Cloning and Expression in *Escherichia coli* of *Acinetobacter calcoaceticus* Genes for Benzoate Degradation

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The catabolic genes necessary for the conversion of benzoate to catechol have been cloned from *Acinetobacter calcoaceticus* into *Escherichia coli*. The cloned genes, *benABCD*, encode both a benzoate 1,2-dioxygenase system, composed of NADH-cytochrome *c* reductase and terminal oxygenase components, and a *cis*-diol dehydrogenase. The dioxygenase system appears to be encoded by three genes, *benABC*, whose products, 53-, 19-, and 38-kilodalton proteins, correspond in size to those of components in other bacterial dioxygenases. The cloned dioxygenase system is expressed at high levels in *E. coli*, enabling the conversion of benzoate to a *cis*-diol, 2-hydro-1,2-dihydroxybenzoate, at a rate comparable to that of fully induced *A. calcoaceticus* cultures. A *cis*-diol dehydrogenase, the product of the *A. calcoaceticus benD* gene, when present in *E. coli* enables this organism to convert the *cis*-diol intermediate to catechol. The dehydrogenase has been partially purified and is a dimer with two identical 31-kilodalton subunits. The *ben* genes are clustered on the *A. calcoaceticus* chromosome with independently regulated genes needed for the dissimilation of catechol. In a 16-kilobase-pair region of the chromosome there are 10 genes for benzoate catabolism, organized in no fewer than three transcriptional units. This kind of arrangement, termed supraoperonic clustering, has been observed previously in pseudomonads.

In many species of bacteria, benzoate degradation takes place by way of the β -ketoadipate pathway (Fig. 1). In this sequence of steps, a nonaromatic *cis*-diol, 2-hydro-1,2-dihydroxybenzoate (DHB), is formed as an intermediate in the conversion of benzoate to catechol (27, 29). Oxygenative cleavage of catechol is then followed by a series of reactions leading to the formation of succinate and acetyl coenzyme A (32). Although many of the steps after catechol formation are well understood, less is known about the genetic organization and the biochemical mechanisms which underlie the initial reactions of benzoate degradation.

Direct studies of such reactions can be difficult, because the enzymes catalyzing them are often unstable and difficult to assay. In the present investigation, a genetic approach was taken, and we isolated the genes responsible for DHB formation and those for its conversion to catechol. *Acinetobacter calcoaceticus*, from which genes for catechol degradation have been cloned previously (22, 31), was used in these studies because it has a natural transformation system, which can greatly facilitate genetic manipulations. Although DHB had not been isolated from this organism, it could be rapidly oxidized by *A. calcoaceticus* cell extracts in an NAD-dependent reaction (27). It seemed likely therefore that in this bacterium, as in others, the first step in benzoate oxidation involved its conversion to DHB.

cis-Diols are formed, generally, by multicomponent aromatic ring dioxygenases, of which some are encoded by chromosomal genes, others are encoded by plasmid genes. Several functionally related plasmids, designated TOL plasmids, which encode enzymes for the degradation of a variety of aromatic hydrocarbons including toluene, have been isolated from pseudomonads (12, 20, 42-44). A broad substrate-specific dioxygenase system, encoded by TOL plasmid pWWO, forms *cis*-diols from many C-3- and C-4-

substituted benzoates, some disubstituted benzoates, and from benzoate itself (15, 25). A TOL plasmid aromatic dioxygenase system expressed in *Pseudomonas putida* BG1 also exhibits relaxed substrate specificity (40). In contrast, chromosome-encoded *Pseudomonas* benzoate 1,2-dioxygenase systems have narrow substrate specificity and do not show significant oxidation of most substituted benzoates (25, 26).

Multicomponent dioxygenases consist of a short electron transport system, having one or more proteins, and an iron-sulfur terminal oxygenase (6). Both flavin and iron-sulfur moieties are involved in the electron transfer from NADH to the terminal oxygenase. In *Pseudomonas arvilla*, there is an NADH-cytochrome *c* reductase component of a benzoate 1,2-dioxygenase system which contains FAD and a 2Fe-2S center on one peptide (45). The benzene, toluene, and naphthalene dioxygenase systems utilize two proteins for the electron transfer, one with a flavin molecule and the other with an iron-sulfur center (2, 11, 33, 34). These multicomponent systems produce reactive *cis*-diols which are readily converted to catechols by NAD-dependent dehydrogenases (1, 24, 28, 40).

After catechol formation, benzoate degradation in *A. calcoaceticus* proceeds by a well-characterized route. The *cat* genes, encoding enzymes for the conversion of catechol to tricarboxylic acid cycle intermediates, are clustered on the chromosome (Fig. 1 and 2) (31). The *catA* gene, encoding catechol 1,2-dioxygenase, is separated from the other *cat* genes by 3.5 kilobase pairs (kbp) of DNA and is not regulated coordinately with them (5, 22).

The possibility that the *A. calcoaceticus* genes for benzoate degradation (*ben* genes; Fig. 1) are linked to *cat* genes had been raised earlier by the isolation of strains carrying large deletions extending through both regions (31). In both *P. putida* and *Pseudomonas aeruginosa* strains, independently regulated genes coding for enzymes having related

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FIG. 1. Metabolites, enzymes, and structural genes associated with dissimilation of benzoate via catechol and the β -ketoadipate pathway. CoA, Coenzyme A.

metabolic functions are clustered, an arrangement described as "supraoperonic" (17, 30, 39). By cloning DNA fragments adjacent to the *catA* gene, we show here that such a chromosomal arrangement also exists in *A. calcoaceticus*. In addition, the newly cloned DNA and the properties of several mutant strains unable to metabolize benzoate have allowed us to identify the *A. calcoaceticus* enzymes used for the conversion of benzoate to catechol and to study their regulation.

MATERIALS AND METHODS

Bacterial strains and plasmids. *A. calcoaceticus* strains were derived from strain BD413 (18, 19), which is designated strain ADP1 in our collection. Strains ADP137 (23), ADP136, and ADP138 are spontaneous mutants of wild-type ADP1 which were selected for their ability to grow with 10 mM succinate in the presence of 5 mM 4-fluorobenzoate (41). This substrate analog is toxic to *A. calcoaceticus* strains that are able to degrade benzoate.

For cloning, vector pUC19 (Ap^r *lac* ϕ) and host *Escherichia coli* JM101 [*supE* *thi* Δ (*lac-proAB*) *F'* *traD36 proAB lacI^qZ Δ M15*] were used (48). Wild-type *E. coli* does not contain genes for the β -ketoadipate pathway. This vector-host system allows direct screening for recombinant plasmids on plates containing 0.006% 5-bromo-4-chloro-3-indolyl- β -D-

galactopyranoside and 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Plasmids carrying cloned *A. calcoaceticus* fragments, some of which have been described before (22, 31), are shown in Fig. 2.

Media and growth conditions. Bacterial growth conditions, including supplements and carbon sources, were described previously (22, 31).

Isotopes, enzymes, and chemicals. *cis,cis*-Muconate and DHB were gifts from the Celgene Corp. All other chemicals and enzymes were obtained commercially at the highest purity available. Restriction enzymes, DNA ligase, and calf intestinal phosphatase were used as suggested by the suppliers (Bethesda Research Laboratories, Boehringer Mannheim Biochemicals, International Biotechnologies Inc., and New England Nuclear Corp.).

DNA manipulations and hybridizations. DNA manipulations, including chromosomal and plasmid purifications, DNA size fractionation, *E. coli* transformation, subcloning, and restriction mapping, as well as hybridizations, including Southern blots and colony hybridizations, were described previously (22, 31).

***Acinetobacter* transformation assay.** *Acinetobacter* DNA, purified in a variety of ways, can transform an *Acinetobacter* recipient strain when the DNA is placed directly onto an appropriate bacterial plate carrying the recipient (22).

Enzyme assays. Cultures for enzyme assays were prepared (31) and disrupted by sonication (22) as described previously, except that cultures to be assayed for NADH-cytochrome *c* reductase activity were disrupted in buffer (50 mM phosphate buffer containing 10% glycerol, pH 7.0) without dithiothreitol. Enzyme induction was examined in cells that were grown on glucose (10 mM) in the presence of benzoate (2 mM). Catechol 1,2-dioxygenase activity was measured spectrophotometrically by monitoring the increase in A_{260} that occurs with *cis,cis*-muconate formation (16). DHB dehydrogenase activity was measured spectrophotometrically by following DHB-dependent NAD reduction at 340 nm (28). NADH-cytochrome *c* reductase activity was determined by following cytochrome *c* reduction at 550 nm (45). Protein concentrations were determined by the method of Lowry et al. (21) with bovine serum albumin as the standard.

Metabolite monitoring by HPLC. Samples containing 0.5 ml of culture medium were filtered through low-protein-binding, 0.20- μm pore size filters (Acrodiscs from Gelman Sciences) to remove whole cells. Metabolites in 5 μl of the filtrates were resolved on a Vydac reverse-phase C_{18} high-performance liquid chromatography (HPLC) column (4.6 mm by 25 cm). Elution at a rate of 0.7 ml/min was carried out with 30% acetonitrile containing 0.1% phosphoric acid, and the eluant was monitored by UV detection at 210 nm. Under these conditions, retention times for DHB and benzoate were approximately 5.3 and 16.5 min, respectively. Culture medium samples were mixed with authentic samples of DHB and benzoate and chromatographed to assure proper peak identification. Peak areas were quantitated with a Shimadzu C-R3A integrator.

To determine the fate of benzoate added to *A. calcoaceticus* mutant strains, 100-ml cultures were grown overnight on 10 mM glucose and diluted to 200 ml, and 10 mM glucose and 5 mM benzoate were added. Samples were collected at intervals during a 48-h period. In studies of benzoate utilization by wild-type strain ADP1 growing on 5 mM benzoate, optical density measurements were used to estimate cell number, and changes were correlated with the amount of benzoate remaining in the medium over a 10-h period. *E. coli*

strains carrying plasmids were grown to the early stationary phase with 0.5% glycerol in the presence of IPTG, which induces the expression of the *lac* promoter. Benzoate (5 mM) was added, and samples were taken at intervals during a 12-h period.

Partial purification of DHB dehydrogenase by HPLC. A crude extract of JM101(pIB1382) was prepared by suspending about 2.5 g of cells in 10 ml of 50 mM sodium ethylenediamine buffer with 1 mM 2-mercaptoethanol (pH 7.3). The cells were broken by sonication (five 30-s pulses), and the solution was centrifuged at $15,000 \times g$ for 15 min. The supernatant fraction was then centrifuged at $100,000 \times g$ for 30 min in an ultracentrifuge. The supernatant fraction was filtered through a 0.22- μ m pore size Millex GV (low binding) unit, and the filtrate was termed crude extract.

A 9-ml sample of the crude extract containing 13.5 mg of protein per ml was chromatographed on a Toya Soda TSK DEAE-5PW anion-exchange HPLC column (21.5 mm by 15 cm). Elution at a rate of 1 ml/min was carried out with a 0 to 0.3 M NaCl gradient over 120 min. The eluant was monitored by UV detection at 280 nm, and 2-ml fractions were collected. Fractions were assayed for DHB dehydrogenase activity, and the five fractions having the highest activities were combined.

The combined fractions were concentrated to a volume of several hundred microliters by using an Amicon centricon 10 microconcentrator. Components within a 100- μ l sample were then separated by gel filtration with two HPLC columns connected in series. A wide-pore (30 nm) Du Pont Zorbax GF-450 column (9.4 mm by 25 cm) and a smaller-pore (15 nm) Du Pont Zorbax GF-250 column (9.4 mm by 25 cm) were coupled. The enzyme preparation was eluted at a rate of 1 ml/min with a mobile phase of 50 mM sodium ethylenediamine buffer with 100 mM NaCl and 1 mM 2-mercaptoethanol (pH 7.3). The eluant was monitored at 280 nm, and 0.5-ml fractions were collected. The three fractions having the highest DHB dehydrogenase activities were combined.

After preparative gel filtration, the DHB dehydrogenase and a mixture of molecular weight markers (Bio-Rad Laboratories), including thyroglobulin (bovine), gamma globulin (bovine), ovalbumin (chicken), myoglobin (horse), and vitamin B₁₂, were separated by gel filtration as described above. The size of the DHB dehydrogenase was estimated by comparing its elution volume with those of the molecular weight standards.

Proteins from the crude extract, combined DEAE fractions, and combined gel filtration fractions were separated by gel electrophoresis on 12% total, 2.7% cross-linked, 0.1% sodium dodecyl sulfate (SDS)-polyacrylamide denaturing gels (38). The gels were stained with Coomassie blue and dried with a gel slab dryer (Bio-Rad model 224).

In vitro transcription-translation. The protein products of cloned *A. calcoaceticus* inserts were identified by using a DNA-directed translation kit from Amersham Corp., a cell-free, *E. coli*-derived, coupled transcription-translation system (7). Proteins produced from plasmids (1 μ g per reaction) were radioactively labeled with L-[³⁵S]methionine according to instructions, with a 50-min incubation period and a 30-min (cold) methionine chase reaction. Samples containing half of the volume of each reaction mixture were analyzed on SDS-polyacrylamide gels as described above. ¹⁴C-labeled protein molecular weight markers, from Bethesda Research Laboratories, were used as size standards. Gels were fixed in 7% acetic acid-5% methanol and dried before autoradiography by using Kodak X-Omat RP film.

TABLE 1. Expression of *benD*^a in *A. calcoaceticus*

Strain	Mutation ^b	Sp act on the following growth substrates:			
		Benzoate	Glucose plus benzoate ^c	<i>cis,cis</i> -Muconate	Glucose ^d
ADP1	None	1.0	0.15	0.14	<0.001
ADP136	<i>benD3136</i>		<0.001	<0.001	<0.001
ADP137	<i>ben-3137</i>		1.35	1.22	<0.001
ADP138	<i>benD3138</i>		<0.001	<0.001	<0.001

^a Expressed as the specific activity of DHB dehydrogenase (micromoles per minute per milligram of protein).

^b The *benD* designation is assigned on the basis of these observations, metabolite accumulation, and genetic analysis. The specific gene mutated in strain ADP 137 cannot be designated, but the *ben-3137* mutation appears to lie within the *benABC* gene cluster.

^c Benzoate was added as an inducer.

^d Similar results were found for succinate and *p*-hydroxybenzoate.

RESULTS

Characterization of *ben* mutants. Mutant strains ADP136, ADP137, and ADP138, selected for their ability to grow in the presence of 4-fluorobenzoate, were not able to grow with benzoate as the sole carbon source. They were, however, able to grow with *cis,cis*-muconate; when grown with this carbon source all expressed wild-type levels of catechol 1,2-dioxygenase. The mutant strains were grown with various carbon sources, and DHB dehydrogenase levels were measured (Table 1). Strain ADP137 had high levels of DHB dehydrogenase activity when induced with either benzoate or *cis,cis*-muconate, whereas these inducers did not elicit measurable levels of the dehydrogenase in either ADP136 or ADP138.

The mutants were then grown with glucose in the presence of benzoate, and metabolites in the culture media were monitored by HPLC during a 48-h time period. Cultures of ADP136 were able to convert 20% of 5 mM benzoate to DHB, and cultures of ADP138 were able to convert 10% of 5 mM benzoate to DHB, under these conditions. No benzoate was removed from the culture medium by cultures of ADP137 (within the limits of detection, which are roughly 2%). On the basis of this and subsequent genetic evidence, two mutations, *benD3136* (in ADP136) and *benD3138* (in ADP138), were assigned to the structural gene for the diol dehydrogenase; the third mutation, *ben-3137* (in ADP137), appears to prevent benzoate oxidation, the function encoded by the *benABC* genes.

Transformation of *ben* mutants by *catA*-carrying clones. Plasmid pIB1362 and its deletion derivative pIB1341 both carry the cloned *A. calcoaceticus catA* gene (Fig. 2) (22). The *Acinetobacter* transformation assay was used to demonstrate that the *benD3136* mutation was transformed to the wild type by pIB1362 but not by pIB1341. Neither plasmid transformed the *benD3138* mutation or the *ben-3137* mutation to the wild type. JM101(pIB1362) did not express DHB dehydrogenase. This evidence, coupled with the failure of pIB1362 to transform the *benD3138* mutation to the wild type, suggested that part but not all of the *benD* gene was carried on pIB1362. To clone the entire *benD* gene, an effort was made to isolate a fragment from the *A. calcoaceticus* chromosome which overlapped the cloned pIB1362 *EcoRI* fragment and extended further into the *ben* region.

Cloning of DNA adjacent to the *catA* region. ADP1 chromosomal DNA was cleaved with the restriction endonuclease *Asp718*, an isoschizomer of *KpnI*. The DNA was size

TABLE 2. Expression of *benD*^a and *catA*^b in *E. coli*.^c

Strain	Level of expression of:	
	<i>benD</i>	<i>catA</i>
JM101(pUC19)	<0.001	<0.001
JM101(pIB1381)	0.41	0.004
JM101(pIB1382)	16.5	0.004
JM101(pIB1383)	0.40	<0.001

^a Expressed as specific activity of DHB dehydrogenase (micromoles per minute per milligram of protein). Expression of *benD* in the fully induced wild type is 1.0.

^b Expressed as specific activity of catechol 1,2-dioxygenase (micromoles per minute per milligram of protein). Expression of *catA* in the fully induced wild type is 0.75.

^c Cultures were grown with L broth and IPTG.

fractionated, and samples of DNA from the fractions were used to transform ADP136 and ADP138. A fraction of DNA ranging in size from approximately 4.5 to 6.0 kbp was able to transform to the wild type both the *benD3136* and the *benD3138* mutations. DNA from this fraction was ligated to linearized, dephosphorylated pUC19 and used to transform JM101. Recombinant plasmid-containing clones were screened by colony hybridization; of 435 colonies screened, 5 hybridized to a probe made from the *EcoRI*-*Sall* fragment at the left end of the pIB1362 insert (Fig. 2).

Restriction analysis of plasmids from the five hybridizing colonies showed that all contained the same 5-kbp insert in the same orientation relative to the *lac* promoter of pUC19. One of the apparently identical clones was designated pIB1381. Restriction and Southern blot analyses were consistent with the interpretation that the pIB1381 insert carried 1.5 kbp of DNA adjacent to the pIB1362 insert on the *A. calcoaceticus* chromosome. A restriction map of pIB1381 was completed (Fig. 2).

Purified pIB1381 DNA transformed *benD3136* and *benD3138* but not *ben-3137* to the wild type. Since *ben-3137* is linked to *benD3138* (M. E. Rae, unpublished data) and appears to lie in the *benABC* region, an attempt was made to isolate these genes by cloning chromosomal DNA adjacent to the *A. calcoaceticus* insert of pIB1381. A DNA size fraction of *EcoRI*-cut ADP1 chromosomal DNA, containing 4.5- to 6.0-kbp fragments, was able to transform *ben-3137* to the wild type. This DNA was ligated to linearized, dephosphorylated pUC19 and used to transform JM101. The *KpnI*-*EcoRI* fragment from the left end of the pIB1381 insert (Fig. 2) was used to probe the recombinant plasmid-containing clones from this transformation. Of 600 colonies screened by colony hybridization, 4 hybridized to the pIB1381 probe.

Restriction analysis showed that the plasmids from the four hybridizing colonies all carried the same 5.3-kbp *EcoRI* insert. Both orientations of the insert relative to the *lac* promoter of pUC19 were isolated, and plasmids having the two opposite orientations were designated pIB1351 and pIB1352. Restriction and Southern blot analyses were consistent with the chromosomal fragments of pIB1351 and pIB1381 being positioned on the chromosome, relative to each other, as shown in Fig. 2. A restriction map of the pIB1351 insert is also shown in Fig. 2. Both pIB1351 and pIB1352 were able to transform the *ben-3137* mutation to the wild type.

Subcloning and expression of *benD*, encoding DHB dehydrogenase. JM101(pIB1381) had high DHB dehydrogenase activity (Table 2). The deletion subclone pIB1383 (Fig. 2), derived from pIB1381, also enabled JM101 to express DHB

dehydrogenase. In addition, the orientation of the pIB1381 insert relative to the *lac* promoter was reversed, and the resulting plasmid was designated pIB1382. JM101(pIB1382) had the highest DHB dehydrogenase activity measured (Table 2). DNAs from both pIB1382 and pIB1383 were able to transform *benD3136* and *benD3138* to the wild type.

The entire *catA* gene is carried in both pIB1381 and pIB1382 on the fragment which overlaps the insert of pIB1362 (Fig. 2) (22). Catechol 1,2-dioxygenase, the product of the *catA* gene, was expressed at extremely low levels in extracts of JM101(pIB1381) and JM101(pIB1382) (Table 2).

Partial purification of DHB dehydrogenase from JM101 (pIB1382). DHB dehydrogenase was partially purified from JM101(pIB1382). The final enzyme preparation had a specific activity of 108 $\mu\text{mol/min}$ per mg of protein, a value somewhat lower than that of the purified *Alcaligenes eutrophus* enzyme, which has a specific activity of 150 $\mu\text{mol/min}$ per mg of protein (28). On a denaturing SDS-polyacrylamide gel, one 31-kilodalton (kDa) band predominated (Fig. 3). The native protein size was determined to be 62 kDa by gel filtration. When pIB1382 DNA was used as template for in vitro transcription-translation, a band corresponding in size to a protein of 31 kDa was observed after SDS-polyacrylamide gel electrophoresis and autoradiography.

Subcloning and expression of *benABC*, encoding the benzoate dioxygenase system. The plasmids pIB1354 and pIB1356 are deletion derivatives of pIB1352 (Fig. 2). Benzoate was added to cultures of JM101(pUC19), JM101(pIB1351), JM101(pIB1352), JM101(pIB1354), and JM101(pIB1356), and the culture media were monitored by HPLC. Benzoate was converted quantitatively to DHB by JM101(pIB1352) and JM101(pIB1354) (Fig. 4), whereas no benzoate was removed from the media by any of the other strains. The rate of benzoate removal by JM101(pIB1354) (0.8 $\mu\text{mol/min}$ per mg of protein) was comparable to that of fully induced wild-type *A. calcoaceticus* growing on benzoate (0.6 $\mu\text{mol/min}$ per mg of protein). The rate with JM101(1352) was slightly lower (0.2 $\mu\text{mol/min}$ per mg of protein).

Both JM101(pIB1352) and JM101(pIB1354) had NADH-

FIG. 3. Partial purification of DHB dehydrogenase from JM101(pIB1382). Samples of the crude extract (lane 1), combined fractions after DEAE column chromatography (lane 2), and combined fractions after gel filtration column chromatography (lane 3) were separated by SDS-polyacrylamide gel electrophoresis. The arrow points to the dehydrogenase.

FIG. 4. Conversion of 5 mM benzoate to 5 mM DHB by JM101(pIB1354). Circles and squares indicate the mole percentage of benzoate and DHB, respectively, in the culture medium as detected by HPLC analysis.

cytochrome *c* reductase activity higher than that of fully induced strain ADP1 (Table 3). Such reductase activity would be expected for strains expressing a cloned benzoate dioxygenase system (Fig. 5). The protein products of pIB1352 and pIB1354 genes were determined by in vitro transcription-translation (Fig. 6). The major bands common to both pIB1352 and pIB1354 are at positions corresponding to 53-, 38-, and 19-kDa proteins.

DISCUSSION

benABC genes and their products, the enzymes that convert benzoate to DHB. In this report we describe the first isolation of chromosomal genes for a multicomponent aromatic dioxygenase system, which should facilitate future genetic, biochemical, and evolutionary comparisons with broad-specificity plasmid-borne systems. Genes carried on pIB1352 and pIB1354 enable *E. coli* to convert benzoate, normally a toxic chemorepellant for this species (36), rapidly and quantitatively to DHB. The 3.5-kbp insert in pIB1354 contains the approximately 3 kbp of DNA required to encode gene products of 19, 38, and 53 kDa. The 19- and 53-kDa proteins correspond in size to the 20- and 50-kDa subunits associated with the terminal oxygenase of the *P. arvilla* benzoate dioxygenase system (46, 47). Also roughly corresponding in size are the 20- and 57-kDa subunits of the broad-specificity TOL plasmid dioxygenase system (15). Other terminal oxygenases of aromatic dioxygenase systems, including those for naphthalene and toluene degradation, have subunits of approximately these sizes (10, 13).

The third protein encoded by pIB1352 and pIB1354 has a size corresponding to 38 kDa and is likely to be the NADH-cytochrome *c* reductase component of the benzoate dioxygenase system (Fig. 5). The analogous component purified from *P. arvilla* is a 38-kDa flavoprotein (45), and the *Pseudomonas cepacia* phthalate oxygenase reductase is a 34-kDa protein containing flavin mononucleotide and a ferredoxin-iron-sulfur type center (3).

TABLE 3. Expression of *benC*^a

Strain	Growth substrate	Level of <i>benC</i> expression
ADP1	Benzoate	0.40
ADP1	Succinate	<0.03
ADP1	<i>cis,cis</i> -Muconate	<0.03
ADP137	<i>cis,cis</i> -Muconate	<0.03
JM101(pUC19)	L broth + IPTG	<0.03
JM101(pIB1352)	L broth + IPTG	0.52
JM101(pIB1354)	L broth + IPTG	1.64

^a Expressed as the specific activity of NADH-cytochrome *c* reductase (micromoles per minute per milligram of protein).

FIG. 5. Diagram of the benzoate dioxygenase system based on that of *P. arvilla* (45–47). The *benAB* genes encode two nonidentical subunits of an oxygenase component, and the *benC* gene encodes a reductase component that mediates electron transfer. The *benC*-encoded component can catalyze NADH-dependent cytochrome *c* reduction.

The *benD* gene and its product, DHB dehydrogenase. *A. calcoaceticus* DHB dehydrogenase does not correspond in size with enzymes performing similar functions in other bacteria. The product of the *A. calcoaceticus benD* gene, partially purified from *E. coli*, is a 62-kDa dimer composed of 31-kDa subunits. The *A. eutrophus* DHB dehydrogenase has a native molecular mass of 94.6 kDa and a single subunit mass of 24 kDa (28). A plasmid-encoded, inducible, NAD-dependent dehydrogenase which oxidizes *cis-p*-toluate diol to 4-methylcatechol in *P. putida* BG1 is a 96-kDa protein (40). The sizes of *Pseudomonas* naphthalene dihydrodiol dehydrogenase (24) and *cis*-benzene dihydrodiol dehydrogenase (1) also differ from the size of the *A. calcoaceticus* dehydrogenase.

Expression of *A. calcoaceticus* genes in *E. coli*. It seems unlikely that the properties of the *A. calcoaceticus benD* gene product are abnormal because it was formed in *E. coli*. This organism generally appears to transcribe and to translate *A. calcoaceticus* genes associated with aromatic catabolism correctly. As shown previously, the size of catechol 1,2-dioxygenase is the same whether it is purified from fully induced *A. calcoaceticus* or from *E. coli* carrying the cloned gene on a recombinant plasmid (22). In addition, the *benABC* genes, when transcribed and translated by *E. coli* extracts,

FIG. 6. Proteins synthesized by in vitro transcription-translation. The *A. calcoaceticus* inserts of pIB1354 and pIB1352 encode 53-, 38-, and 19-kDa proteins as determined by SDS-polyacrylamide gel electrophoresis and autoradiography. The arrow points to the β -lactamase encoded by the pUC19 ampicillin resistance gene (35).

produce proteins that correspond in size and in overall function with those encoded by analogous genes in other organisms. Of 15 *A. calcoaceticus* gene products examined after expression in *E. coli* (9, 22, 31), the only protein that appears to demonstrate discernable host-dependent properties is protocatechuate 3,4-dioxygenase, the product of the *pcaA* gene. This enzyme is relatively inactive when the gene is expressed in *E. coli* at extremely high levels (9). Under these circumstances, the observed difference in catalytic properties may be more readily ascribed to a specific demand of the enzyme for iron rather than attributed to a general difference in mechanisms of macromolecular synthesis.

Regulation of enzyme activity and gene expression. The synthesis of DHB dehydrogenase from pIB1382 is consistent with the assignment of mutations in ADP136 and ADP138 to the *benD* structural gene. DHB, however, only accumulates in small amounts when either ADP136 or ADP138 is grown in the presence of benzoate. In *A. eutrophus* a *benD* mutation allows much greater conversion of benzoate to DHB (29), and this difference may reflect different regulation in the two bacteria. It is possible that in *A. calcoaceticus* DHB inhibits the benzoate dioxygenase system. Mutations in *xylL*, the TOL plasmid diol dehydrogenase gene, also lead to reduced expression of the TOL dioxygenase system (14). NADH depletion or variable cell membrane permeability to DHB could play a role in lowering dioxygenase activity. Since strain ADP1 cannot grow with DHB, there may be a permeability barrier to this compound, or inability to grow with this compound may simply reflect that DHB does not induce the dehydrogenase.

The *ben-3137* mutation in ADP137 prevents conversion of benzoate to DHB and also seems to affect DHB dehydrogenase expression (Table 1). There are higher levels of DHB dehydrogenase in ADP137 than in ADP1 induced with either benzoate or *cis,cis*-muconate. Despite the different levels of expression in ADP137 and ADP1, it appears that both *cis,cis*-muconate and benzoate independently induce expression of *benD*. *cis,cis*-Muconate-grown ADP1 has 10% of the DHB dehydrogenase activity of fully induced, benzoate-grown ADP1, whereas growth with other carbon sources (succinate, glucose, *p*-hydroxybenzoate) does not induce expression of *benD*. *cis,cis*-Muconate does not induce NADH-cytochrome *c* reductase (Table 3), nor do *cis,cis*-muconate-grown wild-type cultures oxidize benzoate (5).

Both *cis,cis*-muconate and benzoate, structurally dissimilar metabolites, can, however, independently induce catechol 1,2-dioxygenase, the product of the *catA* gene (23). Although the *catA* and *benD* genes are located in the same region of the chromosome (Fig. 2), there are at least 1.3 kbp of DNA separating them, and different levels of expression of the two cloned genes suggest that they are transcribed independently (Table 2). The 40-fold higher DHB dehydrogenase levels in JM101(pIB1382) than in JM101(pIB1381) suggest that the *benD* gene is transcribed from left to right (Fig. 2), since the only difference in the two plasmids is the orientation of the insert relative to the *lac* promoter. This is the same direction as *catA* transcription, yet JM101 (pIB1382), which carries the entire *catA* gene, expresses extremely low levels of the dioxygenase. In this strain carrying both the *benD* and *catA* genes, the former is expressed at levels 16-fold higher than in fully induced ADP1, whereas the latter is expressed at levels that are 0.5% of that of fully induced ADP1 (Table 2). The level of *catA* expression is independent of insert orientation relative to the *lac* promoter, suggesting that *catA* is expressed, in these

strains, from an independent promoter and is not coordinately expressed with *benD*. Transcripts originating at the *lac* promoter of pIB1362 do not appear to proceed through the *catA* gene (22).

Supraoperonic clustering. The 10 *ben* and *cat* genes clustered within 16 kbp of DNA on the *A. calcoaceticus* chromosome appear to be organized in at least three transcriptional units. The *catA* gene seems to be transcribed independently of either *benD* or the other *cat* genes. In addition, different effects of *cis,cis*-muconate on *ben* gene expression may indicate that the *benABC* and *benD* genes are subject to different regulatory controls. The significance of supraoperonic clustering of genes associated with benzoate utilization, also observed in pseudomonads (17, 30, 39), is yet to be determined. The plausibility of one interpretation, that physical interaction among different genes is involved in their stabilization or regulation, is heightened by observation of direct involvement of *catE* sequences in the repair of a mutation in the *pcaE* gene (8). The influence of genetic location on the stability and regulation of the *ben* genes could be determined by examination of their properties after transposition to different loci.

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LITERATURE CITED

1. Axcell, B. C., and P. J. Geary. 1973. The purification and some properties of the enzyme *cis*-1,2-dihydroxycyclohexa-3,5-diene (nicotinamide adenine dinucleotide) oxidoreductase (*cis*-benzene glycol dehydrogenase). *Biochem. J.* **136**:927-934.
2. Axcell, B. C., and P. J. Geary. 1975. Purification and some properties of a soluble benzene-oxidizing system from a strain of *Pseudomonas*. *Biochem. J.* **146**:173-183.
3. Batie, C. J., E. LaHaie, and D. P. Ballou. 1987. Purification and characterization of phthalate oxygenase and phthalate oxygenase reductase from *Pseudomonas cepacia*. *J. Biol. Chem.* **262**:1510-1518.
4. Bolivar, R., R. L. Rodriguez, M. C. Betlach, and W. H. Boyer. 1977. Construction and characterization of new cloning vehicles. I. Ampicillin resistant derivatives of the plasmid pMB9. *Gene* **2**:75-93.
5. Canovas, J. L., and R. Y. Stanier. 1967. Regulation of the enzymes of the β -ketoadipate pathway in *Moraxella calcoaceticus*. *Eur. J. Biochem.* **1**:289-300.
6. Dagley, S. 1986. Biochemistry of aromatic hydrocarbon degradation in pseudomonads, p. 527-555. In J. R. Sokatch and L. N. Ornston (ed.), *The bacteria*, vol. 10. Academic Press, Inc., New York.
7. DeVries, J. K., and G. Zubay. 1967. DNA-directed peptide synthesis. II. The synthesis of the alpha-fragment of the enzyme β -galactosidase. *Proc. Natl. Acad. Sci. USA* **57**:1010-1012.
8. Doten, R. C., L. A. Gregg, and L. N. Ornston. 1987. Influence of the *catBCE* genes on the phenotypic reversion of a *pcaE* mutation in *Acinetobacter calcoaceticus*. *J. Bacteriol.* **169**:3175-3180.
9. Doten, R. C., K.-L. Ngai, D. J. Mitchell, and L. N. Ornston. 1987. Cloning and genetic organization of the *pca* gene cluster from *Acinetobacter calcoaceticus*. *J. Bacteriol.* **169**:3168-3174.
10. Ensley, B. D., and D. T. Gibson. 1983. Naphthalene dioxygenase: purification and properties of a terminal oxygenase component. *J. Bacteriol.* **155**:501-511.
11. Ensley, B. D., D. T. Gibson, and A. L. Laborde. 1982. Oxidation of naphthalene by a multicomponent enzyme system from *Pseudomonas* sp. strain NCIB 9816. *J. Bacteriol.* **149**:948-954.

12. Friello, D. A., J. R. Mylroie, D. T. Gibson, J. E. Rogers, and A. M. Chakrabarty. 1976. XYL, a nonconjugative xylene degradative plasmid in *Pseudomonas* Pxy. *J. Bacteriol.* **127**: 1217-1224.
13. Gibson, D. T., W.-K. Yeh, T.-N. Liu, and V. Subramanian. 1982. Toluene dioxygenase: a multicomponent enzyme system from *Pseudomonas putida*, p. 51-62. In M. Nozaki, S. Yamamoto, Y. Ishimura, M. J. Coon, L. Ernster, and R. W. Estabrook (ed.), *Oxygenases and oxygen metabolism*. Academic Press, Inc., New York.
14. Harayama, S., P. R. Lehrbach, and K. N. Timmis. 1984. Transposon mutagenesis analysis of meta-cleavage pathway operon genes of the TOL plasmid of *Pseudomonas putida* mt-2. *J. Bacteriol.* **160**:251-255.
15. Harayama, S., M. Rekik, and K. N. Timmis. 1986. Genetic analysis of a relaxed substrate specificity aromatic ring dioxygenase, toluate 1,2-dioxygenase, encoded by TOL plasmid pWWO of *Pseudomonas putida*. *Mol. Gen. Genet.* **202**:226-234.
16. Hegeman, G. D. 1966. Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. I. Synthesis of enzymes by the wild type. *J. Bacteriol.* **91**:1140-1154.
17. Holloway, B. W., and A. F. Morgan. 1986. Genome organization in *Pseudomonas*. *Annu. Rev. Microbiol.* **40**:79-105.
18. Juni, E. 1972. Interspecies transformation of *Acinetobacter*: genetic evidence for a ubiquitous genus. *J. Bacteriol.* **112**: 917-931.
19. Juni, E., and A. Janik. 1969. Transformation of *Acinetobacter calco-aceticus* (*Bacterium anitratum*). *J. Bacteriol.* **98**:281-288.
20. Kunz, D. A., and P. J. Chapman. 1981. Isolation and characterization of spontaneously occurring TOL plasmid mutants of *Pseudomonas putida* HS1. *J. Bacteriol.* **146**:952-964.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:269-275.
22. Neidle, E. L., and L. N. Ornston. 1986. Cloning and expression of the *Acinetobacter calcoaceticus* catechol 1,2-dioxygenase structural gene, *cataA*, in *Escherichia coli*. *J. Bacteriol.* **168**: 815-820.
23. Neidle, E. L., and L. N. Ornston. 1987. Benzoate and muconate, structurally dissimilar metabolites, induce expression of *cataA* in *Acinetobacter calcoaceticus*. *J. Bacteriol.* **169**:414-415.
24. Patel, T. R., and D. T. Gibson. 1974. Purification and properties of (+)-cis-naphthalene dihydrodiol dehydrogenase of *Pseudomonas putida*. *J. Bacteriol.* **119**:879-888.
25. Reineke, W., and H.-J. Knackmuss. 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of benzoic acid. *Biochim. Biophys. Acta* **542**:412-423.
26. Reineke, W., W. Otting, and H.-J. Knackmuss. 1978. cis-Dihydrodiols microbially produced from halo- and methylbenzoic acids. *Tetrahedron* **34**:1707-1714.
27. Reiner, A. M. 1971. Metabolism of benzoic acid by bacteria: 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid is an intermediate in the formation of catechol. *J. Bacteriol.* **108**:89-94.
28. Reiner, A. M. 1972. Metabolism of aromatic compounds in bacteria. Purification and properties of the catechol-forming enzyme, 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid (NAD⁺) oxidoreductase (decarboxylating). *J. Biol. Chem.* **217**:4960-4965.
29. Reiner, A. M., and G. D. Hegeman. 1971. Metabolism of benzoic acid by bacteria. Accumulation of (-)-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid by a mutant strain of *Alcaligenes eutrophus*. *Biochemistry* **10**:2530-2536.
30. Rosenberg, S. L., and G. D. Hegeman. 1969. Clustering of functionally related genes in *Pseudomonas aeruginosa*. *J. Bacteriol.* **108**:1270-1276.
31. Shanley, M. S., E. L. Neidle, R. E. Parales, and L. N. Ornston. 1986. Cloning and expression of *Acinetobacter calcoaceticus catBCDE* genes in *Pseudomonas putida* and *Escherichia coli*. *J. Bacteriol.* **165**:557-563.
32. Stanier, R. Y., and L. N. Ornston. 1973. The beta-ketoadipate pathway. *Adv. Microb. Physiol.* **9**:89-151.
33. Subramanian, V., T.-N. Liu, W.-K. Yeh, and D. T. Gibson. 1979. Toluene dioxygenase: purification of an iron-sulfur protein by affinity chromatography. *Biochem. Biophys. Res. Commun.* **91**:1131-1139.
34. Subramanian, V., T.-N. Liu, W.-K. Yeh, M. Narro, and D. T. Gibson. 1981. Purification and properties of NADH-ferredoxin TOL reductase. A component of toluene dioxygenase from *Pseudomonas putida*. *J. Biol. Chem.* **256**:2723-2730.
35. Sutcliffe, G. 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. *Proc. Natl. Acad. Sci. USA* **75**:3737-3741.
36. Tso, W.-W., and J. Adler. 1974. Negative chemotaxis in *Escherichia coli*. *J. Bacteriol.* **118**:560-574.
37. Vieira, J., and J. Messing. 1982. The pUC plasmids, and M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**:259-268.
38. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**:4406-4412.
39. Wheelis, M. L., and R. Y. Stanier. 1970. The genetic control of dissimilatory pathways in *Pseudomonas putida*. *Genetics* **66**: 245-266.
40. Whited, G. M., W. R. McCombie, L. D. Kwart, and D. T. Gibson. 1986. Identification of cis-diols as intermediates in the oxidation of aromatic acids by a strain of *Pseudomonas putida* that contains a TOL plasmid. *J. Bacteriol.* **166**:1028-1039.
41. Wigmore, G. J., and D. W. Ribbons. 1980. *p*-Cymene pathway in *Pseudomonas putida*: selective enrichment of defective mutants by using halogenated substrate analogs. *J. Bacteriol.* **143**: 816-824.
42. Williams, P. A., and K. Murray. 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (arvilla) mt-2: evidence for the existence of a TOL plasmid. *J. Bacteriol.* **120**:416-423.
43. Williams, P. A., and M. J. Worsey. 1976. Ubiquity of plasmids in coding for toluene and xylene metabolism in soil bacteria: evidence for the existence of new TOL plasmids. *J. Bacteriol.* **125**:818-828.
44. Wong, C. L., and N. W. Dunn. 1974. Transmissible plasmid coding for the degradation of benzoate and m-toluene in *Pseudomonas arvilla* mt-2. *Genet. Res.* **23**:227-232.
45. Yamaguchi, M., and H. Fujisawa. 1978. Characterization of NADH-cytochrome *c* reductase, a component of benzoate 1,2-dioxygenase system from *Pseudomonas arvilla* C-1. *J. Biol. Chem.* **253**:8848-8853.
46. Yamaguchi, M., and H. Fujisawa. 1980. Purification and characterization of an oxygenase component in benzoate 1,2-dioxygenase system from *Pseudomonas arvilla* C-1. *J. Biol. Chem.* **255**:5058-5063.
47. Yamaguchi, M., and H. Fujisawa. 1982. Subunit structure of oxygenase component in benzoate 1,2-dioxygenase system from *Pseudomonas arvilla* C-1. *J. Biol. Chem.* **257**:12497-12502.
48. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103-119.