Cloning and Expression of *pca* Genes from *Pseudomonas putida* in *Escherichia coli*

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β -Keto adipate elicits expression of five structural *pca* genes encoding enzymes that catalyse consecutive reactions in the utilization of protocatechuate by *Pseudomonas putida*. Three derivatives of *P. putida* PRS2000 were obtained, each carrying a single copy of Tn5 DNA inserted into a separate region of the genome and preventing expression of different sets of *pca* genes. Selection of Tn5 in or near the *pca* genes in these derivatives was used to clone four structural *pca* genes and to enable their expression as inserts in pUC19 carried in *Escherichia coli*. Three of the genes were clustered as components of an apparent operon in the order *pcaBDC*. This observation indicates that rearrangement of the closely linked genes accompanied divergence of their evolutionary homologues, which are known to appear in the order *pcaDBC* in the *Acinetobacter calcoaceticus* *pcaEFDBCA* gene cluster. Additional evidence for genetic reorganization during evolutionary divergence emerged from the demonstration that the *P. putida* *pcaE* gene lies more than 15 kilobase pairs (kbp) away from the *pcaBDC* operon. An additional *P. putida* gene, *pcaR*, was shown to be required for expression of the *pca* structural genes in response to β -keto adipate. The regulatory *pcaR* gene is located about 15 kbp upstream from the *pcaBDC* operon.

INTRODUCTION

Six *pca* structural genes encode the enzymes that convert protocatechuate to citric acid cycle intermediates via the β -keto adipate pathway (Fig. 1). The pathway is distributed widely among bacteria, and comparative studies indicate that structural genes for the pathway in biologically distant prokaryotic groups have evolved from a single ancestral set (Durham & Ornston, 1980; Durham *et al.*, 1980; Yeh *et al.*, 1980*a, b*; Yeh & Ornston, 1981). The divergent genes have fallen under different forms of transcriptional control (Canovas *et al.*, 1967; Ornston & Ornston, 1972; Stanier & Ornston, 1973; Ornston & Parke, 1977; Cain, 1980; Parke & Ornston, 1986) and thus gene rearrangement appears to have played a significant role in the evolution of the pathway.

Recent investigations have established that genes for protocatechuate metabolism in *Acinetobacter calcoaceticus*, expressed coordinately in response to protocatechuate (Canovas & Stanier, 1967), are clustered within a single transcriptional unit in the order *pcaEFDBCA* (Doten *et al.*, 1987*a*; C. Hartnett, personal communication). Published evidence concerning the *pca* genes in *Pseudomonas putida* indicates that they are organized quite differently. The *pcaA* gene is the only *P. putida* gene expressed in response to protocatechuate, with β -keto adipate eliciting expression of the other *pca* genes in this organism. The consequences of an apparent polarity mutation (Ornston, 1966) and frequent cotransduction of the *pcaB* and *pcaD* genes (Wheelis & Stanier, 1970) suggested that *P. putida* genes are cotranscribed in the order *pcaBDC*. The inference that these genes formed a transcriptional unit was strengthened by the demonstration that regulatory mutations caused their constitutive expression (Parke & Ornston, 1976).

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Fig. 1. Enzymes and structural genes associated with the central reactions of the β -ketoadipate pathway in *P. putida*.

Separate transcriptional control of the *pcaE* gene was indicated by its inducible control in the constitutive *pcaBDC* mutant strains (Parke & Ornston, 1976), yet this gene was cotransducible with genes from the *pcaBDC* cluster in the wild-type strain (Wheelis & Stanier, 1970).

In this article we describe the use of Tn5 mutagenesis and subsequent cloning into *Escherichia coli* to elucidate the molecular organization of structural genes that respond to the inducer β -ketoadipate in *P. putida*.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype* or phenotype†	Source or reference‡
<i>P. putida</i>		
PRS2000	Wild-type	Parke & Ornston (1976)
PRS3004	<i>pcaE</i> ::Tn5 Km ^r Pob ⁻ Ben ⁻	This study (PRS2000)
PRS3015	<i>pcaR</i> ::Tn5 Km ^r Pob ⁻ Ben ⁻	This study (PRS2000)
PRS3016	<i>pcaB</i> ::Tn5 Km ^r Pob ⁻ Ben ⁻	This study (PRS2000)
<i>E. coli</i>		
JM109	<i>recA endA gyrA96 thi hsdR17 supE44 relA lacY</i> (Δ lac-proAB) [F' <i>traD36 proAB lacI^a lacZ M15</i>]	Yanisch-Perron <i>et al.</i> (1985)
Neco100	<i>leu thr thi trp recA lacY</i>	Youvan <i>et al.</i> (1982)
Plasmids		
pUC19	Ap ^r lac p/o	Yanisch-Perron <i>et al.</i> (1985)
pRKTV14	Sm ^r Km ^r Tn903::Tn7::Tn5	Gift from N. J. Panopoulos
pSUP1021	Tc ^r Km ^r Cm ^r	Simon <i>et al.</i> (1986)
pPX40	Ap ^r Km ^r <i>pcaR</i> ::Tn5 <i>pcaB</i> ⁺	17.7 kbp, partial <i>SalI</i> digest of PRS3015
pPX43	Ap ^r Km ^r <i>pcaB</i> ::Tn5 <i>pcaC</i> ⁺ <i>pcaD</i> ⁺	11.0 kbp, partial <i>SalI</i> digest of PRS3016
pPX44	Ap ^r Km ^r <i>pcaB</i> ::Tn5 <i>pcaC</i> ⁺ <i>pcaD</i> ⁺	4.8 kbp, partial <i>SalI</i> digest of PRS3016
pPX45	Ap ^r Km ^r <i>pcaB</i> ::Tn5 <i>pcaC</i> ⁺ <i>pcaD</i> ⁺	~18 kbp, partial <i>SalI</i> digest of PRS3016
pPX53	Ap ^r Km ^r <i>pcaB</i> ::Tn5 <i>pcaC</i> ⁺ <i>pcaD</i> ⁺	~13 kbp, <i>EcoRI</i> subclone of pPX43
pPX57	Ap ^r <i>pcaC</i> ⁺	0.6 kbp, <i>SalI</i> subclone of pPX44
pPX81	Ap ^r <i>pcaB</i> ⁺	5.8 kbp, <i>SalI</i> digest of PRS2000
pPX200	Ap ^r Km ^r <i>pcaE</i> ::Tn5	6.8 kbp, partial <i>SalI</i> digest of PRS3004
pPX202	Ap ^r <i>pcaE</i> ⁺	7.8 kbp, <i>EcoRI</i> digest of PRS2000

* Genetic designations in the text or in referenced text.

† Km, kanamycin; Ap, ampicillin; Sm, streptomycin; Cm, chloramphenicol; Ben, benzoate; Pob, *p*-hydroxybenzoate.

‡ kbp, kilobase pairs, which refers to the size of the insert DNA fragment.

METHODS

Bacterial strains and plasmids. These are listed in Table 1. Strains of *P. putida* described herein were derived from the biotype A strain 90 (ATCC 12633) of Stanier *et al.* (1966). *E. coli* Neco100(pRKTV14) was kindly provided by N. Panopoulos (Youvan *et al.*, 1982).

Media and growth conditions. *P. putida* and *E. coli* were grown at 30 °C and 37 °C, respectively, in either L-broth (Miller, 1972) or defined mineral medium (Ornston & Stanier, 1966) supplemented with Hutner's 'Metals 44' (Cohen-Bazire *et al.*, 1957). When required, this medium was solidified using 1.5% (w/v) Bacto-Agar. Unless otherwise specified, aromatic compounds were supplied at a concentration of 5 mM and nonaromatic compounds at 10 mM. All carbon sources were added aseptically to growth media immediately prior to inoculation. For enzyme analysis, *P. putida* strains were grown to late exponential phase in culture volumes of 200 ml in one-litre Erlenmeyer flasks. Strains of *E. coli* were grown overnight in 50 ml L-broth and then diluted fourfold with fresh L-broth and incubated for a further 4 h. When required, isopropyl β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Where indicated, kanamycin was added to media to a final concentration of 100 μ g ml⁻¹ for *P. putida* and 50 μ g ml⁻¹ for *E. coli*. Ampicillin was used at a final concentration of 50 μ g ml⁻¹. For preparation of chromosomal or plasmid DNA, cultures were grown in L-broth. In all cases, baffled shake flasks were used and aeration was achieved by shaking at 200 r.p.m. in a New Brunswick controlled-environment incubator.

Isolation of transposon Tn5 insertion mutants. After overnight growth in L-broth, 0.1 ml volumes of *E. coli* Neco100(pRKTV14) and *P. putida* PRS2000 were mixed on L-broth agar and grown overnight at 30 °C. The resulting growth was suspended in 5 ml basal medium and plated onto basal medium containing 0.5 mM-succinate, kanamycin, and either benzoate or *p*-hydroxybenzoate so as to give about 100 colonies per plate. Following incubation at 30 °C for 3 d, plates were examined for small colonies among a population of predominantly large colonies. Approximately 5–10% of the small colonies tested were unable to grow with the appropriate aromatic compound.

Enzyme assays. Procedures for preparation of extracts and enzyme assays have been described previously (Doten *et al.*, 1987a). One unit of enzyme activity is defined as the amount of enzyme required to convert 1.0 μ mol substrate to product in 1 min, under the assay conditions used. Protein concentrations were determined by the Lowry method. Indicator assays for detection of enol-lactone hydrolase, the *pcaD* gene product, in whole cells have been described (Patel *et al.*, 1975).

Isolation of chromosomal and plasmid DNA. Chromosomal DNA was isolated by the method described by Berns & Thomas (1965). Plasmid DNA was purified by the cleared lysate procedure (Schleif & Wensink, 1981), using caesium chloride/ethidium bromide density gradient centrifugation.

In vitro DNA manipulation. Restriction endonuclease digestions were performed according to the manufacturer's specifications. For partial digestions, approximately 10 µg chromosomal DNA was digested with 0.05 units of either *Eco*RI or *Sal*I for 1 h and mixed with 1 µg of appropriately digested and dephosphorylated pUC19 plasmid DNA. The method used was that described by Maniatis *et al.* (1982).

DNA transformation. Transformation of *E. coli* was by the method of Hanahan (1983). The presence of recombinant plasmids was screened by identification of white colonies on agar plates containing L-broth, 0.1 mM IPTG and 2% (w/v) 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal).

Analysis of recombinant plasmids. Southern hybridizations were performed according to established procedures (Southern, 1975). Purified DNA fragments were labelled by nick-translation with DNA polymerase and [α -³²P]dTTP. The procedure of Maniatis *et al.* (1982) was used for colony DNA-DNA hybridization.

Chemicals and reagents. All chemicals used were of the highest quality available and, with the exception of β-carboxy-*cis,cis*-muconate (Meagher *et al.*, 1972) and (+)-muconolactone (Ornston & Stanier, 1966), were obtained commercially.

RESULTS

Cloning and mapping of pcaE

Transposon Tn5 insertion mutagenesis of *P. putida* strain PRS2000 yielded strain PRS3004, which was unable to grow at the expense of benzoate or *p*-hydroxybenzoate. Enzymic analyses revealed that this mutant strain expressed inducibly other structural genes of the β-ketoadipate pathway, but failed to express *pcaE* (Table 2).

No *Eco*RI restriction sites lie within Tn5, and restriction of PRS3004 chromosomal DNA with *Eco*RI yielded a single *Eco*RI restriction fragment that hybridized with DNA from Tn5 (Fig. 2, lane 2). Exposure of the mutant strain to *p*-hydroxybenzoate selected spontaneous revertants that invariably were kanamycin sensitive. Thus, strain PRS3004 appears to have acquired a single copy of Tn5 inserted in or near the *pcaE* structural gene.

Chromosomal DNA from strain PRS3004 was partially digested with *Sal*I, ligated into pUC19 and transformed into *E. coli* strain JM109, followed by selection for kanamycin resistance. A selected strain carrying a 6.8 kbp *Sal*I insert in pUC19 was designated JM109(pPX200). The 6.8 kbp fragment was then used as a probe to screen an *Eco*RI-generated pUC19 genomic library of PRS2000 for clones containing DNA that hybridized with *pcaE* DNA. Approximately 5000 colonies were screened, and two hybridized strongly to the probe. After growth in the absence of IPTG, these strains expressed the *pcaE* gene at levels corresponding to 0.02 µmol min⁻¹ (mg protein)⁻¹. Addition of IPTG to the growth medium caused the cells to produce the *pcaE* gene product at levels ranging from 0.05 to 0.1 µmol min⁻¹ (mg protein)⁻¹, indicating that the structural gene was under control of the *lac*

Table 2. *Expression of pca genes in Tn5 derivatives of P. putida grown with glucose plus adipate*

Strain	Level of gene expression*			
	<i>pcaB</i>	<i>pcaC</i>	<i>pcaD</i>	<i>pcaE</i>
PRS2000	100	100	100	100
(wild-type)	(0.98)	(3.18)	(1.54)	(0.04)
PRS3004	211	111	186	<1
(<i>pcaE</i> ::Tn5)				
PRS3015	12	13	9	5
(<i>pcaR</i> ::Tn5)				
PRS3016	<1	20	8	183
(<i>pcaB</i> ::Tn5)				

* Expressed as a percentage of the observed activity, relative to fully induced cells of wild-type *P. putida* grown in the presence of the gratuitous inducer, adipate. Experimental values of fully induced expression are given in parentheses as µmol substrate used or product formed min⁻¹ (mg protein)⁻¹. The levels observed in strain PRS3015 after growth with glucose plus adipate were the same as those observed with uninduced wild-type cells.

Fig. 2. Southern hybridization of chromosomal DNA from Tn5 mutant strains of *P. putida* showing a single insertion of Tn5 into the chromosome of each of the mutant strains. Lanes 1–4, DNA digested with *Eco*RI; lanes 5–8, DNA digested with *Sal*I. Restricted DNA was electrophoresed on a 0.7% agarose gel and hybridized to the 2.7 kbp *Bgl*II fragment of Tn5. The *Bgl*II fragment was prepared from pSUP1021 and, after electrophoresis, isolated from low-melting-point agarose. Lanes 1 and 5, PRS2000; lanes 2 and 6, PRS3004; lanes 3 and 7, PRS3015; lanes 4 and 8, PRS3016. *Eco*RI does not cut within Tn5 and gives rise to a single hybridizing band with *Eco*RI-restricted DNA from strains carrying chromosomal Tn5 inserts. *Sal*I cuts once within Tn5 and therefore gives rise to two hybridizing fragments with *Sal*I-restricted DNA from the strains carrying Tn5 inserts.

promoter. Expression of the *pcaE* gene was not enhanced when adipate, a physiologically effective analogue of the inducer β -keto adipate in *P. putida* (Parke & Ornston, 1976), was included in the *E. coli* growth medium.

The plasmid from one of the strains was designated pPX202. Subsequent analysis of this plasmid indicated that it contained a single 7.8 kbp *Eco*RI insert in pUC19. Cleavage of pPX202 with *Hind*III yielded 3.5 and 6.6 kbp fragments. The latter contained pUC19 DNA and, upon ligation and introduction into JM109, did not express *pcaE*. The 3.5 kbp insert also did not express *pcaE* when placed under the *lac* promoter of pUC19, and so it appears that the *Hind*III restriction site lies within the *pcaE* gene. The *pcaE* gene can be deduced to be about 1.2 kbp in length (Yeh & Ornston, 1981); the approximate location of the gene can be deduced as being within 1 kbp of the *Hind*III site. All the subclones carrying each of the internal pPX202 *Sal*I fragments inserted into pUC19 were prepared. None of the subclones expressed *pcaE*. Therefore it appears likely that the *Sal*I site proximal to the *Hind*III site, which effectively bisects the pPX202 insert, also lies within the *pcaE* gene.

Cloning and mapping of *pcaB* : : Tn5, *pcaC* and *pcaD*

Transposon Tn5 mutagenesis of *P. putida* PRS2000 gave rise to mutant strain PRS3016 which, in response to β -keto adipate, was unable to express *pcaB* but expressed slightly less than basal levels of *pcaC* and *pcaD*. Under these conditions, *pcaE* was detected at levels comparable to those detected in PRS2000 which had been similarly induced. DNA isolated from PRS3016 yielded only one *Eco*RI fragment that hybridized with DNA from Tn5 (Fig. 2, lane 4). The basal levels of expression of the *pcaC* and *pcaD* genes are consistent with the genetic evidence provided by Wheelis & Stanier (1970) which showed that *pcaB* and *pcaD* are tightly linked on the *P. putida* chromosome. These results would further suggest that this linkage is such that the three genes form a transcriptional unit and that the insertion of a single copy of Tn5 into or near the

Fig. 3. Restriction endonuclease digestion of *pca* clones with *Sal*I. Digested DNA was electrophoresed on a 0.7% agarose gel. Lane 1, λ DNA digested with *Hind*III (resulting in fragments of the sizes indicated); lane 2, pPX43; lane 3, pPX44; lane 4, pPX45; lane 5, pPX57; lane 6, pPX40; lane 7, pPX202.

pcaB structural gene produced a polarity mutation reducing the expression of the neighbouring *pcaC* and *pcaD* genes. It would therefore appear likely that DNA bordering *pcaB*::Tn5 might contain the *pcaD* gene. In order to explore this possibility, chromosomal DNA from strain PRS3016 was partially digested with *Sal*I, ligated into pUC19 and transformed into *E. coli* strain JM109. Ten kanamycin-resistant colonies were isolated, three of which gave positive indicator tests for enol-lactone hydrolase, the product of the *pcaD* gene. Enzymic analysis of these three transformants, JM109(pPX43), JM109(pPX44) and JM109(pPX45), not only revealed expression of *pcaD*, but also of *pcaC*, both at levels comparable to those found in fully induced *P. putida* cultures. Adipate failed to influence the expression of either of the genes. Addition of IPTG to the growth medium increased expression of the genes approximately threefold, indicating that expression was being directed from the *lac* promoter. Although it is difficult to rationalize the expression of the plasmid-borne *pcaC* and *pcaD* genes in *E. coli*, it is possible that transcription from the *lac* promoter is sufficient to overcome the polarity mutation imposed by the Tn5 insertions. None of the *E. coli* transformants expressed any of the other *pca* structural genes.

Determination of the gene order pcaB::Tn5, pcaD, pcaC

Electrophoresis of *Sal*I digests of the aforementioned recombinant plasmids revealed that the respective inserts in pPX43, pPX44 and pPX45 are approximately 11 kbp, 4.8 kbp and 18 kbp (Fig. 3). All three plasmids contain, in addition to the 2.7 kbp pUC19 fragment, *Sal*I segments

Fig. 4. Restriction and genetic map of the *pcaBDC* region of *P. putida*. Also shown are the relevant restriction maps of the pPX43, pPX44, pPX45 and pPX57 constructs, which depict the site of Tn5 insertion into *pcaB* and their orientation with respect to the *pcaBDC* region. Not shown in detail are the numerous *SalI* restriction sites that border the *pcaBDC* operon in plasmid pPX45. Transcription occurs from left to right, as determined by the *lac* promoter of pUC19.

of 3.8, 0.6 and 0.4 kbp (Figs 3 and 4). A common terminus of the pPX44 and pPX45 segments at the internal *SalI* site of Tn5 was identified by demonstrating that they, unlike pPX43, lacked the characteristic internal 3.3 kbp *HindIII* and 2.7 kbp *BglII* fragments that encompass the single *SalI* site within the transposon. Therefore, both the pPX44 and the pPX45 inserts contain at one end the portion of Tn5 beginning at the internal *SalI* restriction site containing the kanamycin resistance gene (Fig. 4). The inserts extend 2.1 kbp and approximately 15 kbp, respectively, into *P. putida* DNA containing and lying beyond the *pcaC* and *pcaD* genes.

Of the three inserts shared by pPX43, pPX44 and pPX45, the 3.8 kbp *SalI* fragment is the only one large enough to contain the kanamycin resistance gene of Tn5 (Fig. 3), and this fragment failed to express *pcaC* or *pcaD* when placed under the *lac* promoter of pUC19 in *E. coli* JM109. Thus it appeared that the *SalI* cuts giving rise to the 0.4 kbp and 0.6 kbp fragments inactivated or removed the two *pca* genes. Subsequent restriction analysis showed the sizes of the cloned *SalI* fragments to be in the order 3.8, 0.4 and 0.6 kbp. The order of the genes was shown to be *pcaB*, *pcaD*, and *pcaC* by subcloning the 0.6 kbp *SalI* fragment into pUC19. When carried in *E. coli*, the resulting plasmid, pPX57, expressed *pcaC* at high levels but did not express *pcaD*. Thus the *pcaC* gene, which can be deduced to be approximately 0.4 kbp in size (Patel *et al.*, 1975; Yeh *et al.*, 1980a), lies within the 0.6 kbp *SalI* fragment.

Cloning of *pcaB*

The recombinant plasmid pPX43, containing the entire region of Tn5, was digested with *EcoRI*, ligated into pUC19 and transformed into *E. coli* JM109 with selection for kanamycin resistance. Since *EcoRI* cuts outside Tn5, we anticipated that plasmids in the resulting subclones would contain at least part of *pcaB*::Tn5 in a region bordered by *EcoRI* restriction sites. One such subclone, carrying the plasmid designated pPX53, expressed high levels of *pcaC* and *pcaD* in response to induction by IPTG. No expression of these two genes was found in strains bearing recombinant plasmids in which the *EcoRI* fragment had been cloned in the reverse orientation.

Restriction analysis of pPX53 revealed that Tn5 had inserted into a 5.8 kbp *SalI* fragment which presumably contained *pcaB*. The inserted *EcoRI* fragment from pPX53 was used to probe

a *SalI*-generated genomic DNA library of *P. putida* PRS2000 in *E. coli* JM109. Two of 7000 screened colonies contained DNA which hybridized to the *EcoRI* insert of pPX53. One of these strains, JM109(pPX81), containing only this 5.8 kbp *P. putida* *SalI* fragment, expressed high levels of *pcaB*, but neither *pcaC* nor *pcaD*. Restriction mapping showed that the *SalI* fragment in pPX81 was the same as that of pPX53 with the exception that the former did not contain Tn5 inserted into *pcaB*.

The restriction and genetic map of the *pcaBDC* region is shown in Fig. 4. Not depicted fully on the map are *SalI* sites on the approximately 15 kbp of *P. putida* DNA carried on pPX45 (Fig. 3). This DNA contains the portion of the *pcaB* gene transcribed beyond its internal *SalI* restriction site, the *pcaD* gene, the *pcaC* gene, and about 13 kbp of DNA extending downstream from this region. This DNA does not hybridize with the 7.8 kbp *EcoRI* fragment containing the *pcaE* gene (data not shown). Therefore we may conclude that the *pcaE* gene does not lie in the region extending 15 kbp downstream from the *pcaBDC* cluster.

Identification and mapping of the pcaR gene

The initial screening of Tn5-inactivated mutants yielded one strain, PRS3015, that expressed low levels of *pcaE* and basal levels of the *pcaBDC* genes after growth in the presence of *p*-hydroxybenzoate. Expression of *pcaA*, induced by protocatechuate, was not affected in this strain. Adipate, an inducer of the *pcaBDC* genes, the *pcaE* gene and the *cat* genes in wild-type cells (Parke & Ornston, 1976), elicited expression of the *cat* genes but not the *pca* genes in strain PRS3015 (Table 2). Therefore, the failure to express the *pca* genes cannot be attributed to a mutation blocking permeation of adipate into strain PRS3015. DNA from this strain produced only one *EcoRI* fragment that hybridized to Tn5 DNA (Fig. 2, lane 3), and thus the mutation in PRS3015 appeared to be a single insertion in a gene required for expression of the *pca* structural genes. Comparison of *EcoRI* and *SalI* restriction fragments carrying Tn5 (Fig. 2) showed that the fragment containing the mutated gene, designated *pcaR*, is physically distinct from the respective *EcoRI* fragments containing the *pcaE* gene and the *pcaBDC* cluster.

Selection for growth with *p*-hydroxybenzoate gave rise to two classes of phenotypic revertants of strain PRS3015. Representatives of one class appeared to have lost the Tn5 insertion from *pcaR* in that they were indistinguishable from wild-type cells with respect to both sensitivity to kanamycin and rate of growth with *p*-hydroxybenzoate. Representatives of the other class retained kanamycin resistance and formed small colonies on *p*-hydroxybenzoate. These strains expressed the *pcaBDC* genes at the basal level found in the parental strain PRS3015 and expressed constitutively the *pcaE* gene at levels comparable to those found in fully induced wild-type *P. putida* cells. Thus mutations increasing expression of *pcaE* seem to be sufficient to allow slow growth of *pcaR* mutants with *p*-hydroxybenzoate.

Partial digestion of DNA from strain PRS3015 with *SalI* and subcloning of the resulting fragments yielded a pUC19 derivative, designated pPX40, that conferred kanamycin resistance and, in response to IPTG, expressed *pcaB*, but not *pcaD* or *pcaC*, at levels comparable to those found in fully induced *P. putida* cultures. The other *pca* structural genes were not expressed from pPX40. In the absence of IPTG, *E. coli* JM109(pPX40) expressed *pcaB* at a low level which was not increased by addition of adipate to the growth medium.

Digestion of pPX40 with *Bgl*II and *Hind*III showed that it lacked the characteristic internal fragments of Tn5 and thus it appeared that the *SalI* segment at one end of the insert in this plasmid extends from the internal *SalI* site of the transposon through its kanamycin resistance marker and into *P. putida* DNA containing part of the *pcaR* gene. The identity of the *SalI* segment at the other end of the insert was indicated by the fact that *SalI* digests of the plasmid yielded the 5.8 kbp fragment that carries the *pcaB* gene but did not produce the 0.4 and 0.6 kbp *SalI* fragments associated with the *pcaD* and *pcaC* genes. Other fragments produced by digestion of pPX40 with *SalI* account for approximately 15 kbp of *P. putida* DNA that lies between *pcaR*:Tn5 and *pcaB*. This DNA does not hybridize with the 7.8 kbp *EcoRI* fragment containing the *pcaE* gene (data not shown). It therefore appears that *pcaE* is not in the region extending 15 kbp upstream from *pcaB*.

DISCUSSION

Organization of pca structural genes

The conclusion that the *P. putida* *pca* genes form a transcriptional unit in the order *pcaBDC* is in accord with earlier observations of polar expression (Ornston, 1966), unified transcriptional control (Parke & Ornston, 1976) and genetic linkage (Wheelis & Stanier, 1970). The sizes of the *Sal*I restriction fragments produced from the plasmid pPX44 indicate that a portion of the *pcaB* gene, the *pcaD* gene and the *pcaC* gene are encompassed in no more than 2.1 kbp of DNA. The sizes of the products of the latter two genes (Parke, 1979; Yeh & Ornston, 1981; Yeh *et al.*, 1980a) indicate that they require at least 1.4 kbp of DNA sequence, so it is reasonable to conclude that the three genes are closely clustered. Amino acid sequence comparisons have shown that *pcaD* (Yeh *et al.*, 1980b) and *pcaC* (Yeh *et al.*, 1980a) genes from *P. putida* are homologous with their counterparts from *A. calcoaceticus*, and therefore it is remarkable that, in the latter species, the *pcaB*, *pcaD* and *pcaC* genes are tightly linked in a different sequence as part of an operon transcribed in the order *pcaEFDBCA* (Doten *et al.*, 1987a; C. Hartnett, personal communication). Evidently gene rearrangements exceeded those demanded for the different transcriptional controls exercised over *pca* genes in the two bacterial species.

The results of this investigation indicate that the *pcaE* structural gene lies more than 15 kbp from the *pcaBDC* operon on *P. putida*. These findings reduce, but do not eliminate, ambiguities that emerged from an earlier investigation of the linkage of *pcaB* and *pcaE* in *P. putida*. A single *pcaB* donor yielded respective cotransduction frequencies of 13% and 69% when tested with two different *pcaE* recipients (Wheelis & Stanier, 1970). The *pcaE* gene product is composed of two different protein subunits (Yeh & Ornston, 1981), and one interpretation to account for the discrepancy in transduction frequencies is that the recipients carried mutants in physically distant structural genes for the two different subunits. This interpretation is rendered unlikely by our observation that the *pcaE* gene can be expressed in its entirety from a single 7.8 kbp restriction segment.

Wheelis & Stanier (1970) suggested that many of the ambiguities observed in transductional analysis of *P. putida* *pca* genes and neighbouring genes could be resolved by construction of a circular map. At face value, this interpretation might seem to be inconsistent with the genes residing as components of a linear chromosome. A possible resolution of this inconsistency might lie in physical interactions among DNA sequences that are distant in primary structure. Such interactions might lead to relatively frequent packaging of two separate DNA segments in the same transducing phage coat. Evidence that unlinked genes may interact is given by demonstration of repair of a *pcaE* mutation by *catBCE* sequences in *A. calcoaceticus* (Doten *et al.*, 1987b).

Transcriptional control exerted by pcaR in P. putida

The *pcaR* regulatory gene, which has been found to lie approximately 15 kbp upstream from the *pcaBDC* operon, is required for expression of physically separate genes transcribed by different promoters that respond inducibly to β -ketoadipate in *P. putida*. Earlier evidence for different promoters, such as the constitutive expression of the *pcaBDC* genes in mutants that remained inducible for *pcaE* (Parke & Ornston, 1976), is fortified by our finding that mutations causing enhanced transcription of *pcaE* did not influence expression of the *pcaBDC* cluster. Benzoate chemotaxis, an additional inducible response to ketoadipate (Harwood *et al.*, 1984), is lost as a consequence of the *pcaR* mutation in *P. putida* (C. Harwood, personal communication). Therefore the *pcaR* gene may exert positive control over a number of different transcriptional units in *P. putida*.

Several taxa deserve special attention in comparative studies directed at the elucidation of processes underlying divergence of the *pcaR* regulatory gene. *Pseudomonas aeruginosa* resembles *P. putida* in many respects and elaborates closely homologous structural genes (Patel & Ornston, 1976) that appear to be organized and transcribed in a roughly similar fashion (Kemp & Hegeman, 1968). *Azotobacter* species possess *pca* structural genes that are homologous to their *P. putida* counterparts, and β -ketoadipate elicits a similar inducible response in these two

biological groups (Durham & Ornston, 1980). Therefore it will be of interest to determine if homologues of *pcaR* govern gene expression in *Azotobacter*. Benzoate is not metabolized via β -ketoadipate in *Azotobacter* (Hardisson *et al.*, 1969; Sala-Trepat & Evans, 1971), and it will be interesting to determine how benzoate chemotaxis, if it is demonstrated by members of this genus, is controlled. β -Ketoadipate serves no known function as an inducer in *Acinetobacter calcoaceticus*, and it seems reasonable to propose that representatives of this species do not contain a functional counterpart of *pcaR*.

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