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Cloning and Expression of *Acinetobacter calcoaceticus catBCDE* Genes in *Pseudomonas putida* and *Escherichia coli*

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This report describes the isolation and preliminary characterization of a 5.0-kilobase-pair (kbp) *Eco*RI DNA restriction fragment carrying the *catBCDE* genes from *Acinetobacter calcoaceticus*. The respective genes encode enzymes that catalyze four consecutive reactions in the catechol branch of the β-ketoadipate pathway: *catB*, muconate lactonizing enzyme (EC 5.5.1.1); *catC*, muconolactone isomerase (EC 5.3.3.4); *catD*, β-ketoadipate enol-lactone hydrolase (EC 3.1.1.24); and *catE*, β-ketoadipate succinyl-coenzyme A transferase (EC 2.8.3.6). In *A. calcoaceticus*, *pcaDE* genes encode products with the same enzyme activities as those encoded by the respective *catDE* genes. In *Pseudomonas putida*, the requirements for both *catDE* and *pcaDE* genes are met by a single set of genes, designated *pcaDE*. A *P. putida* mutant with a dysfunctional *pcaE* gene was used to select a recombinant pKT230 plasmid carrying the 5.0-kbp *Eco*RI restriction fragment containing the *A. calcoaceticus catE* structural gene. The recombinant plasmid, pAN1, complemented *P. putida* mutants with lesions in *catB*, *catC*, *pcaD*, and *pcaE* genes; the complemented activities were expressed constitutively in the recombinant *P. putida* strains. After introduction into *Escherichia coli*, the pAN1 plasmid expressed the activities constitutively but at much lower levels that those found in the *P. putida* transformants or in fully induced cultures of *A. calcoaceticus* or *P. putida*. When placed under the control of a *lac* promoter on a recombinant pUC13 plasmid in *E. coli*, the *A. calcoaceticus* restriction fragment expressed *catBCDE* activities at levels severalfold higher than those found in fully induced cultures of *A. calcoaceticus*. Thus there is no translational barrier to expression of the *A. calcoaceticus* genes at high levels in *E. coli*. The genetic origin of the cloned *catBCDE* genes was demonstrated by the fact that the 5.0-kbp *Eco*RI restriction fragment hybridized with a corresponding fragment from wild-type *A. calcoaceticus* DNA. This fragment was missing in DNA from an *A. calcoaceticus* mutant in which the *cat* genes had been removed by deletion. The properties of the cloned fragment demonstrate physical linkage of the *catBCDE* genes and suggest that they are coordinately transcribed.

The β-ketoadipate pathway is used widely in microbial dissimilation of aromatic compounds (33). The central reactions of the pathway are shown in Fig. 1: an analogous series of reactions, catalyzed by products of the *pca* and *cat* genes, convert protocatechuate and catechol, respectively, to tricarboxylic acid cycle intermediates. Representative strains of *Acinetobacter calcoaceticus* are noteworthy in that they exert extensively unified transcriptional controls over the *cat* and *pca* genes (26). For example, one regulatory gene governs expression of the *catBCDE* genes and another regulatory gene controls transcription of the *pcaABCDE* genes (7, 30). The unified control patterns demand genes encoding isofunctional enzymes in *A. calcoaceticus*: the *catE* and the *pcaE* structural genes encode different β-ketoadipate succinyl-coenzyme A (CoA) transferases. A third enzyme with β-ketoadipate succinyl-CoA transferase activity is encoded by a gene associated with the adipate pathway (6).

In contrast to *A. calcoaceticus*, *Pseudomonas putida* elaborates a single β-ketoadipate succinyl-CoA transferase, encoded by the *pcaE* structural gene, which is required for utilization of either benzoate (via the catechol pathway; Fig. 1) or *p*-hydroxybenzoate (via the protocatechuate pathway; Fig. 1). Similarly, a single enol-lactone hydrolase, the product of the *pcaD* structural gene (Fig. 1), is found in *P. putida* (33). The *catB* and *catC* genes are coordinately controlled and tightly linked in this species. More remarkably, these genes are clustered on the chromosome with independently regulated genes for catabolic pathways that give rise to catechol in *P. putida* (36). The possibility that *A. calcoaceticus catBCDE* genes are linked to each other and linked to the independently transcribed *ben* genes (encoding enzymes that convert benzoate to catechol; Fig. 1) was raised by the isolation of the *A. calcoaceticus* mutant strain ADP141: this organism carries a deletion mutation extending from the *ben* into the *cat* region (M. E. Rae, unpublished observation).

To further characterize the *ben* and *cat* regions from *P. putida* and *A. calcoaceticus*, we attempted to clone the genes on the broad-host-range plasmid pKT230 (2). Our initial effort, an attempt to select for recombinant plasmids containing the *P. putida cat* genes in *P. putida* mutants carrying deletions, gave rise to unstable derivatives, probably because of rapid recombination between homologous regions on the plasmid and the chromosome. This difficulty would not be expected when cloning *A. calcoaceticus cat* genes in the heterologous host, since genes for isofunctional enzymes from *A. calcoaceticus* and *P. putida* have diverged so widely that homologous recombination between them is unlikely (28). It appeared probable that selection for *A. calcoaceticus* genes on a recombinant pKT230 plasmid in a *P. putida* mutant carrying a mutation in the *pcaE* gene would give stable derivatives. In this report we describe the use of this procedure to isolate a strain carrying the *catBCDE* genes from *A. calcoaceticus*. We present a restriction map for the 5.0-kilobase-pair (kbp) fragment carrying the genes. In addition, we offer direct evidence that the genes can be transcribed as a unit and are physically absent in the *A. calcoaceticus* strain carrying the *ben-cat* deletion.

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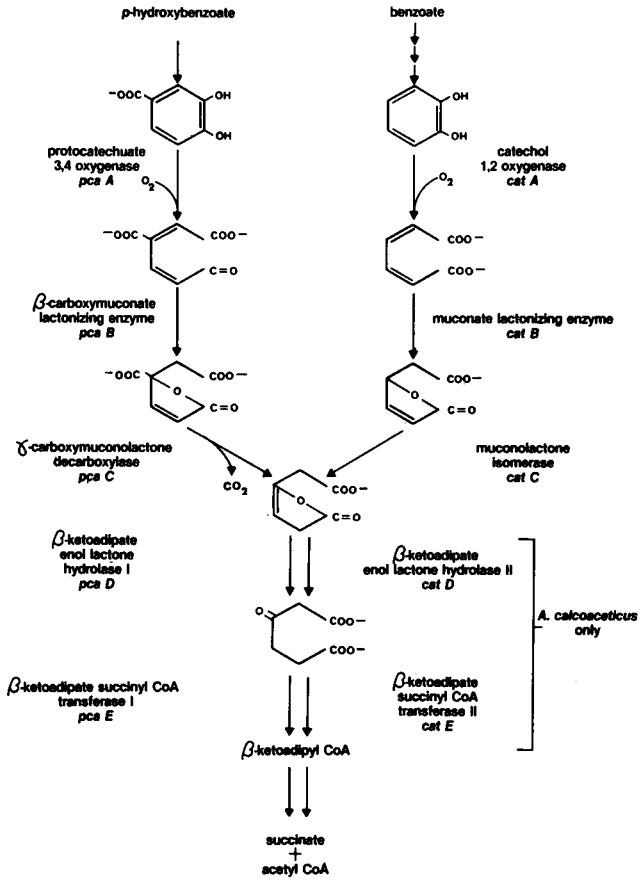

FIG. 1. The β-ketoadipate pathway for the dissimilation of aromatic compounds. Genetic designations for the enzymes are shown. A single hydrolase (*pcaD*) and a single transferase (*pcaE*) are synthesized in *P. putida*.

MATERIALS AND METHODS

Chemicals and reagents. All restriction enzymes, T4 DNA ligase, and calf intestine phosphatase were obtained commercially and were used and stored according to the manufacturers' recommendations. *cis,cis*-Muconate was a gift from the Celanese Research Company. Muconolactone was synthesized enzymatically and purified as previously described (27). All other chemicals were of reagent grade purity or were the highest quality available.

Bacterial strains and plasmids. The bacterial strains used here are listed with their genotypes and sources in Table 1. The table also presents the relevant genotypes of the plasmids used in this study.

Media and growth conditions. Cultures of *A. calcoaceticus* and *P. putida* were grown and maintained on minimal medium plates (27) supplemented with Hutner's Metals 44 (9) solidified with 1.5% agar. Streptomycin and kanamycin were used in selective media at a concentration of 150 μg per ml; ampicillin and tetracycline were used at respective concentrations of 40 and 25 μg/ml. Cultures to be used for the preparation of chromosomal or plasmid DNA were grown in L broth (17) supplemented with a selective antibiotic where appropriate. *P. putida* was cultured at 30°C; *A. calcoaceticus* and *E. coli* were cultured at 37°C. Cultures grown for the investigation of the regulation of enzyme synthesis were incubated in 100 ml of medium in 500-ml baffled shake flasks at 200 rpm in a New Brunswick controlled-environment incubator shaker. *E. coli* was cultured in L broth, on L agar plates, or in defined minimal medium (1) as indicated.

Purification of DNA. Chromosomal *A. calcoaceticus* DNA was purified from 50 ml of a fully grown culture according to established procedures (4). Plasmid DNA was purified by the cleared-lysate method (26) using cesium chloride-ethidium bromide density gradient centrifugation (3).

Restriction endonuclease digestion, ligation, and transformation. Chromosomal *A. calcoaceticus* DNA (2 μg) was digested with *Eco*RI under conditions recommended by the supplier to yield a complete digest. This DNA was mixed with 1 μg of *Eco*RI-cut pKT230 and ligated overnight at 4°C with T4 DNA ligase. The ligated preparation was then used to transform cells which had been made competent by treatment with 0.1 M $CaCl_2$ according to the method of Dagert and Ehrlich (10). Transformants were selected for growth on minimal benzoate medium.

Analysis of recombinant plasmids. Potential *P. putida* transformants were examined for the presence of plasmid DNA by a rapid screening procedure in which 10 ml of a saturated culture was lysed in a Tris-sucrose-EDTA solution with lysozyme (11). The DNA was electrophoresed on 0.7% horizontal submerged agarose gels in 89 mM Tris-borate buffer (pH 8.3) with 2.5 mM EDTA (19). After electrophoresis the DNA was visualized by staining with ethidium bromide and viewed under UV light. Subcloning of recombinant DNA in *E. coli* was performed by ligating either 1 μg of digested plasmid DNA or purified fragments with 1 μg of appropriately cut and dephosphorylated vector. *E. coli* transformants were plated on selective media and screened for the presence of insert DNA by electrophoresis of rapid plasmid screening preparations and comparison of their migration to that of standard plasmids. Where possible, insertional inactivation of either antibiotic resistance markers or β-galactosidase was also used to screen for the

TABLE 1. Bacterial strains and plasmids

Strains and plasmids	Genotype or phenotype	Source
P. putida		
PRS5	*pcaD*	This study
PRS2000	Wild type	This study
PRS2015	*catB*	This study
PRS2191	*catC*	This study
PRS2241	*pcaE*	This study
A. calcoaceticus		
ADP1	Wild type (BD413)	E. Juni
ADP141	Δ(*ben-cat*) *3141 pcaE3125*	This study
E. coli		
HB101	*pro leu thi lacY hsdR hsdM recA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44*	B. Bachmann
JM101	Δ(*lac-pro*) *supE* F' *traD36 proAB lacI^{q}ZΔM15*	J. Messing
Plasmids		
pKT230	Km^r Sm^r	K. N. Timmis
pBR322	Ap^r Tc^r	R. Bolivar
pUC13	Ap^r *lac*p/o	J. Messing

presence of insert DNA. Subclones were identified by characteristic plasmid restriction patterns and by the presence of enzyme activities corresponding to the *catBCDE* genes. Restriction analysis of a number of different subclones was then used to construct a restriction map of the cloned *A. calcoaceticus* DNA.

Southern hybridizations were performed according to established procedures (32). Chromosomal DNA was digested to completion and electrophoresed through 0.7% agarose gels. The DNA was acid nicked (35) and transferred to nitrocellulose membrane filters. Purified fragments were labeled by nick translation with DNA polymerase and [α-^{32}P]dATP; hybridization was carried out in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.4) at 30°C. Filters were washed in 0.2× SSC with 0.2% sodium dodecyl sulfate added for 30 min at 40°C and then for 2 h at 55°C before being exposed.

Measurement of enzyme activity. Cultures for enzyme assays were grown to a cell density between 120 and 160 Klett units as measured in a Klett-Summerson colorimeter (no. 54 filter) and collected by centrifugation at 10,000 × *g* for 5 min. Cells were either frozen overnight or immediately broken by sonication. The enzymes were stable for extended periods when stored in frozen cell pellets. The cells were broken in 50 mM Tris hydrochloride (pH 7.5)–10% (vol/vol) glycerol–5 mM ammonium sulfate–5 mM magnesium chloride–1 mM EDTA–1 mM dithiothreitol. Cell debris was removed by centrifugation at 17,500 × *g* for 30 min, and all enzyme activities were determined for each extract immediately. Activities of enzymes encoded by the *catBCDE* genes were determined by established procedures (24, 25, 39). Protein was measured by the method of Lowry et al. (18). Specific activities are reported as micromoles of product formed per minute per milligram of protein.

RESULTS

Selection of the *A. calcoaceticus catE* gene in a *pcaE* mutant *P. putida* strain. Expression of *pcaE*, the structural gene for the single β-ketoadipate succinyl-CoA transferase of *P. putida*, is blocked in strain PRS2241: the nonreverting mutation in this organism prevents its growth with either *p*-hydroxybenzoate or benzoate (Fig. 1). DNA prepared from *A. calcoaceticus* ADP1 (15) was digested with *Eco*RI and ligated into pKT230 (2) that had been purified from wild-type *P. putida* PRS 2000 and cut with *Eco*RI (Fig. 2). The resulting ligation mixture was used to transform strain PRS2241, and, after growth for 3 h, the culture was plated onto minimal benzoate medium to select for transformants that grew with benzoate. Only 1 of 18 examined transformants possessed the antibiotic resistance phenotype characteristic of the parent plasmid. It is likely that the other benzoate-positive strains were derived by transformation with wild-type chromosomal DNA copurified with the pKT230 plasmid from the wild-type strain; these transformants did not contain a plasmid. The antibiotic-resistant transformant contained a plasmid which, after purification in a cesium chloride-ethidium bromide gradient, efficiently transformed the original *pcaE* mutant to a benzoate-positive phenotype. The purified plasmid was designated pAN1 and, after restriction endonuclease digestion and subsequent electrophoresis with standard-molecular-weight DNAs, was judged to carry a single 5.0-kbp insert at the *Eco*RI site of pKT230 (Fig. 2).

Complementation of *catB*, *catC*, and *pcaD* mutations in *P. putida*. Plasmid pAN1 purified from the *pcaE* mutant PRS2241 was used to transform a set of *P. putida* mutants in order to determine other *A. calcoaceticus* genes carried on the 5.0-kbp insert. Wild-type transformants were obtained with high efficiency when the plasmid preparation was introduced into mutants carrying mutations in the *catB*, *catC*, and *pcaD* structural genes. However, between 20 and 60% of the transformants contained neither the drug resistance markers nor the plasmid after gaining the ability to grow with benzoate. This observation reinforced the interpretation that transformation by the recombinant plasmid was obscured by a high background of transformation by linear DNA derived from the donor strain. This possibility was tested by transforming the *P. putida* mutants with plasmid pAN1 purified from *E. coli*, a species that lacks genes for the β-ketoadipate pathway. The recombinant plasmid was transformed into *E. coli*, and the kanamycin resistance gene was selected. After purification from *E. coli*, the plasmid was introduced by transformation into *catB*, *catC*, *pcaD*, and *pcaE* mutant *P. putida* strains. Selection for growth with benzoate always produced kanamycin-resistant strains. Selection for kanamycin resistance invariably yielded recombinants that had acquired the ability to grow with benzoate. Thus, in the absence of chromosomal DNA encoding *catBCDE* functions, the plasmid-borne *cat* and kanamycin resistance genes demonstrated 100% linkage. Plasmid purified from *E. coli* was less than 1% as efficient as plasmid purified from *P. putida* in transforming the mutant

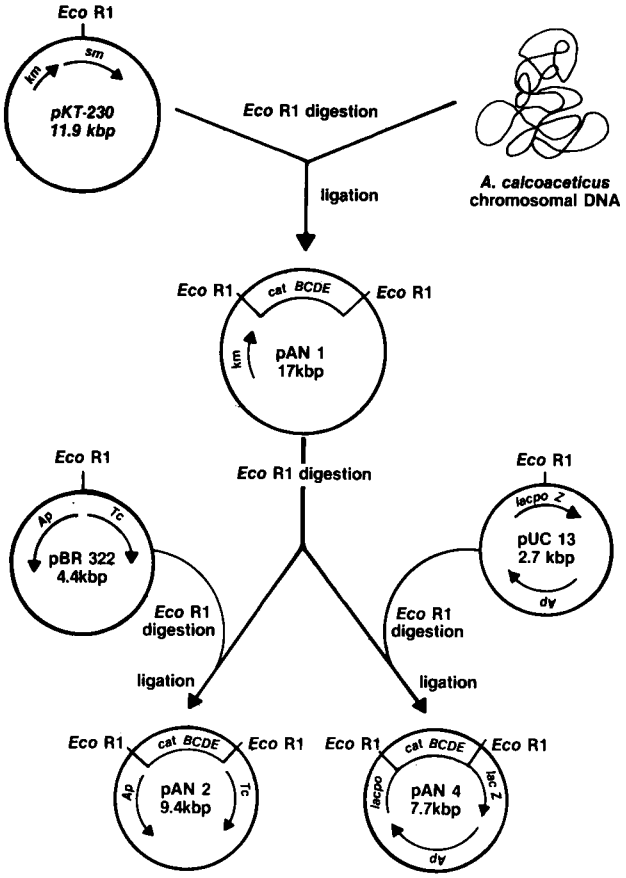

FIG. 2. Derivation of the *A. calcoaceticus catBCDE* subclones. *A. calcoaceticus* DNA was digested with *Eco*RI and ligated into pKT230 to give the plasmid pAN1. The 5.0-kbp *Eco*RI insert was then subcloned into complementary sites on pBR322 to yield pAN2 and pAN3 and into pUC13 to yield pAN4.

TABLE 2. Complementation of dysfunctional *P. putida* genes by constitutively expressed *A. calcoaceticus* genes carried on pAN1

Strain	Dysfunctional *P. putida* gene	Expression of complementing gene[a] in:	
		Benzoate-grown (induced) cells	Succinate-grown (uninduced) cells
PRS2015(pAN1)	*catB*	1.04	0.57
PRS2191(pAN1)	*catC*	0.36	1.25
PRS5(pAN1)	*pcaD*	1.76	3.43
PRS2241(pAN1)	*pcaE*	2.70	3.02

[a] Expressed as the ratio of the observed specific activity of the gene product to the corresponding specific activity found in extracts of fully induced (muconate-grown) *A. calcoaceticus*. The latter activities were 0.425 (*catB*), 2.8 (*catC*), 1.08 (*catD*), and 0.047 (*catE*) μmol/min per mg of protein.

strains. The difference in efficiency probably is due to the effective restriction barriers known to exist in *P. putida* (2).

Constitutive expression of the *A. calcoaceticus catBCDE* genes in *P. putida*. Enzymes of the β-ketoadipate pathway are inducible in *A. calcoaceticus* and *P. putida*: growth with an inducing substrate produces enzyme levels more than 50-fold higher than those found in uninduced cells (29). When strain PRS2241(pAN1) was grown in the absence of inducer, the *catBCDE* gene products were produced at elevated levels, comparable to those found in induced cultures (Table 2). In principle, the constitutive expression of the *catBCD* genes in PRS2241(pAN1) could be due to transcription of chromosomal *P. putida* genes. This possibility was eliminated by examining the expression of each plasmid-borne *A. calcoaceticus cat* gene in *P. putida* mutants lacking the corresponding enzymatic activity. As shown in Table 2, each *A. calcoaceticus cat* gene is expressed constitutively in *P. putida*.

Expression of the *catBCDE* genes in *E. coli*. When carried on the recombinant plasmid pAN1, the *catBCDE* genes were expressed constitutively in *E. coli*: specific activities ranged from 7 to 30% of fully induced levels (Table 3), substantially lower than the level of synthesis when the pAN1 plasmid was expressed constitutively in *P. putida* (Table 2). As was observed with *P. putida*, the level of expression of the *catBCDE* genes on the pAN1 plasmid was not increased when cells were exposed to an inducer (Table 3).

The *A. calcoaceticus Eco*RI insert DNA from pAN1 was subcloned into pBR322 (5) to yield plasmids pAN2 and pAN3, which differ only in the orientation of the insert (Fig. 2). When carried in *E. coli*, the recombinant plasmids did not express the *catB* gene, expressed the *catC* gene at low constitutive levels, and expressed the *catDE* genes at levels comparable to those found in induced *A. calcoaceticus* cultures (Table 3). This observation strengthens the conclusion, drawn from the properties of *A. calcoaceticus* regulatory mutant strains, that, under some circumstances, the *catCDE* genes may be transcribed from a promoter independent of the promoter that normally governs coordinate expression of all four genes (30). Growth of *E. coli* in the presence of glucose caused the constitutively expressed enzymes to be repressed to levels ranging from 23 to 63% of the levels found in cells grown on L broth (Table 3). Therefore it appears that catabolite repression can be exerted on the *A. calcoaceticus* promoter in *E. coli*.

When placed under the control of the *lac* promoter-operator of the plasmid pUC13 (34), the *catBCDE* genes were expressed at very high levels in *E. coli* carrying the resulting plasmid pAN4. As shown in Table 4, growth with the *lac* inducer isopropyl-β-D-thiogalactoside produced levels of enzyme ranging from 1.8 to 5.3% of the cell protein, and the *catBCDE* gene products accounted for more than 10% of the soluble protein in the cells. Omission of the inducer from the growth medium decreased the level of enzyme production by no more than 25% (Table 4), and growth in the presence of glucose lowered the specific activity of the enzymes to levels ranging between 50 and 70% of the specific activities found in fully induced cells (Table 4).

Catechol oxygenase, the *catA* gene product, and β-ketoadipyl-CoA thiolase, the product of the *catF* gene, were not present at detectable levels in *E. coli* strains carrying pAN4. The absence of β-ketoadipyl-CoA thiolase in these strains is also indicated by the fact that they rapidly convert muconate to β-ketoadipate but do not grow at the expense of muconate (J. Parales, unpublished observation).

Subcloning and construction of a restriction map. Plasmids pAN2 and pAN3 contain the 5.0-kbp *Eco*RI insert placed in the two possible orientations into the complementary site of pBR322 (Fig. 2). Four of the five *Hin*dIII fragments contained in the parent plasmid, pAN1, were subcloned into the corresponding site of pBR322, generating pAN12, pAN13, pAN14, and pAN15 (Fig. 3). Plasmid pAN12 (Fig. 3) contains 500 base pairs of the insert DNA and the 2-kbp fragment of pKT230 that connects the *Eco*RI site of insertion to the *Hin*dIII site of pKT230. The fifth *Hin*dIII fragment, containing the majority of the parent plasmid pKT230 and only 350 base pairs of *A. calcoaceticus* DNA, was not subcloned. The subclones were subjected to standard restriction analysis, and a physical map of the DNA was generated. The results of this study are summarized in Fig. 3.

Hybridization with DNA from the wild type and from a deletion mutant strain. The four subcloned *Hin*dIII restric-

TABLE 3. Constitutive expression of *catBCDE* genes in *E. coli*

Strain	Growth substrate	Level of expression[a] of:			
		catB	*catC*	*catD*	*catE*
HB101(pAN1)	L broth	0.08	0.04	0.30	0.30
HB101(pAN1)	L broth + muconate	0.08	0.03	0.25	0.26
HB101(pAN2)	L broth	<0.002	0.05	0.64	1.28
HB101(pAN2)	L broth + glucose	<0.002	0.02	0.15	0.38
HB101(pAN3)	L broth	<0.002	0.02	0.44	1.19
HB101(pAN3)	L broth + glucose	<0.002	0.01	0.12	0.32

[a] Expressed as the ratio of the observed specific activity to the specific activity found in fully induced (muconate-grown) cultures of *A. calcoaceticus*.

TABLE 4. Expression of *catBCDE* genes under a *lac* promoter in *E. coli* strain JM101(pAN4)

Growth substrate	Level of expression[a] of:			
	catB	*catC*	*catD*	*catE*
L broth	3.72	4.79	21.2	10.2
L broth + glucose	1.91	3.18	17.1	8.7
L broth + IPTG[b]	3.86 (1.8%)[c]	5.86 (1.8%)	24.7 (5.3%)	13.6 (2.7%)

[a] Expressed as the ratio of the observed specific activity to the specific activity found in fully induced (muconate-grown) cultures of *A. calcoaceticus*.

[b] IPTG, isopropyl-β-D-thiogalactoside.

[c] Values in parentheses represent enzyme level as the percentage of total cell protein.

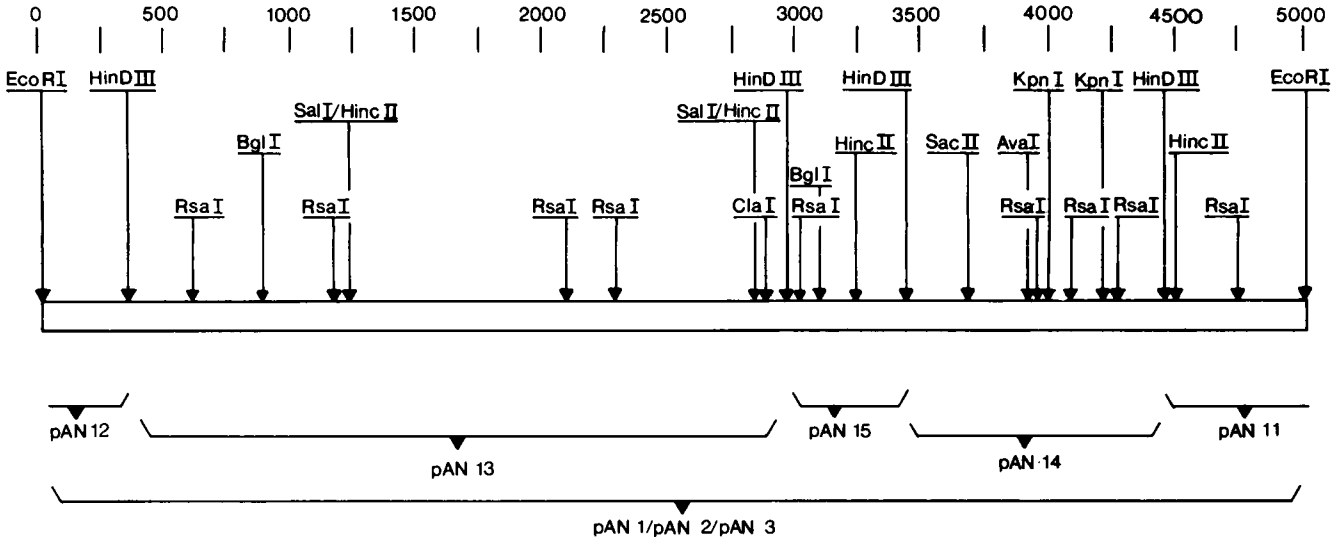

FIG. 3. Restriction map of the *catBCDE* gene region. Distances are given in base pairs from the *Eco*RI site, and restriction sites are indicated. No cut sites were found for the restriction enzymes *Bam*HI, *Pst*I, *Bgl*II, *Pvu*II, *Bcl*I, and *Xho*I. *Hin*dIII subclones pAN12 through pAN15 are indicated below. Plasmid pAN12 includes 2 kbp of pKT230 DNA from the *Hin*dIII site of that plasmid.

tion fragments and the 5.0-kbp *Eco*RI fragment were used as hybridization probes to analyze restricted chromosomal DNA from wild-type *A. calcoaceticus* and from strain ADP141, a deletion mutant that has lost the ability to express the *ben* and *cat* genes. Wild-type DNA was shown by Southern blot analysis (32) to strongly hybridize to *Eco*RI and *Hin*dIII restriction fragments with sizes identical to those that would be predicted on the basis of the restriction map shown in Fig. 3. These restriction fragments were not present in DNA from the deletion mutant strain. Figure 4 shows a Southern blot of deletion mutant ADP141 and wild-type chromosomal DNA digested with *Eco*RI and hybridized to a labeled *Hin*dIII fragment from plasmid pAN13. DNA from the deletion mutant strain clearly lacks the strongly hybridizing 5.0-kbp *Eco*RI fragment present in the wild-type chromosome. DNA from both strains produced an 11-kbp *Eco*RI fragment that hybridized less strongly to the *Hin*dIII fragment (Fig. 4). A fragment of identical length was revealed when other probes from the various subclones were hybridized with *Eco*RI digests of chromosomal DNA from the two strains.

DISCUSSION

Genes present and absent on the cloned 5.0-kbp *A. calcoaceticus* DNA fragment. Three lines of evidence indicate that the cloned *Eco*RI fragment contains the *catBCDE* genes from *A. calcoaceticus*. First, *P. putida* strains with mutations in the corresponding genes are complemented by the recombinant plasmid pAN1 in which the genes are expressed constitutively. Second, when placed under control by a suitable promoter the *catBCDE* genes are expressed in *E. coli*, an organism that does not utilize the β-ketoadipate pathway. Third, DNA corresponding to the 5.0-kbp *Eco*RI restriction fragment is present in wild-type *A. calcoaceticus* and missing in a mutant *A. calcoaceticus* strain in which the *catBCDE* genes have been removed by deletion. The fact that the deletion appears to extend into the *ben* genes suggests that these genes and the *cat* genes are tightly clustered in *A. calcoaceticus*.

The subunit sizes of the *catBCDE* gene products demand 3.0 kbp of structural gene information. Therefore, if the genes do not overlap, their sequence information occupies 60% of the fragment. We did not detect activities corresponding to the *catF* gene product (Fig. 1) in extracts of cells in which the *catBCDE* genes were expressed constitutively. This evidence does not preclude the possibility that the fragment may contain, in whole or in part, the *catF* gene; its lack of expression may be due to the absence of controls required to effect its transcription.

Hybridization with different *Hin*dIII probes derived from the 5.0-kbp *Eco*RI fragment consistently revealed an 11-kbp *Eco*RI DNA fragment that was present in both wild-type cells and the mutant strain from which the *catBCDE* genes had been deleted. This evidence indicates that the two *Eco*RI fragments are homologous over a substantial dis-

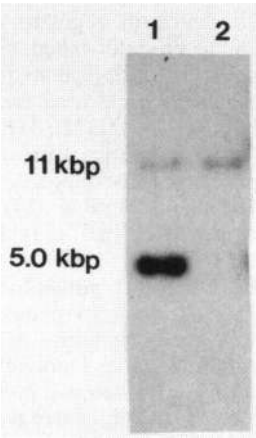

FIG. 4. Southern hybridization analysis of *A. calcoaceticus* strains. The chromosomal DNA was digested to completion with *Eco*RI, run out on a 0.7% agarose gel, and hybridized to the 3.0-kbp *Hin*dIII fragment of the pAN13 subclone. Lane 1 contains wild-type DNA, and lane 2 contains DNA from the *ben-cat* deletion strain ADP141.

tance. The *pcaCD* gene products are known to be homologous to their *catCD* counterparts (20, 37, 38), and so it is possible that the 11-kbp fragment encodes the *pca* genes (Fig. 1).

Transcription of the cloned *catBCDE* genes. The available evidence, based upon the properties of regulatory mutant strains, suggests that two different promoters may participate in expression of the *A. calcoaceticus catBCDE* genes. Most *cat* constitutive mutant strains express the *catBCDE* genes in the absence of inducer (30). In some mutant strains, the *catCDE* genes are expressed constitutively and the *catB* gene product is formed inducibly (30).

The two patterns of constitutive expression are mimicked by *E. coli* strains carrying the 5.0-kbp *Eco*RI restriction fragment. Promoters external to the fragment appear to cause constitutive expression of all four genes in strains carrying the plasmids pAN1 and pAN4. Expression of the *catBCDE* genes on pAN1 may result from its insertion downstream from a β-lactamase promoter on the parental plasmid pKT230. Streptomycin resistance in pKT230 is expressed from this promoter, and the *Eco*RI site of insertion for the cloned *catBCDE* gene fragment is between the promoter and the structural gene for streptomycin resistance (2). Expression from the β-lactamase promoter could explain why the *catBCDE* genes do not exhibit normal repression or induction when carried on pAN1. Expression of the *catBCDE* genes from pKT230 is substantially higher in *P. putida* than in *E. coli*, further demonstrating that there are few barriers to the expression of foreign genes in fluorescent *Pseudomonas* species (8, 21). The relatively low rates of expression in *E. coli* probably are due to lower efficiency of transcription from the β-lactamase promoter in these organisms. Transcription from an external *lac* promoter caused synthesis of substantial quantities of the *catBCDE* gene products in *E. coli*: the four proteins accounted for 10% of the cell protein and *catE* expression was 20-fold above the level in fully induced *A. calcoaceticus* cells (Table 4).

The *catBCDE* genes in pAN2 and pAN3 are independent of external promoters after insertion into the *Eco*RI site of pBR322. This genetic arrangement leads to expression of the *catCDE* genes but not the *catB* gene in *E. coli* (Table 3). Evidently, an internal promoter within the *catBCDE* cluster elicits transcription in *E. coli*. The relationship of this promoter to the promoter that causes constitutive expression of the *catCDE* genes in regulatory mutants of *A. calcoaceticus* remains to be determined.

Genes from *P. putida*, including genes from dissimilatory aromatic pathways, have been cloned and are also poorly expressed in *E. coli* (8, 12, 13, 22, 31). DNA sequence analysis of xylene catabolic genes from *P. putida* reveals that they lack *E. coli* consensus sequences associated with polymerase recognition and binding (14). The cloned *A. calcoaceticus* genes may also lack a typical (*E. coli*-like) promoter region.

Effectiveness of pKT230 and *P. putida* for cloning catabolic genes. Cloning of *A. calcoaceticus* genes in *P. putida* on pKT230 demonstrates the suitability of this host-vector system for cloning catabolic genes from heterologous organisms. It is likely that this approach will be used extensively, and we think it appropriate to emphasize two cautions. First, the restriction systems of *P. putida* can substantially reduce the effectiveness of transformation with a plasmid from a heterologous host. Second, procedures used to introduce plasmids into *P. putida* appear to allow substantial transformation of the recipient by linear DNA carried along with the plasmid (23). These problems can be circumvented most readily by using pKT230 purified from the recipient mutant strain as a vector in transformations designed to select for a recombinant plasmid carrying a heterologous gene.

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