gene on the clone has been verified by DNA sequence analysis (not shown). A subclone

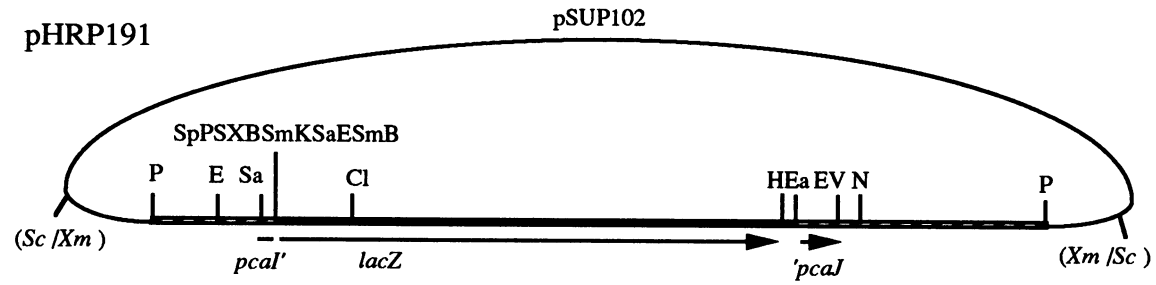
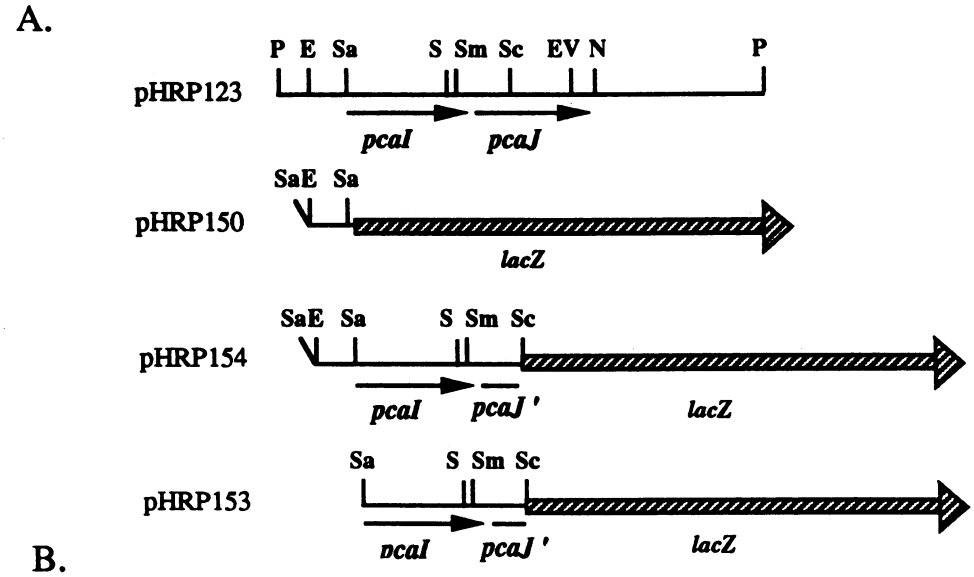


FIG. 2. Suicide vector used for delivery of the *pcaI-lacZ* fusion to the *P. putida* chromosome. See Materials and Methods for details of the construction. Abbreviations: B, *Bam*HI; Cl, *Cla*I; E, *Eco*RI; Ea, *Eag*I; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; N, *Nru*I; P, *Pst*I; Sa, *Sal*I; Sc, *Sca*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I; Xm, *Xmn*I.

of pHRP200, plasmid pHRP202, was digested with *Sac*I, and the 3.4-kb *Sac*I fragment from pHRP302 carrying a gentamicin resistance gene was inserted into the *Sac*I site within the *catR* coding sequence. The resulting plasmid, pHRP210, was cut with *Hin*dIII and *Eco*RI, and the *catR*-gentamicin resistance gene fragment was ligated with pRK415, forming pHRP211. Although pRK415 is a broad-host-range vector that replicates in *P. putida*, we have found that it is very unstable in the absence of selective pressure, as is the parent vector, pRK404 (6). For this reason, pRK415 can be used as a suicide delivery vector. To replace the wild-type *catR* gene with its insertionally inactivated counterpart, a triparental mating was done with DH5α(pHRP211) as donor, PRS2000 as recipient, and HB101(pRK2013) as the mobilizing strain. The mating mixture was plated onto minimal medium containing succinate and gentamicin. Gentamicin-resistant colonies were patched onto the same medium, as well as onto minimal medium containing succinate and tetracycline, and a gentamicin-resistant, tetracycline-sensitive strain was identified. As expected, this strain (PRS4020) was found to be incapable of growth with benzoate as the sole carbon source, since CatR is required for the induction of the



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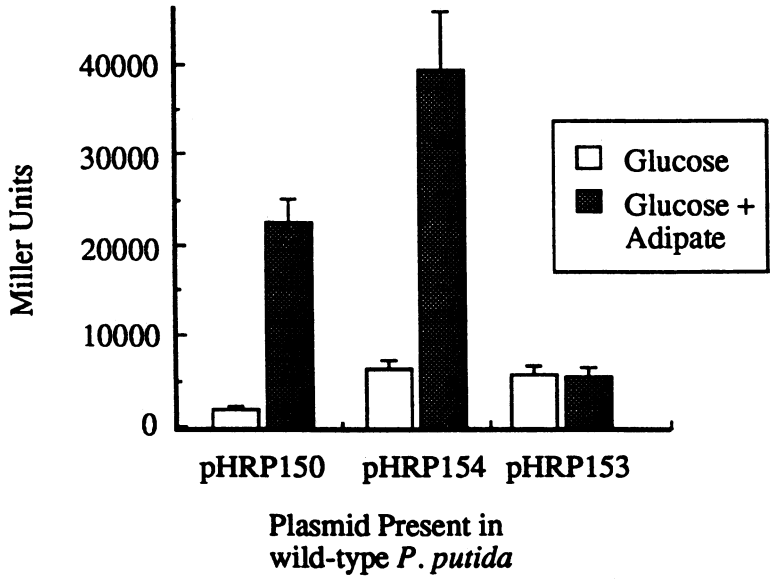

FIG. 3. Use of *lacZ* fusions to determine if *pcaI* and *pcaJ* are cotranscribed. (A) Restriction map of the *pcaIJ* region and diagrams depicting the three *lacZ* fusions used in this study. Abbreviations: E, *Eco*RI; EV, *Eco*RV; N, *Nru*I; P, *Pst*I; S, *Sma*I; Sa, *Sac*I; Sc, *Sca*I. (B) β-Galactosidase levels in PRS2000 carrying the three *lacZ* fusion plasmids after growth under noninducing (10 mM glucose) or inducing (10 mM glucose plus 20 mM adipate) conditions.

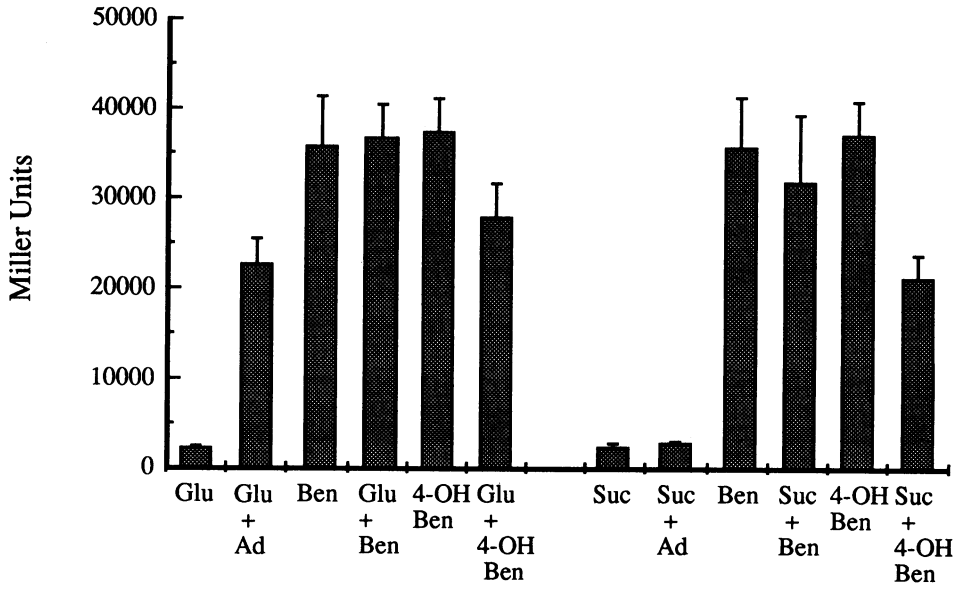

FIG. 4. Induction of *pcaI-lacZ* fusion (on plasmid pHRP150) expression in wild-type *P. putida* PRS2000. Results of β-galactosidase assays are shown. Abbreviations: Glu, 10 mM glucose; Ad, 20 mM adipate; Ben, 5 mM benzoate; 4-OH Ben, 5 mM 4-hydroxybenzoate; Suc, 10 mM succinate.

catBCA gene cluster. A *pcaR catR* double mutant was constructed in the same way by mobilizing pHRP211 into the *pcaR* transposon mutant PRS3015, forming PRS4021.

RESULTS

***pcaIJ* is an operon.** Comparisons of β-galactosidase levels in *P. putida* carrying the three transcriptional fusions depicted in Fig. 3A indicated that *pcaI* and *pcaJ* constitute an operon. Plasmid pHRP150 carries a 350-bp DNA fragment from upstream of the *pcaI* gene fused to a promoterless *lacZ* gene on the broad-host-range vector pHRP309 (32). Plasmid pHRP154 carries this same region plus all of *pcaI* and the first third of *pcaJ*. pHRP153 is similar to pHRP154 but has the entire *pcaI* promoter region deleted up to the *Sac*I site within the putative Shine-Dalgarno sequence preceding *pcaI* (31). When pHRP150 or pHRP154 was present in wild-type *P. putida*, β-galactosidase was induced in cultures that were grown in the presence of adipate. However, β-galactosidase was completely uninducible by adipate in cells carrying pHRP153 (Fig. 3B). This indicates that an adipate-regulated promoter is not present directly upstream of *pcaJ* but rather that *pcaI* and *pcaJ* are cotranscribed from an adipate-inducible promoter upstream of *pcaI*.

Identification of the *pcaIJ* operator-promoter region. As described above, pHRP150 carries the 350-bp region from upstream of *pcaI*. This plasmid was mobilized into the wild-type *P. putida* strain, PRS2000, and cells were grown under various conditions. A 10- to 18-fold induction of β-galactosidase activity was seen when cells were grown with benzoate, 4-hydroxybenzoate, or glucose plus adipate, compared with growth with glucose alone (Fig. 4). This level of induction corresponds well with the levels of induction of β-ketoadipate:succinyl-CoA transferase. Typically, a 9- to 18-fold increase in transferase activity is seen when PRS2000 is grown under inducing conditions (31).

Neither glucose nor succinate appears to cause significant catabolite repression. When wild-type cells carrying pHRP150 were grown with glucose or succinate in the presence of the inducer adipate or aromatic compounds that are metabolized to the physiological inducer β-ketoadipate, no dramatic catabolite repression effect was seen (Fig. 4). Slightly more repression was seen when cells were grown with benzoate or 4-hydroxybenzoate in the presence of succinate. The unexpected uninducibility when cells were grown with succinate and adipate may be explained by inhibition of adipate uptake by succinate (see Discussion).

5′ deletion analysis to identify the smallest fully inducible promoter fragment. A combination of *Bal* 31 treatment and polymerase chain reaction techniques was used to isolate a series of deletions from the 5′ end of the 350-bp *pcaI* promoter-containing fragment (see Materials and Methods) (Fig. 5B). These deletion fragments were fused to *lacZ* and mobilized into wild-type *P. putida*. Promoter constructs with deletions up to −33 relative to the +1 transcriptional start site retained fully inducible β-galactosidase activities (Fig. 5C).

PcaR (but not CatR) is required for *pcaI* induction. The *pcaI-lacZ* fusion plasmid pHRP150 was mobilized into the *pcaR* mutant PRS3015, the *catR* mutant PRS4020, and the double mutant (*pcaR catR*) PRS4021. CatR is the positive regulator of the *catBC* operon (37) and is required for growth with benzoate and catechol (Fig. 1). PcaR is hypothesized to be the positive regulator of the *pca* genes (15). PRS3015 and PRS4021 are unable to grow on either benzoate or 4-hydroxybenzoate, and PRS4020 is incapable of growth on benzoate. PRS3015 is uninducible for *pcaBDC* and *pcaIJ* (15). PRS3015 and PRS4021 were uninducible for β-galactosidase when carrying the *pcaI-lacZ* fusion, while β-galactosidase levels in PRS4020 were similar to those seen in the wild-type

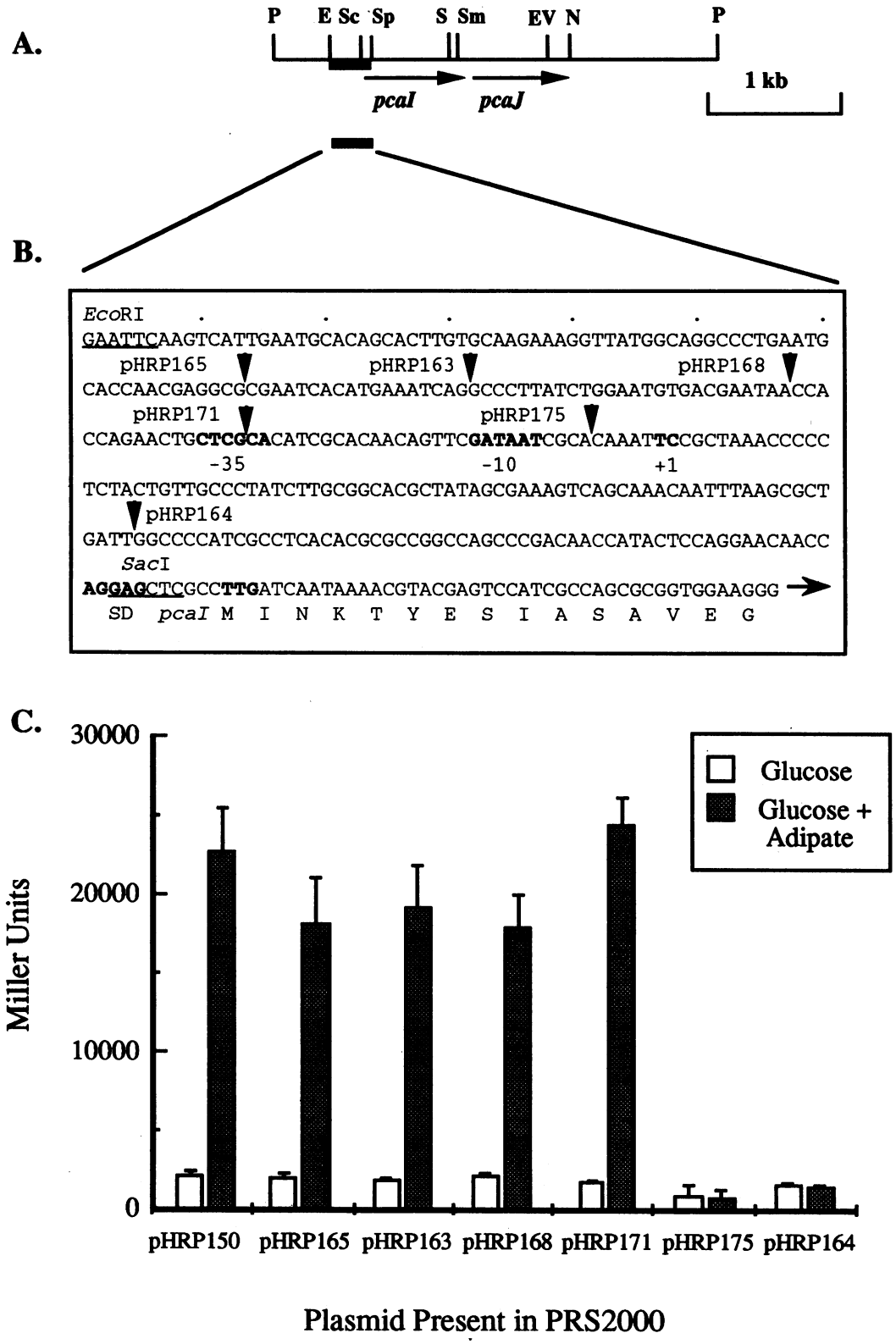

FIG. 5. Deletion analysis of the region upstream of *pcaI* to identify DNA sequences specifically required for full induction by adipate. (A) Restriction map of the *pcaIJ* region. Sp, *Sph*I; other abbreviations are as in the legend to Fig. 3. (B) Sequence of the *pcaI* promoter region and start of the gene. A series of 5′ deletions was constructed as described in Materials and Methods and fused to *lacZ* at the same point after the 15th codon of *pcaI* (horizontal arrow). Arrowheads indicate the deletion endpoint of each plasmid. Plasmid pHRP150 contains the entire boxed region fused to *lacZ* at the same point. The −35, −10, +1, Shine-Dalgarno, and translational start sites are in boldface, and the *Eco*RI and *Sac*I sites are indicated for orientation. (C) β-Galactosidase levels in PRS2000 carrying the entire *pcaI* promoter region fused to *lacZ* (pHRP150) and fusion constructs with successively smaller fragments. Cells were grown under inducing and noninducing conditions as described in the legend to Fig. 3.

TABLE 3. β-Galactosidase activities in *P. putida* regulatory mutants carrying the *pcaI-lacZ* fusion on pHRP150

Organism	β-Galactosidase activity (Miller units)[a] when grown with[b]:		Induction ratio[c]
	Glucose	Glucose + adipate	
PRS2000 (wild type)	2,078 (355)	22,721 (2,651)	10.9
PRS3015 (pcaR::Tn*5*)	1,251 (52)	1,331 (49)	1.1
PRS4020 (*catR*::Gm^r)	2,536 (122)	23,268 (1,258)	9.2
PRS4021 (*pcaR*::Tn*5* *catR*::Gm^r)	1,217 (5)	1,221 (15)	1.0

[a] Standard deviations are given in parentheses.
[b] Cells were grown with 10 mM glucose (uninduced) or 10 mM glucose plus 20 mM adipate (induced).
[c] Fold induction (growth with glucose plus adipate/growth with glucose).

strain (Table 3). These results confirm that PcaR is required for *pcaI* induction and show that CatR is unnecessary.

Construction and analysis of a chromosomal *pcaI-lacZ* fusion. In order to verify the results with the plasmid-encoded *lacZ* fusion, a *pcaI-lacZ* fusion suicide delivery plasmid (pHRP191) was constructed (Fig. 2) and mobilized into wild-type *P. putida*. The mating mixture was plated onto minimal medium plates containing succinate and 40 μg of X-Gal per ml. A single blue colony was isolated and tested for the presence of pSUP102 by screening for tetracycline resistance and the ability to grow on benzoate and 4-hydroxybenzoate. This strain, PRS4050, was tetracycline resistant and wild type for growth on aromatic compounds, indicating that a single crossover event had caused the insertion of the entire plasmid into the *P. putida* chromosome, apparently a common occurrence when pSUP102 is used (21, 40). The insertion was found to be very stable, and we were unable to isolate strains that had lost tetracycline resistance. The patterns of induction of β-galactosidase activity in the chromosomal fusion strain PRS4050 resembled those in PRS2000 carrying pHRP150 (Fig. 6; compare with Fig. 4). Growth with adipate, β-ketoadipate, or aromatic compounds caused a 10- to 20-fold induction of β-galactosidase activity. Absolute enzyme levels in PRS4050 were approximately 10-fold lower than in both induced and uninduced PRS2000 carrying the *pcaI-lacZ* fusion on plasmid pHRP150, suggesting a plasmid copy number of about 10.

Analysis of the *pcaI-lacZ* fusion in *E. coli* and *P. aeruginosa*. The plasmid pHRP150 was mobilized from *E. coli* S17-1 into *P. aeruginosa*. Cultures of *P. putida*, *P. aeruginosa*, and *E. coli* DH5α carrying pHRP150 were grown in the presence and absence of adipate. Results of β-galatosidase assays with these cultures indicated that the *pcaI-lacZ* fusion is regulated by adipate in *P. aeruginosa* but not in *E. coli* (Table 4). *E. coli* does not possess the β-ketoadipate pathway and presumably does not have an activator protein corresponding to PcaR. Therefore, one would not expect the fusion to be regulated in this organism. *P. aeruginosa* β-ketoadipate:succinyl-CoA transferase is known to be induced by β-ketoadipate (20), and the fact that the fusion is regulated in *P. aeruginosa* indicates that this organism also has a PcaR protein. The PcaR proteins from the two organisms are probably very similar, since the *P. aeruginosa* protein appears to recognize the *P. putida pcaI* promoter.

The *pcaI* promoter is not recognized by the alternative σ factor RpoN. The *P. putida pcaI* promoter region has nucleotide sequences that are characteristic of a typical *E. coli* σ^{70}-specific promoter (12). A 5′-GATAAT-3′ sequence in the −10 region agrees with the *E. coli* −10 consensus sequence (5′-TATAAT-3′) at five positions, and a sequence (5′-CTCGCA-3′) with three matches of six to the *E. coli* −35 consensus sequence (5′-TTGACA-3′) is located 16 nucleotides upstream of the *P. putida* −10 hexamer. Matches to the σ^{54} consensus sequence were also seen in the *pcaI* promoter region, however. We tested whether RpoN is required for induction of the *pcaI* promoter by mobilizing the *pcaI-lacZ* fusion plasmid (pHRP150) into the *P. aeruginosa* RpoN mutant, strain N1, and its parent strain, PAK-SR. The results of β-galactosidase assays indicated that RpoN is unnecessary for expression of *pcaI* (Table 4).

DISCUSSION

Adipate (or β-ketoadipate) and the *pcaR* regulatory gene were shown to be required for induction of the *pcaI* promoter by using both plasmid and chromosomal *pcaI-lacZ* fusions. Fusion analysis also indicated that *pcaI* and *pcaJ* are cotranscribed from a promoter upstream of *pcaI*. This result was not unexpected, since the two genes are separated by only 8 bp (31). The enzyme that these genes encode, β-ketoadipate:succinyl-CoA transferase, is an $\alpha_2\beta_2$ tetramer, and one would predict that the genes might be cotranscribed and, possibly, translationally coupled.

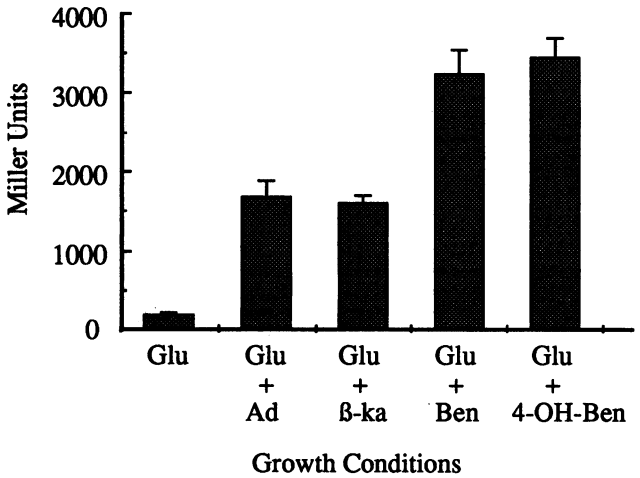

FIG. 6. β-Galactosidase activities of the *pcaI-lacZ* fusion when present in a single copy on the *P. putida* chromosome. Strain PRS4050 carries a chromosomally encoded *pcaI-lacZ* fusion (see text). This strain was grown under various conditions, and results of β-galactosidase assays are shown. Abbreviations: Glu, 10 mM glucose; Ad, 20 mM adipate; Ben, 5 mM benzoate; 4-OH-Ben, 5 mM 4-hydroxybenzoate; β-ka, 5 mM β-ketoadipate.

TABLE 4. β-Galactosidase activities in organisms carrying the *pcaI-lacZ* fusion on pHRP150

Organism	β-Galactosidase activity (Miller units)[a] when grown with[b]:		Induction ratio[c]
	Glucose	Glucose + adipate	
P. putida PRS2000	2,078 (355)	22,721 (2,651)	10.9
P. aeruginosa PAO1	3,479 (519)	42,412 (6,460)	12.2
E. coli DH5α	338 (33)	348 (35)	1.0
P. aeruginosa PAK-SR	2,824 (585)	20,917 (2,864)	7.4
P. aeruginosa N1 (RpoN$^-$)	1,754 (223)	14,500 (1,704)	8.3

[a] Standard deviations are given in parentheses.
[b] Cells were grown with 10 mM glucose (uninduced) or 10 mM glucose plus 20 mM adipate (induced).
[c] Fold induction (growth with glucose plus adipate/growth with glucose).

It is unknown at this time what type of σ factor recognizes the *pcaI* promoter, but the most likely candidate is σ^{70} (RpoD). It has been demonstrated that *Pseudomonas* σ^{70} promoters are likely to be very similar to their *E. coli* counterparts (8, 10), and the region upstream of *pcaI* has similarity to the *E. coli* σ^{70} consensus promoter sequences (12, 31). Sequence similarity in the −35 region is poor, but this is not uncommon in positively regulated promoters (36). It was previously shown that some of the TOL plasmid catabolic genes for the degradation of aromatic compounds required the RpoN σ factor for expression (21), but we have demonstrated here that expression from the *pcaI* promoter does not require RpoN.

A careful study of catabolite repression of the *pcaB* and *pcaC* gene products has indicated that glucose caused a slight decrease in enzyme levels and that succinate caused a more substantial decrease (28). According to one study (33), synthesis of β-ketoadipate:succinyl-CoA transferase was catabolite repressed when cells were grown with 4-hydroxybenzoate and glucose compared with cells grown with 4-hydroxybenzoate alone. In our studies, we have not noticed that the presence of either glucose or succinate causes a significant reduction in transferase levels, and these observations are in accord with results obtained by using the *pcaI-lacZ* fusion. The transferase assay gives somewhat variable results: fully induced cell extracts can have from 30 to 100 mU/mg, depending on the extract. The variability is probably due in part to the thermolability of the enzyme (46). β-Galactosidase assays are much more reproducible and can easily be repeated many times. For this reason, the use of *lacZ* fusions to study catabolite repression is likely to yield more accurate results.

One unexpected result of the catabolite repression studies is important to note. The *pcaI* promoter was completely uninducible in cultures grown with succinate plus adipate (Fig. 3). Previous work has shown that addition of succinate to cells that had been preloaded with [^{14}C]adipate led to loss of accumulated adipate (29), and succinate was later shown to be a competitive inhibitor of adipate uptake (27). Taken together, these results indicate that adipate probably does not accumulate in cells in the presence of succinate. When cells are grown with benzoate or 4-hydroxybenzoate, β-ketoadipate is produced internally and does not have to be transported in order for induction to take place.

Definitive assignment of the *cis*-acting nucleotide sequences required for *pcaIJ* activation will require the biochemical determination of the binding site for PcaR, and such experiments are in progress (14). Direct determination of the PcaR DNA-binding site will also be required to rule out the formal possibility that the plasmid DNA which replaced the normal upstream region in each of the P_{pcaI} deletions contains a sequence which can serve as an alternative PcaR-binding site. If such a site does exist, our results would indicate that its spacing from *pcaI* can be varied considerably without any discernible effect on the level of adipate-inducible β-galactosidase activity.

Given the above caveats, the deletion analysis of the *pcaI* promoter reported here indicates that no specific sequences upstream of the −35 region of the *pcaI* gene are required for full induction by adipate and the PcaR regulatory protein. Our results suggest that the PcaR protein binds unusually closely to the +1 site, either downstream of the RNA polymerase-binding region or within it, possibly on the opposite face of the DNA strand. This type of activator binding has been observed in only a few instances, notably in the mercury resistance operon (26). Although the most extreme promoter deletion that retained full activity was positioned at nucleotide −33 relative to the transcription initiation site, the plasmid DNA that replaced the deleted *P. putida* DNA created a new −35 hexamer (5′-ATCCCA-3′) that retained the same degree of similarity to the *E. coli* −35 consensus sequence as the unaltered version in the *pcaI* promoter. Thus, the −33 deletion would not necessarily be expected to alter the binding affinity of RNA polymerase.

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