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Cloning and Genetic Organization of the *pca* Gene Cluster from *Acinetobacter calcoaceticus*

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The β-ketoadipate pathway of *Acinetobacter calcoaceticus* comprises two parallel metabolic branches. One branch, mediated by six enzymes encoded by the *cat* genes, converts catechol to succinate and acetyl coenzyme A (acetyl-CoA); the other branch, catalyzed by products of the *pca* genes, converts protocatechuate to succinate and acetyl-CoA by six metabolic reactions analogous or identical to those of the catechol sequence. We used the expression plasmid pUC18 to construct expression libraries of DNA from an *A. calcoaceticus* mutant strain from which the *cat* genes had been deleted. Immunological screening with antiserum to the *pcaE* gene product, β-ketoadipate:succinyl-CoA transferase I, resulted in the isolation of a cloned 11-kilobase-pair (kbp) fragment which inducibly expressed all six *pca* genes under control of the *lac* promoter on pUC18. The induced *Escherichia coli* cells formed the six *pca* gene products at levels 10- to 30-fold higher than found in fully induced *A. calcoaceticus* cultures, although protocatechuate 3,4-dioxygenase (the iron-containing product of the *pcaA* gene) from the recombinant strain possessed a relatively low turnover number. An *E. coli* culture expressing the cloned *pca* genes quantitatively converted protocatechuate to β-ketoadipate; failure of the organism to metabolize the latter compound can be most readily ascribed to relatively low pool levels of succinyl-CoA, a required substrate for β-ketoadipate:succinyl-CoA transferase, in *E. coli*. The gene order and direction of transcription were determined to be *pcACBDFE* by identification of enzymes expressed in subclones, by using natural transformation to identify subclones carrying DNA corresponding to dysfunctional alleles in mutant *A. calcoaceticus* strains, and by restriction mapping of both the 11-kbp fragment and derivatives of the 11-kbp fragment containing Tn*5* in the *pcaA*, *pcaB*, *pcaC*, *pcaD*, and *pcaE* genes. The fragment containing the *pca* gene hybridized strongly and specifically to a previously cloned fragment containing *A. calcoaceticus cat* genes.

The *pca* structural genes encode six enzymes that convert protocatechuate to citric acid cycle intermediates via β-ketoadipate (Fig. 1; 27). Protocatechuate induces coordinate synthesis of the enzymes in *Acinetobacter calcoaceticus* (3), and their unified transcriptional control has been suggested by their constitutive formation in regulatory mutants (4, 22). The physical organization of the *A. calcoaceticus pca* genes has not been explored.

The *A. calcoaceticus cat* genes encode six enzymes that convert catechol to citric acid cycle intermediates by reactions analogous or identical to those catalyzed by the *pca* gene products (Fig. 1). The *cat* genes have been cloned, and the *catA* gene (19) has been shown to lie near a separate transcriptional unit containing the *catBCDE* genes (23). The latter genes are carried on a 5-kilobase-pair (kbp) *Eco*RI fragment that hybridizes to some extent with an 11-kbp *Eco*RI segment from *A. calcoaceticus* DNA (23). The known amino acid sequence homology of the *catD* gene and its *pcaD* counterpart (30) raised the possibility that the 11-kbp *Eco*RI segment carried some or all of the *pca* genes.

We have observed that deletion of *catBCE* sequences from the genetic background of an *A. calcoaceticus pcaE* mutant decreases the frequency of phenotypic reversion of the *pcaE* mutation more than 300-fold, and the phenotypically reverted *pcaE* genes are unstable in the presence of the *catBCE* sequences (7a). This evidence suggests that the phenotypic reversion events involved genetic recombination introducing *cat* sequences into the *pcaE* gene.

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To establish a basis for analysis of possible interaction between *cat* and *pca* genes in *A. calcoaceticus*, we constructed expression libraries of *A. calcoaceticus* DNA and used immunological screening to identify a clone that expressed the *pcaE* gene. The cloned DNA was an 11-kbp *Eco*RI fragment that hybridized with the 5-kbp *Eco*RI fragment containing the *catBCDE* genes and possessed the genes for protocatechuate catabolism clustered in the order *pcaACBDFE*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this investigation are listed in Table 1. *Escherichia coli* JM109 cultures were grown in L broth (18). *A. calcoaceticus* cultures were grown in minimal medium (21) supplemented with Hutner's Metals 44 (5) and 5 mM *p*-hydroxybenzoate. During transposon mutagenesis, *E. coli* EM3001(pZR1) cultures were grown in YM broth (7). *E. coli* JM109 and *A. calcoaceticus* cultures were grown at 37°C, and *E. coli* EM3001(pZR1) cultures were grown at 30°C; all cultures were grown aerobically. Ampicillin was added at a final concentration of 75 μg/ml, and kanamycin was added at a concentration of 20 μg/ml. In induction studies, isopropyl-β-D-thiogalactoside (IPTG) was added at a final concentration of 1 mM. Solid media were solidified by addition of 1.5% agar.

Isolation of chromosomal and plasmid DNA. Chromosomal DNA was isolated by the method described by Berns and Thomas (1). Plasmid DNA was purified by the cleared lysate procedure (16), using cesium chloride-ethidium bromide density gradient centrifugation.

Immunological screening of transformants for transferase production. *A. calcoaceticus* chromosomal DNA was di-

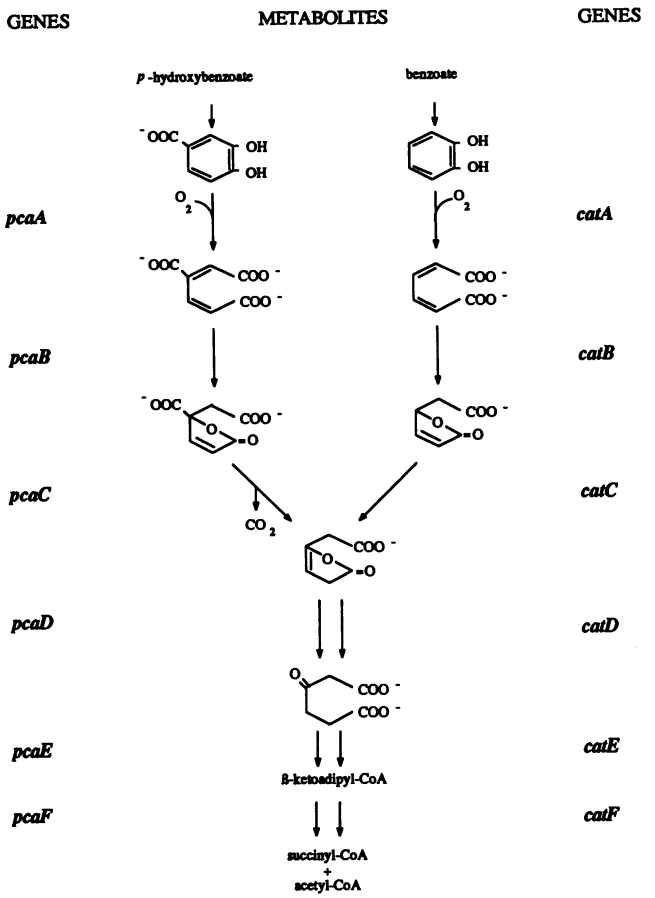

FIG. 1. The β-ketoadipate pathway of *A. calcoaceticus*. Enzymes encoded by genes are: *pcaA*, protocatechuate 3,4-dioxygenase; *pcaB*, β-carboxy-*cis*,*cis*-muconate lactonizing enzyme; *pcaC*, carboxymuconolactone decarboxylase; *pcaD*, β-ketoadipate enol-lactone hydrolase I; *pcaE*, β-ketoadipate-succinyl-CoA transferase I; *pcaF*, β-ketoadipyl-CoA thiolase I; *catA*, catechol 1,2-dioxygenase; *catB*, muconate-lactonizing enzyme; *catC*, muconolactone isomerase; *catD*, β-ketoadipate enol-lactone hydrolase II; *catE*, β-ketoadipate-succinyl-CoA transferase II; and *catF*, β-ketoadipyl-CoA thiolase II.

gested with *Bam*HI or *Eco*RI or partially digested with *Sau*3A to give an average fragment size of 8 kbp. The DNA was ligated into phosphatase-treated pUC18 as described by Maniatis et al. (17), and the ligation mixtures were used to transform *E. coli* JM109 by the method of Hanahan (9). Transformants were spread on L broth-ampicillin-IPTG plates to give approximately 300 colonies per plate and then were replicated onto nitrocellulose filters, lysed, and screened with antibody for production of β-ketoadipate:succinyl-coenzyme A (CoA) transferase I (10). The filters were then incubated with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase. The filters were washed as described by Helfman et al. (10) and developed in a solution of 4-chloro-1-napthol (0.5 mg/ml) and hydrogen peroxide (0.01%). Positive clones were identified by their dark purple color.

Cell extracts and enzyme assays. Cell-free extracts were prepared by sonication and centrifugation as previously described (23). Activities of the following enzymes of the β-ketoadipate pathway were determined by the indicated spectrophotometric procedures: *p*-hydroxybenzoate hydroxylase (EC 1.14.13.2; 28); protocatechuate 3,4-dioxygenase (EC 1.99.2.3; 8); β-carboxy-*cis*,*cis*-muconate cycloisomerase (EC 5.5.1.2; 20) γ-carboxymuconolactone decarboxylase (EC 4.1.1.44; 20); β-ketoadipate enol-lactone hydrolase (EC 3.1.1.24; 20); and β-ketoadipate:succinyl-CoA transferase (EC 2.8.3.6;33). Activity of β-ketoadipate thiolase was determined by measuring the decrease in absorbance at 305 nm due to β-ketoadipyl-CoA:Mg^{2+} complex; the complex was generated with ketoadipate, succinyl-CoA, and purified β-ketoadipate:succinyl-CoA transferase (33). β-Ketoadipyl-CoA for thiolase assays was prepared in a total volume of 1 ml containing 200 μmol of Tris hydrochloride (pH 8.0), 4 μmol of succinyl-CoA (33), 15 μmol of β-ketoadipate, and 1 U of purified β-ketoadipate:succinyl-CoA transferase from *A. calcoaceticus* ADP1. The mixture was incubated at room temperature for 1 h before being centrifuged through an Amicon Centricon-10 to remove the transferase. Thiolase assays were conducted in a total volume of 1 ml containing 200 μmol of Tris hydrochloride (pH 8.0), 20 μmol of $MgCl_2$, 0.1 μmol of CoA, and 0.1 μmol of β-ketoadipyl-CoA. Disappearance of substrate was determined by measuring the decrement of absorbance at 305 nm. Protein concentration was determined by the method of Bradford (2), using reagents obtained from Bio-Rad Laboratories. A unit of activity is the amount of enzyme that transforms 1 μmol of substrate per min.

HPLC separation of *pca*-encoded enzymes. *pca*-Encoded enzymes were separated by high-pressure liquid chromatography (HPLC) as follows. Crude extracts of IPTG-induced cultures of strain JM109(pZR1) containing 250 mg of protein in 10 ml of EDA buffer (10 mM sodium ethylenediamine, pH 7.3, 1 mM $MnCl_2$, and 1 mM 2-mercaptoethanol) were dialyzed against the buffer, centrifuged at 100,000 × *g* for 1 h, and then filtered through a 0.45-μm-pore-size membrane filter. Filtrate was injected onto a TSK DEAE-5PW anion-exchange column (2.15 by 15 cm; Bio-Rad Laboratories), and protein was eluted with EDA buffer containing a 0 to 0.25 M linear gradient of NaCl at a flow rate of 5 ml/min over 120 min. Elution of protein was monitored by measurement of absorbance at 280 nm. Fractions were collected and assayed for enzyme activity.

Conversion of protocatechuate to ketoadipate. IPTG-induced cultures of strain JM109(pZR1) were washed and suspended in 100 ml of basal medium to a final concentration corresponding to about 1 mg of soluble protein per ml. Sodium protocatechuate (1 M) was added to a final concentration fo 100 mM, and the mixture was stirred at 37°C; the pH was maintained between 6.5 and 7.5 by addition of 1 M NaOH. Concentrations of protocatechuate and ketoadipate were measured in samples which were clarified by centrifugation and separated by reverse-phase chromatography on a Vydac 201 HS column (0.46 by 25 cm; 10-μm particle size) at room temperature. Respective mobile phases A and B were 0.1% phosphoric acid in water and 0.1% phosphoric acid in acetonitrile. The flow rate was 1 ml/min, and the gradient extended from 0 to 100% phase B over 30 min. β-Ketoadipate concentrations were measured at 214 nm, and protocatechuate concentrations were determined at 254 nm.

Tn*5* mutagenesis of the cloned *pca* gene cluster. *E. coli* EM3001 was made competent by the method of Dagert and Ehrlich (6), transformed with pZR1, and mutagenized with λ NK467, a Tn*5* vector, by the method of de Bruijn and Lupski (7). Mutagenized cells were pooled, and plasmid was purified by the method of Ish-Horowicz (17). Purified plasmid was transformed into *E.coli* JM109 with selection on L broth-ampicillin-kanamycin plates to isolate plasmids containing a Tn*5* insertion. The *pca* gene that had been inactivated by

TABLE 1. Strains and plasmids used in this study

Strains and plasmids	Relevant description	Source or reference
A. calcoaceticus		
ADP1	Wild type (BD413)	15
ADP6	*pcaA3006*	This laboratory
ADP125	*pcaE3125*	This laboratory
ADP141	*pcaE3125 Δben–cat-3141*	This laboratory
ADP157	*pobA3157 Δben–cat-3144*	This laboratory
ADP161	*Δben–cat-3141*	ADP141 × ADP1
E. coli		
JM109	*recA1 Δlac-pro endA1 gyrA96 thi-1 hsdR17 supE44 relA1* F′ *traD36 proAB*$^+$ *lacI*^{q}*ZΔM15*	29
EM3001	*met-100 hsdR11 gal-151 recA56*, DE46$^-$, λ^s	E. A. Elsinghorst
Plasmids		
pUC18		29
pZR1	*pcaACBDFE* on 11-kbp *Eco*RI fragment in pUC18	This study
pZR2	*pcaAC* on 2.5-kbp *Hin*dIII fragment in pUC18	Subclone of pZR1
pZR3	*pcaD* on 2.8-kbp *Hin*dIII fragment in pUC18	Subclone of pZR1
pZR4	*pcaCBDF* on 5.6-kbp *Sal*I fragment in pUC18	Subclone of pZR1
pZR5	1.3-kbp *Sal*I-*Eco*RI fragment in pUC18	Subclone of pZR1
pZR6	*pcaBDF* on 4.1-kbp *Hin*cII fragment in pUC18	Subclone of pZR1
pZR10	*pcaA101*::Tn*5* of pZR1	Tn*5* mutagenesis
pZR11	*pcaB102*::Tn*5* of pZR1	Tn*5* mutagenesis
pZR12	*pcaC103*::Tn*5* of pZR1	Tn*5* mutagenesis
pZR13	*pcaD104*::Tn*5* of pZR1	Tn*5* mutagenesis
pZR14	*pcaE105*::Tn*5* of pZR1	Tn*5* mutagenesis

transposon insertion was determined by enzyme assay of crude extracts from IPTG-induced cultures. The location of the Tn*5* inserts was deduced by comparison of the size restriction fragments formed after digestion of pZR1 and transposon-containing derivatives with *Sal*I, *Hin*cII, or *Hin*dIII.

DNA analysis. The alkaline lysis procedure of Ish-Horowicz (17) was used for rapid isolation of plasmid DNA. DNA was digested with endonucleases and electrophoresed through agarose gels as described previously (23). For hybridization with radiolabeled probes, DNA was transferred to nitrocellulose by the method of Southern (25). Purified DNA fragments were labeled by nick translation with DNA polymerase I and [^{32}P]dCTP; hybridization was carried out as described by Johnson et al. (13). The natural transformation procedure of Juni (14) with selection of recombinants on 5 mM *p*-hydroxybenzoate was used to identify DNA fragments containing functional alleles corresponding to *pca* mutations in *A. calcoaceticus*.

Reagents. Muconolactone and β-carboxy-*cis*,*cis*-muconate were synthesized enzymatically and purified as previously described (21). Goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase was purchased from Cooper Biomedical, Inc.

RESULTS

Isolation of cloned *pca* genes. In a previous study (33), antiserum to *A. calcoaceticus* β-ketoadipate:succinyl-CoA transferase I, product of the *pcaE* gene, was prepared. *E. coli* does not form this enzyme, and extracts of wild-type *E. coli* do not cross-react with the antiserum. We used the expression plasmid pUC18 to construct in *E. coli* three genomic libraries of *A. calcoaceticus* ADP161 (from which the *cat* genes have been deleted [23]). One library was created by using a *Sau*3A partial digest of DNA from strain ADP161, and the other two were constructed with *Bam*HI and *Eco*RI complete digests. Approximately 4,000 insert-containing transformants from each library were screened for expression of the CoA transferase. One clone, obtained from the *Eco*RI library, produced material that cross-reacted strongly with the antiserum and produced substantial levels of the CoA transferase activity. The plasmid from this transformant was isolated by cesium chloride-ethidium bromide density gradient centrifugation and termed pZR1.

Restriction endonuclesae digestion revealed that pZR1 contained a single 11-kbp insert in the *Eco*RI site of pUC18. Southern hybridization revealed that the cloned fragment hybridized to both a 5-kbp and an 11-kbp *Eco*RI fragment of wild-type *A. calcoaceticus* chromosomal DNA; the 5-kbp fragment corresponded to the previously cloned *Eco*RI segment containing the *cat* genes (23). Neither cloned fragment of *A. calcoaceticus* DNA hybridized with *E. coli* DNA.

Transformation of *A. calcoaceticus* mutant strains ADP6 (*pcaA3006*) and ADP141 (*pcaE3125*) with the cloned 11-kbp fragment yielded a high frequency of natural transformants that had acquired the wild-type *pca* phenotype. Digestion of the 11-kbp fragment with the restriction enzyme *Hin*cII inactivated the wild-type *pcaA* allele, and digestion of the fragment with *Nco*I inactivated the wild-type *pcaE* allele (Fig. 2). The wild-type allele corresponding to the *pcaA3006* and *pcaE3125* mutations were inactivated slightly or not discernably by a number of other restriction enzymes (Fig. 2).

The 11-kbp fragment did not transform strain ADP157 (*pobA3157*) to wild type. The *pobA* mutation prevents expression of *p*-hydroxybenzoate hydroxylase, an enzyme that gives rise to protocatechuate, and thus it appears likely that the complete *pobA* gene does not lie on the 11-kbp *Eco*RI fragment.

Expression of the *pca* genes in *E. coli*. Evidence presented in the following sections demonstrates that *A. calcoaceticus* genes for protocatechuate catabolism are clustered in the order *pcaACBDFE*, and we shall make reference to the genes in this sequence as we describe their expression. Products of the *pcaABCDFE* genes were not present at

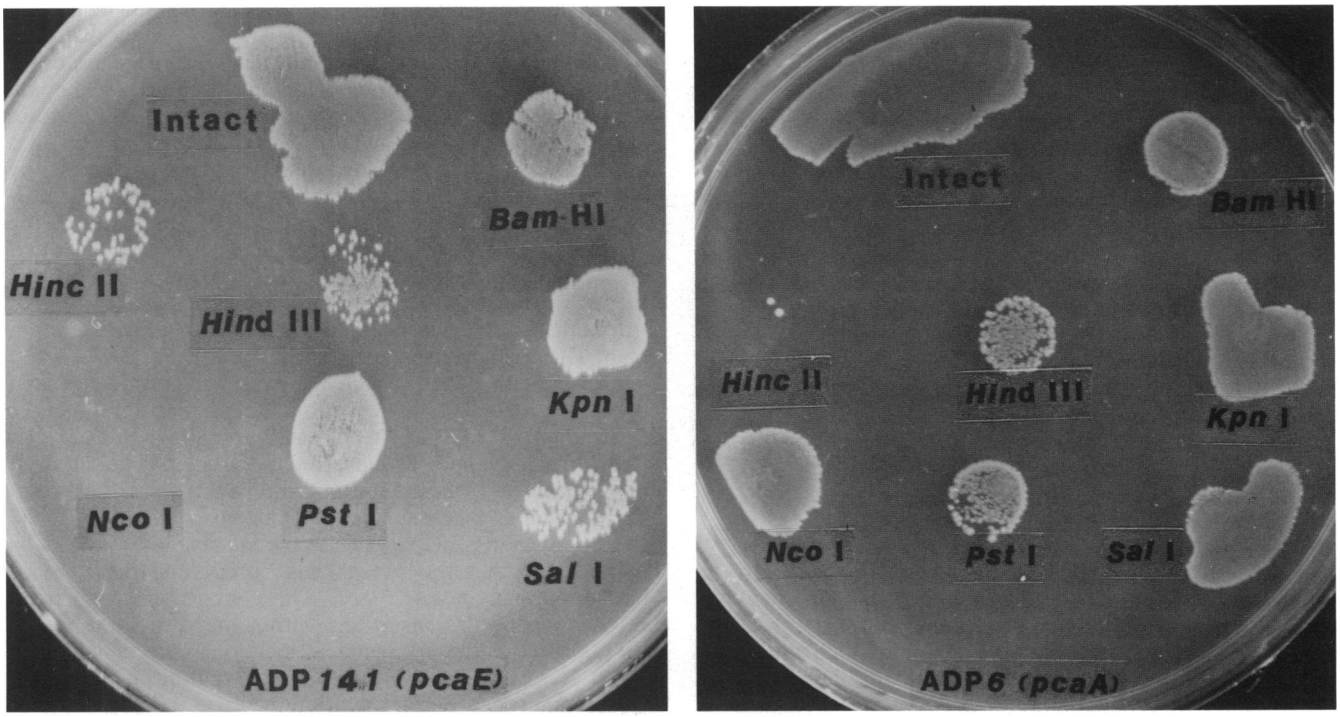

FIG. 2. Transformation of *A. calcoaceticus* strains ADP141 (*pcaE3125*) (left) and ADP6 (*pcaA3006*) (right) with the cloned 11-kbp *Eco*RI fragment and with the fragment digested with several restriction enzymes.

detectable levels in *E. coli* JM109 grown either in the presence or absence of the inducer IPTG. As expected on the basis of the preceding genetic evidence, the *pobA* gene product was not detected in extracts of strain JM109(pZR1). The organism expressed the full set of the *pcaACBDFE* genes at levels exceeding the specific activities found in fully induced cultures of *A. calcoaceticus*, and addition of IPTG to the growth medium increased levels of expression in JM109(pZR1) three- to fourfold (Table 2). The subunit sizes of the enzymes require about 6.2 kbp of DNA sequence, and the presence of the enzyme activities in strain JM109(pZR1) reveals that this information is carried on the 11-kbp *Eco*RI insert in pZR1. Induction of the enzymes encoded on pZR1 in response to IPTG indicates that the *pcaACBDFE* genes can be transcribed under control of a single promoter.

Comparison of the high levels of *pcaACBDFE* expression in IPTG-induced cultures of JM109(pZR1) with specific activities of the purified gene products indicated that the enzymes formed roughly half the soluble cell protein. This conclusion was fortified by demonstration that crude extract of the organisms was resolved by HPLC into six enzyme activities corresponding to five sharp peaks of protein concentration (Fig. 3). The material eluting in the protocatechuate oxygenase (product of the *pcaA* gene) peak was judged to be nearly homogeneous by its behavior on different HPLC columns, but its specific activity of 4 μmol/min per mg of protein was low compared with the specific activity of 20 μmol/min per mg of protein found with homogeneous protocatechuate oxygenase purified from *A. calcoaceticus* (11).

IPTG-induced cultures of *E. coli* JM109(pZR1) contain enzyme activity sufficient to convert protocatechuate to succinate and acetyl-CoA intermediates at a rate of roughly 0.05 μmol/min per mg of soluble cell protein at room temperature. *E. coli* grows at the expense of succinate or acetate, and so it was somewhat surprising that protocatechuate at 2 mM, a concentration established not to be toxic, supported no growth of strain JM109(pZR1) in the presence of IPTG. Closer examination revealed that the bacteria convert protocatechuate to β-ketoadipate. At 37°C, a washed suspension of the cells at a concentration corresponding to about 1 mg of soluble cell protein per ml

TABLE 2. Activity of *pca*-encoded enzymes

Gene	Sp act (μmol/min per mg of protein)			Ratio	
	ADP1	pZR1[a]	pZR1 + IPTG[b]	pZR1 + IPTG/pZR1	pZR1 + IPTG/ADP1
pcaA	0.05	0.11	0.14	1.2	2.7
pcaB	2.18	26.6	72.0	2.7	33.0
pcaC	0.79	6.7	20.1	3.0	25.4
pcaD	1.25	5.0	19.4	3.9	15.5
pcaE	0.09	0.27	0.9	3.3	10.0
pcaF	0.07	0.58	2.2	3.8	31.4

[a] *E. coli* JM109(pZR1).
[b] *E. coli* JM109(pZR1) induced with IPTG.

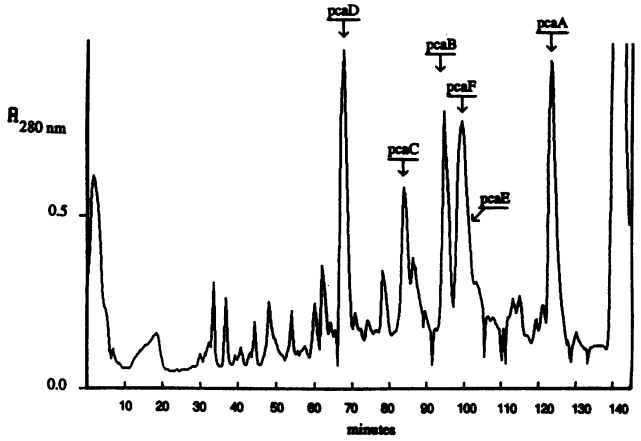

FIG. 3. HPLC separation of crude extract from *E. coli* JM109(pZR1).

quantitatively converted 100 M protocatechuate to β-ketoadipate in about 200 min, a rate corresponding to about three times the rate that would be predicted for the transformation at room temperature. The relatively high rate probably is due to the fact that the enzymatic conversion by whole cells was conducted at 37°C.

Tn*5* mutagenesis of the cloned *pca* gene cluster. Mutagenesis of pZR1 with transposon Tn*5* resulted in the isolation of a number of plasmids with Tn*5* in the *pca* gene cluster. Plasmids carrying a Tn*5* insertion were transformed into *E. coli* JM109, and transformants were assayed for *pca*-encoded enzymes. The modified plasmids were designated as follows: *pcaA*::Tn*5*, pZR10; *pcaB*::Tn*5*, pZR11; *pcaC*::Tn*5*, pZR12; *pcaD*::Tn*5*, pZR13; *pcaE*::Tn*5*, pZR14. As has been observed by others examining the properties of multicopy plasmids with Tn*5* insertions (7), polarity effects were insufficient to allow identification of genes transcribed distal to the site of insertion.

Genetic organization in the *pca* cluster. A restriction map of the 11-kbp *Eco*RI fragment carrying the *pca* genes was prepared (Fig. 4), and a preliminary indication of the gene order was given by the enzymes expressed in strain JM109 derivatives carrying subclones with *pca* genes expressed under the *lac* promoter in pUC18. The 5.6-kbp *Sal*I-*Sal*I fragment, extending from 4.1 to 9.7 kbp in the restriction map (Fig. 4), expressed the *pcaBCDF* genes but not the *pcaA* or the *pcaE* gene. Thus it appeared that restriction at the *Sal*I sites cut within or near the latter two genes. The *pcaA* gene was expressed with *pcaC* on the subcloned 2.5-kbp *Hin*dIII-*Hin*dIII fragment that extends from 2.7 to 5.2 kbp (Fig. 4); the other *pca* genes were not expressed from this fragment. The presence of part of the *pcaA* gene at a position below 4.1 kbp on the restriction map was deduced from the ability of the 4.1-kbp *Eco*RI-*Sal*I fragment to transform the *pcaA3006* mutation and by mapping the *pcaA*::Tn*5* insertion to this fragment. The fragment did not express the *pcaA* gene. The *pcaE* gene was not expressed from the 1.3-kbp *Sal*I-*Eco*RI fragment extending from 9.7 to 11 kbp (Fig. 4), but the presence of part of the *pcaE* gene on the fragment was inferred by its ability to transform the *pcaE3125* mutation in *A. calcoaceticus* ADP141 to wild type and by mapping the *pcaE*::Tn*5* insertion to the fragment. Neither the *pcaA3006* mutation nor the *pcaE3125* mutation was transformed by the 5.6-kbp *Sal*I-*Sal*I fragment lying between 4.1 and 9.7 kbp on the restriction map (Fig. 4).

The foregoing information, coupled with knowledge that the *pcaA* gene requires 1.2 kbp of DNA sequence, allowed us to conclude that the genes are transcribed in the order *pcaAC(BDF)E* at a location beginning at or above 2.9 kbp on the restriction map. The *pcaB* and *pcaF* genes each require about 1.2 kbp of DNA, and the *pcaD* gene requires about 1 kbp. Thus a total of 3.4 kbp of DNA is associated with the *pca(BDF)* genes which are expressed on the 4.1-kbp *Hin*cII-*Hin*cII fragment extending from 5.2 to 9.3 kbp on the restriction map. The presence of *pcaD* between the *pcaB* and *pcaF* genes was deduced by expression of *pcaD*, but not the other two genes, from the 2.8-kbp *Hin*dIII-*Hin*dIII fragment lying between 6.3 and 9.1 kbp on the restriction map. This evidence suggests that the *Hin*dIII site at 6.3 kbp lies within either the *pcaB* or the *pcaF* structural gene, and the 9.1-kbp *Hin*dIII site lies within the other structural gene. Restriction of pZR11, the plasmid containing the *Eco*RI-*Eco*RI fragment with Tn*5* inserted in the *pcaB* gene, allowed us to locate the insertion at 6.3 kbp in the restriction map. These results, together with the known orientation of the 11-kbp insert in pZR1 relative to the *lac* promoter, allows us to conclude that the overall gene order and direction of transcription are *pcaACBDFE*. This conclusion was fortified by location of Tn*5* insertions within the respective *pcaA*, *pcaC*, *pcaD*, and *pcaE* genes at locations corresponding to 3.2, 4.6, 7.6, and 10.1 kbp on the restriction map (Fig. 4). Observation that the *Sal*I restriction site at 9.1 kbp lies near or within the 1.2-kbp *pcaE* gene indicates that the approximately 6.2 kbp of DNA associated with the *pcaACBDFE* gene cluster lies between 2.9 kbp and 10.3 kbp on the restriction map (Fig. 4).

DISCUSSION

Organization of the *pca* genes in *A. calcoaceticus*. The results show that the approximately 6.2 kbp of DNA required for the *pca* genes is clustered within a region of no more than 7.4 kbp. The genes are expressed together under control of the *lac* promoter in *E. coli*. They are coordinately induced in response to protocatechuate in wild-type *A.*

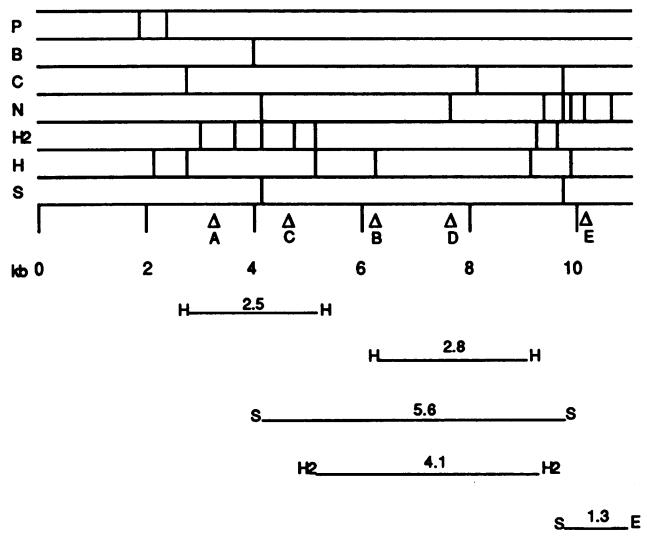

FIG. 4. Partial restriction map of the cloned 11-kbp *Eco*RI fragment containing the *pca* genes, showing the location of Tn*5* insertions and subclones isolated. Symbols: P, *Pst*I; B, *Bam*HI; C, *Cla*I; N, *Nco*I; H2, *Hin*cII; H, *Hin*dIII; S, *Sal*I. Tn*5* insertions are indicated by Δ.

calcoaceticus (4), and the constitutive expression of the tightly clustered genes in regulatory mutant strains (4, 22) strongly suggests that they form an operon.

The order of the clustered *A. calcoaceticus* genes is *pcaACBDFE*, an arrangement quite dissimilar to the organization of *pca* genes in *Pseudomonas putida*. Most of the latter genes are expressed inducibly in response to β-ketoadipate rather than protocatechuate, and the *pcaE* gene is separated by more than 20 kbp from a cluster containing genes in the order *pcaBDC* (J. Hughes and J. Houghton, personal communication). The latter genes, known to be homologous on the basis of the amino acid sequences of their products (31, 32), appear to have rearranged while maintaining tight linkage during their divergence in *A. calcoaceticus* and *P. putida*.

In principle, the arrangement of all of *pca* genes in a single cluster in the *A. calcoaceticus* chromosome might be regarded as a demonstration of genetic simplicity, but this view overlooks a subtle demand placed by maintenance of the *pcaDFE* genes on the same chromosome as a cluster containing the *catDEF* genes. The latter genes encode enzymes that are isofunctional with their *pca* counterparts. Amio acid sequence comparisons indicate that the isofunctional *A. calcoaceticus* enzymes are homologous (30–34), and this conclusion is strengthened by our observation of hybridization between *A. calcoaceticus* DNA fragments containing the *cat* and *pca* gene clusters. Legitimate recombination can cause deletion of closely homologous DNA sequences, and stable maintenance of both *cat* and *pca* genes, observed with wild-type *A. calcoaceticus*, demands that they be divergent to a degree sufficient to prevent frequent recombination between them. Indeed, selection for recombinants that apparently have acquired *cat* sequences in the *pca* region can result in dramatic instability of the *pca* genes (7a). Selective demand for sequence divergence to avoid the loss of genes through recombination may have contributed to sequence rearrangements that have been observed within and among genes (32, 34).

Natural transformation as an aid in characterizing cloned genes. Natural transformation allows swift identification of cloned DNA corresponding to dysfunctional alleles in *A. calcoaceticus* mutant strains (19). In the present investigation, transformation allowed us to locate the *pcaA3006* allele on DNA contained within the 4.1-kbp *Eco*RI-*Sal*I fragment and the *pcaE3125* allele on DNA contained within the 1.3-kbp *Sal*I-*Eco*RI fragment (Fig. 4). Furthermore, transformation with DNA fragments that have undergone digestion with restriction enzymes may give an indication of restriction sites that lie near structural genes. Treatment of the 11-kbp *Eco*RI-*Eco*RI fragment with *Hin*cII destroyed its ability to transform the *pcaA3006* mutation (Fig. 2), and a cluster of *Hin*cII sites lie in the region that contains the *pcaA* gene (Fig. 4). Similarly, digestion of the fragment with *Nco*I inactivated DNA that transformed the *pcaE3125* mutation (Fig. 2), and a number of *Nco*I sites lie in the region that contains the *pcaE* gene (Fig. 4).

General insertion of Tn*5* into sites within *A. calcoaceticus* DNA. *A. calcoaceticus* BD413, the strain from which the organisms used in this investigation were derived, provides a remarkable exception to the normal pattern of general insertion of Tn*5* into numerous sites within procaryotic genomes. Singer and Finnerty (24) observed two hotspots for insertion when *A. calcoaceticus* BD413 was mutagenized with Tn*5* by using the suicide plasmid pJB4JI as the delivery vector. Our success in introducing Tn*5* into five of the six *A. calcoaceticus pca* genes cloned in *E. coli* indicates that, in the absence of the hotspots, there are no barriers to generalized mutagenesis of *A. calcoaceticus* DNA with Tn*5*. The ease with which *A. calcoaceticus* can undergo natural transformation should make it possible to introduce genes containing Tn*5* insertions into the organism's chromosome.

Expression and function of *pca* genes in *E. coli*. In this and previous investigations (19, 23), we have observed constitutive expression of cloned *A. calcoaceticus* genes at high levels in *E. coli*. Therefore expression of *A. calcoaceticus* genes in *E. coli* may permit overall metabolic transformations to be achieved rapidly. Such aspirations must take into account two caveats that emerged from this investigation. One warning arises from the relatively low specific activity of protocatechuate oxygenase, product of the *pcaA* gene, in *E. coli* JM109(pZR1). The other is raised by the failure of this strain to metabolize β-ketoadipate, a metabolic deficiency dramatized by the organism's quantitative formation of this metabolite from protocatechuate.

Integration of the HPLC peak for protocatechuate oxygenase (Fig. 3) indicates that a relatively inactive form of the enzyme accounts for about 8% of the soluble cell protein in IPTG-induced cultures of JM109(pZR1). The reason for the relatively low specific activity of the enzyme is not known, but the enzyme requires ferrous iron, and it is possible that insufficient iron is incorporated into the oxygenase during its assembly in *E. coli*.

Failure of the *E. coli* JM109(pZR1) cultures to metabolize β-ketoadipate cannot be attributed to lack of the compound or of the enzymes that act upon it, because these materials were present in abundance. Representatives of *E. coli* use acetyl-CoA as a donor for CoA transferase reactions (26), and metabolic levels of acetyl-CoA substantially exceed levels of succinyl-CoA in *E. coli* (12). Thus the limitation of β-ketoadipate metabolism in *E. coli* may be imposed by succinyl-CoA pool levels insufficient to drive effective thioester transfer.

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